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Research and Development of Opioid-Related Ligands

Edited by
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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Research in the opioid field continues apace, with ongoing developments in our understanding of the underlying biology through to the clinical consequences of modulating opioid receptors. The sheer volume of research being carried out in this field precludes a thorough bench-to-bedside review and has necessitated a somewhat focused approach within this volume, in particular the use and potential uses of ligands that activate one, or more, of the receptors. Contributions to *Research and Development of Opioid-Related Ligands* include chapters describing current research into the main clinical uses of opioids, analgesia and opioid abuse treatment, as well as what the editors consider to be key areas of pre-clinical development. Not surprisingly the identification of a fourth opioid-like receptor, the NOP receptor, has provided the stimulus for many studies with the aim of determining the potential therapeutic value of modulating the activity of this receptor. A number of chapters within this volume reflect the current interest in this new member of the family.

The 16 chapters of *Research and Development of Opioid-Related Ligands* are arranged into 5 themes, starting with the clinical studies of pain and opioid abuse treatment and followed by chapters on new ligand development, novel assays and concepts, classical opioid pharmacology and NOP receptor pharmacology. An emphasis is placed on translational science and how our increased knowledge may lead to new medicines. The development and use of ligands selective for one receptor or another continues to be of substantial interest, and indeed, the availability of such ligands has allowed the pharmacology of the NOP receptor to be studied in detail from soon after its discovery. Interestingly, ligands induce distinct receptor conformations and produce different signaling cascades, indicating that ligand-directed signaling or biased agonism may have important therapeutic implications. In addition we have now reached a point where selectively promiscuous ligands (ligands that bind to more than one receptor but with defined efficacy at each) can be designed. The rationale for, and progress in, targeting such ligands is made in a number of the chapters.

It is hoped that the volume will provide a useful reference resource but also stimulate further research and debate within the opioid research community.

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Chapter 1

Commentary on the Current Status of Clinically Used Analgesics

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Pain is universally experienced and necessary for prevention of physical injury and, ultimately, for survival. The experience of pain has a price. The medical community has gained an increasing awareness of adverse physical, economic and social consequences of pain. Pain and its consequences are widespread. Estimates are that up to 1.5 billion people worldwide experience chronic pain (*1*). The chronic pain population in the United States alone is estimated at approximately 115 million and data show associated costs for pain in terms of treatment and lost productivity far exceed the annual costs of heart disease or cancer (*2*). Acute pain associated with accidental injury or surgery adds an additional, and substantial, burden to these estimates. Although acute pain following injury or surgery usually resolves, there is an increasing awareness chronic pain is a frequent consequence of surgery occurring in up to 50% of patients following common surgeries (*3*). Undertreatment of acute pain, especially dynamic pain, is prevalent with approximately two-thirds of postoperative patients experiencing moderate to severe or extreme pain after surgery (*4*). The imperative imposed by these observations is underscored by recent data to show that effective treatment of acute pain after surgery improves recovery and long-term outcomes (*5*, *6*).

For centuries, opiate medications have been used to treat human pain due to their potency, effectiveness and availability. Opiates remain the mainstay for treatment of severe acute and, increasingly, chronic pain. However, the side effect profile of opiates is substantial with at least 25% of acute pain patients experiencing clinically significant side effects, even when their pain is undertreated (*4*), and with a substantial percentage of chronic pain patients at risk for the same side effects and opiate dependence. Adjuvant analgesics can improve the management of pain, usually defined as reduced need for opiates and occasionally as improved quality of

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pain control. Unfortunately, all currently available adjuvant agents have limited effectiveness compared to opiates and each has significant side effects that can limit use such as bleeding, sedation, dysphoria, or hepato-renal toxicity.

Against this backdrop, no truly new class of drugs for the treatment of moderate to severe pain has been introduced into clinical medicine since the development of indomethacin as the first non-steroidal anti-inflammatory drug in the 1960s. With our emerging understanding regarding the frequency of ineffective pain control, the adverse consequences of opiate side effects, and the benefits of more effective pain control, there is a clear need for the development of new drugs to treat human pain. The clinical and research environment are in need of a fresh and timely examination of alternative opiate medications. The present volume, with contributions from many experts in the field of alternative opiate drugs, is an important review of current and future drug development. It will be welcomed by scientists and clinicians alike who seek to improve the care of the many patients who need more effective analgesia.

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Chapter 2

Commentary on the Current State of Opioid-Related Research

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Morphine has been the "gold standard" of clinical analgesics since its isolation from opium in the middle of the C19th. Though its unequivocal structure was not determined until 1925, the search for improvements lacking morphine's principle side effects, dependence/tolerance and respiratory depression, has been continuous but not greatly successful. During the last nearly 50 years when I have been in, or close to the field, major advances in terms of the identification of three types of opioid receptor – mu (MOP), kappa (KOP) and delta (DOP) – and their cloning have been made. More recently (1994) the identification of a fourth "opioid-like" receptor and its natural ligand N/OFQ has given fresh impetus to discovery research programmes. These events are reflected in the balance of chapters in the current volume in which precedence is given to ligands having NOP activity and its potential to produce non-rewarding analgesics and substance abuse treatments.

The major efforts by the pharmaceutical industry to exploit selective KOP and DOP agonists as "non-addicting" analgesics have not yielded much success, though butorphanol and nalbuphine, mixed MOP/KOP partial agonists have found limited clinical use. Interest in KOP agonists lacking CNS penetration as peripheral analgesics for the treatment of visceral pain continues as it does for DOP ligands in bifunctional opioids with MOP agonists. These aspects are covered in chapters in the current volume.

An extremely useful timeline for the history of N/OFQ and selective peptide and non-peptide NOP agonists and antagonists is shown in Calo's chapter, which is primarily concerned with peptide ligands for NOP. Though there is no chapter devoted to non-peptide NOP agonists and antagonists there are recent reviews of this topic and their structure – activity relationships and pharmacology are covered in this volume in the chapters of Zaveri, Toll, Whiteside and Ko. In the latter, the important differences between non-human primates and rodents in terms of supraspinal and systemic effects of NOP agonists are discussed. The agonists in

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rodents are either pronociceptive (i.c.v.) or marginally active (s.c.) whereas by both spinal and systemic routes of administration in primates pronounced activity against thermal nociception, allodynia, and hyperalgesia was demonstrated. Thus the potential of NOP agonists as non-addictive analgesics can be recognised.

However, recent focus has been on bifunctional MOP/NOP agonist ligands based on the hypothesis that since NOP agonists block the development of tolerance and dependence in morphine, ligands having both NOP and MOP agonism should maintain analgesic activity, but show less tolerance and addiction liability. Design strategies for the mixed agonist ligands have started from leads with MOP or NOP agonist selectivity, into which the alternative affinity/efficacy is grafted. The Zaveri and Toll chapters predominantly relate to series originating with NOP selectivity. They also refer to mixed ligands related to a lead with MOP (partial) agonist selectivity, buprenorphine, which was shown to have low efficacy, modest potency partial NOP agonist activity, as well as the established MOP partial agonism and KOP, DOP antagonism. With variable efficacy for all the opioid receptor types, the orvinols to which etorphine and diprenorphine as well as buprenorphine belong, have proved amenable to the introduction of NOP activity equal to, or superior to, that of buprenorphine. Discovery programmes with the aim of further improving NOP affinity and efficacy whilst retaining buprenorphine's KOP antagonism in combination with either MOP partial agonism or MOP antagonism are discussed in the Husbands' chapter.

The use of methodone and buprenorphine as opioid abuse medications is comprehensively reviewed in Saxon's chapter. Sufficient up to date information is provided to satisfy the needs of those entering the field. This subject is also covered in Comer's chapter, which primarily addresses the abuse of MOP agonist analgesics, a serious problem as these opioids have become increasingly prescribed for chronic pain.

In conclusion, this volume provides the reader with a picture of the state of opioid science in the second decade of the twenty first century. Clinical use of opioids as analgesics is still overwhelmingly confined to MOP agonists. The prospects for selective KOP and DOP agonists are not great but there is hope that NOP agonists, particularly in bifunctional alliance with MOP agonism, will eventually reach clinical practice as analgesics and possibly as substance abuse medications. Buprenorphine provides a lead for such developments.

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Chapter 3

The Clinical Importance of Conditioning Pain Modulation: A Review and Clinical Implications

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Conditioning pain modulation (CPM) is one of two dynamic test paradigms which measure an individual's ability to dampen pain centrally. Diffuse noxious inhibitory control (DNIC) is inhibitory pain modulation measured through the ability of a conditioning pain to dampen capacity on a test pain in an anatomically different location from the conditioning pain ("heterotropic pain inhibiting pain", also known as counterirritation). The primary target of DNIC is dorsal horn afferent neuron and wide dynamic range neuron. The critical medullary site for DNIC is the sub-nucleus reticularis dorsalis (SRD), located in the medulla. There is interplay between the SRD and rostral ventromedial medulla (RVM). Activation of the RVM by morphine impairs DNIC. There is also an important interplay between the SRD and the anterior cingulate gyrus. The spino-bulbo-spinal loop of DNIC is dependent upon serotonergic neurotransmission. DNIC can be imaged via EEG and functional MRI as well as measured clinically through CPM. DNIC decreases with age and is less efficient in females. Certain pain processing disorders such as fibromyalgia and irritable bowel syndrome are more frequently associated with impaired DNIC. Duloxetine improves DNIC while morphine impairs DNIC. Clinically testing for DNIC through CPM may

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allow clinicians to better prescribe analgesics, as recently demonstrated in adaptive designed trials.

Introduction

Pain reflexes are important survival responses to potentially life-threatening tissue damage. As an early warning system, pain promotes bodily integrity (*1*, *2*). The absence of pain threatens life and limb (*3*, *4*). Painful sensations are mediated by high threshold peripheral afferent neurons which transmit information via the dorsal horn to the brain. Injury causes a temporary hypersensitivity, which results in behaviors that protect the injured part. For most individuals, this is a reversible adaptive response. A shift in balance between inhibitory and facilitatory central nervous (CNS) processes at the level of the spinal cord, brain or brainstem leads either to resolution of pain or persistent chronic maladaptive pain (*5*). Inhibitory mechanisms, which include stress-induced analgesia, conditioning pain modulation (CPM) and placebo analgesia from release of endogenous opioids, reverse hypersensitivity (*6–8*). Facilitatory mechanisms which can lead to chronic pain involve temporal summation and long term potentiation of wide dynamic range neurons within the dorsal horn. This, in turn, changes the pain processing pathways through the cortex to periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) (*9*, *10*). Pain facilitation involves multiple mechanisms: increased expression of glutamate receptors, ion channels with activation of microglia and up-regulation of chemokine expression. Central sensitization may involve peripheral sensitization of nociceptors, central reorganization caused by peripheral input, disinhibition of antinociception through loss of GABAergic inhibitory interneurons and sympathetically maintained pain facilitation (*11*, *12*). The balance of inhibition to facilitation under normal conditions favors inhibition, while in certain circumstances an imbalance occurs which favors facilitation. With the latter, maladaptive chronic pain is seen clinically (*13*, *14*).

CPM is summed up in the statement "pain inhibits pain". A painful stimulus intensity (a “test” stimulus in the case of CPM) is reduced by application of a second stimulus (a “heterotropic” or “conditioning” stimulus) when applied at a site distant from the painful site (*15*, *16*). CPM differs from simple distraction, which also reduces pain (*17*). The conditioning pain can be mechanical, ischemic, chemical, thermal or electrical. The same phenomena is seen in animals using objective measures such as C-fiber firing rates under the influence of the conditioning stimulus and as a pain behavioral response to a conditioning pain. In animals this is called diffuse noxious inhibitory control or DNIC while in humans the same phenomena is presumably tested by CPM (*5*). While DNIC is clearly established in animals, it can only be speculated upon in humans

Clinical Characteristics

Though different types of conditioning stimuli produce CPM, cold pressor pain is a more reliable conditioning stimulus when compared with ischemic

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stimulus (*18*). Some investigators have stated that the conditioning stimulus does not necessarily need to be painful to produce analgesia at the test site (*19*). Non-painful but strong stimulus may produce weak analgesia at the test site if the conditioning stimulus is applied long enough (*20*). Others have contended that the conditioning stimulus must be painful to produce analgesia at the test site (*21*). The greater the intensity of stimulus at the conditioning site, the greater the degree of analgesia at the test site (*22–26*). Heterotropic painful thermal conditioning stimuli produces increased thresholds to spinal nociceptive knee flexion (RIII reflexes) and higher pain thresholds when the test pain is in the distribution of the sural nerve. This is a common method for measuring CPM. Visceral nociception can also be tested by CPM, since visceral pain as a conditioning stimulus blocks somatic test pain (*24*). As with somatic conditioning pain, the degree of analgesia experienced at the somatic test pain site appears to be directly proportional to the intensity of the conditioning visceral stimulus (*27*, *28*).

The test site analgesia effects of CPM are maximal during the conditioning stimulus and quickly diminish once the conditioning stimulus is extinquished. Pain thresholds at the test site return to normal within minutes of discontinuing the conditioning stimulus (*20*, *29–31*). Duration of DNIC analgesia is directly dependent on the intensity of conditioning stimulus and secondarily on the type of stimulus (*32*).

The presence of chronic pain could potentially act as a conditioning stimulus. In the experimental setting when two conditioning stimuli are applied there is less than additive analgesia (*22*). Theoretically, then chronic pain could act as a second conditioning and lead to less than additive analgesia to the test pain. However, there is no evidence that chronic pain alone impairs CPM, though the evidence is weak (*33*).CPM is more efficient after experimentally induced tissue injury and acute inflammation but is impaired with chronic inflammation (*34–36*).

Temporal Summation and CPM

Increased temporal summation is prevalent among patients with idiopathic pain syndromes such as fibromyalgia . Temporal summation thresholds are more likely to be reduced in individuals who experience severe postoperative pain (*37–41*), though not all investigators agree on this point (*42*). A subset of diabetics with painful peripheral neuropathy have increased temporal summation associated with impaired CPM (*43*). There are gender differences in the relationship between CPM and temporal summation. Robust CPM efficiency and increased temporal summation thresholds are more likely to occur in men than in women (*44*).

CPM and Distraction, Insomnia, Catastrophizing and Ethnic Origin

CPM efficiency does not correlate with analgesia experienced due to distractibility, and the ability to obtain pain relief from distraction does not predict efficient CPM (*45*, *46*). Changes in location of cortical electrical activity related to CPM and distraction related analgesia are distinctly different (*47*).

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Insomnia is associated with reduced pain thresholds and loss of CPM efficiency (*48–51*). Expectations of response from CPM (whether one anticipates increased or decreased pain at the test site) influence the degree of analgesia from CPM. Expectations of analgesia enhance CPM related analgesia at the test site, whereas expectations of hyperalgesia or pain at the test site dampen CPM efficiency (*52*). Expectations of improved test site pain with CPM in patients with fibromyalgia reduce pain significantly but not spinal hyperexcitability as measured the RIII reflex. Analgesia experienced by CPM, therefore, does not depend on descending projections in the spinal cord which would dampen spinal reflexes (*53*). This suggests that analgesia from CPM is largely determined by supra-spinal pathways. A catastrophizing personality is associated with lower pain thresholds and impaired CPM, though there are conflicting findings regarding the influence of the catastrophizing personality trait on CPM efficiency. Two studies have found impaired CPM with catastrophizing traits, while a third study found enhanced CPM efficiency (*25*, *54–56*). Individual differences in catastrophizing traits are reported to alter the blocking activity of naltrexone on CPM. Endogenous opioids are important mediators of CPM efficiency in normal individuals; opioid receptor blockade by naltrexone abolishes CPM in subjects without catastrophizing traits. However, CPM responses are unaffected by naltrexone in high catastrophizers (*57*). There are also ethnic differences in CPM efficiency. African Americans are more sensitive to pain and more often have reduced CPM efficiency compared with non-Hispanic whites (*58–61*).

Age and CPM

Chronic pain is more common among older individuals (*62*, *63*). There are age related decrements in CPM efficiency (*64*, *65*). Reduced CPM efficiency is reported to begin at the age of 40-55 years and CPM responses can be absent in those greater than 60 years old (*66*). Conversely, pain thresholds are lower in younger individuals and increase with age (*66*). Those of the elderly who maintain CPM efficiency will more frequently have less chronic pain and better physical function as measured by the Short Form-36 health survey (*65*). Elderly individuals who lose CPM responses altogether may actually experience increasing pain or facilitation of pain at the conditioning site with the conditioning stimulus (*67*).

Gender and CPM

CPM efficiency changes during the menstrual cycle. It is less efficient during the luteal and menstrual phases (*68*). CPM efficiency is stable across the mid-follicular to late luteal phase and becomes more efficient during ovulation (*69*, *70*). Oral contraceptives reduce CPM efficiency (*71*). Many studies have compared CPM analgesia between genders but have not taken into account the menstrual cycle or the use of oral contraceptives (*20*). However, in general, CPM is more efficient in men than women (*21*, *44*, *72*). Most, but not all, studies have found CPM more efficient in men and no study has found CPM more efficient in women than men (*22*, *25*, *73–76*). It is of interest that certain chronic idiopathic pain

syndromes such as fibromyalgia and irritable bowel syndrome are more frequently found in women who would have less efficient CPM (*77–79*). Women, in general, have lower pain thresholds and pain tolerance than men (*80*, *81*). How this relates to CPM efficiency has not been fully explored.

Impaired CPM and Chronic Pain

There is conflicting evidence regarding the association of CPM impairment as a predictor of acute postoperative pain severity. Preoperative impaired CPM is shown to be predictive of chronic surgical pain in multiple studies (*15*, *45*, *82*, *83*). Higher postoperative pain and poorly controlled postoperative pain predict the development of chronic surgical pain with an odds ratio of 1.8 independent of CPM efficiency. The combination of preoperative impaired CPM plus severe uncontrolled acute postoperative pain predicts a very high risk for chronic surgical pain (*20*). CPM is impaired in osteoarthritis and restored once the painful joint is replaced (*31*). This implies that impaired CPM is maintained by certain chronic pain syndromes and reversed once the painful condition is treated by nonpharmacological measures; impaired CPM in these individuals is an effect rather than a cause of chronic pain.

Neuropathic Pain

The degree of sensory or motor loss in neuropathy does not solely explain the severity of pain experienced by individuals (*84–87*). It is possible that enhanced excitatory input into the central nervous system resulting from reduced thresholds and temporal summation or impaired CPM is a cause of chronic pain, which can be targeted by analgesics such as gabapentin, venlafaxine or duloxetine (*43*, *88–90*). Impaired CPM and reduced temporal summation thresholds are not mutually exclusive but, in certain situations, inter-related. In a small group of patients with peripheral neuropathy from chemotherapy, those with significant pain had enhanced temporal summation and impaired CPM. Enhanced temporal summation correlated with impaired CPM (*88*). In a subset of diabetics with painful peripheral neuropathy and inefficient CPM, lower temporal summation thresholds were found. Those with impaired CPM selectively responded to the selective norepinephrine serotonin reuptake inhibitor (SNRI), duloxetine. Pain improvement with duloxetine was directly related to the degree to which CPM efficiency improved. Those with lower temporal summation thresholds plus impaired CPM had elevated temporal summation thresholds with duloxetine (*43*). The selective influence of CPM on RIII reflexes and sural test pain has been carried out in a group of patients with traumatic peripheral nerve injury and pain. The phenotype of pain influenced the effect of CPM on sural pain and the RIII reflex. Those with dynamic allodynia had reduced test pain but not RIII reflex in response to CPM, while those with static mechanical allodynia had reduced test pain and inhibited RIII reflex responses with CPM. Hence, analgesia associated was largely related to supraspinal pathways (*91*). In nerve injured rats, DNIC increases with sensitization of the nerve injured fibers and/or sprouting of

nerve terminals (*92*). Therefore, the type of nerve injury and response to injury influences CPM efficiency. Neuropathic pain phenotype appears to predict CPM responses (*93–95*)

Certain CNS disorders also impair CPM. Individuals with a stroke that involves the retro-olivary portion of the brainstem medulla (Wallenberg stroke) lose CPM efficiency (*27*, *96*). Post stroke pain develops in 25% of individuals with a Wallenberg stroke. Pain thresholds on the contralateral side remain normal in those with pain but are abnormal in those without pain. The pain from a Wallenberg stroke is responsive to amitriptyline which may improve CPM efficiency similar to duloxetine (*97*, *98*). Central nervous system diseases which involve altered dopamine neurotransmission (Parkinson's disease and schizophrenia) do not impair CPM (*99*, *100*).

CPM and Chronic Idiopathic Pain Syndromes

CPM is frequently impaired in idiopathic pain disorders(fibromyalgia, chronic fatigue syndrome, chronic tension headaches, migraines, temporomandibular disorder, atypical trigeminal neuralgia and irritable bowel syndrome), which are known to be poorly responsive to morphine (*77–79*, *101–115*). Impaired CPM may account for morphine treatment related headaches in those with migraines and tension headaches, since morphine is known to impair CPM analgesia (*116*). Impaired CPM inefficiency in these disorders may not be irreversible but improve with medical management . Duloxetine and amitriptyline are effective treatments for fibromyalgia, tension and migraine headaches, since both medications improve CPM efficiency (*98*, *117–119*). Dr Yarnitsky and colleagues have correlated impaired CPM with pain relieved by duloxetine in individuals with diabetic neuropathic pain. CPM inefficiency is an important mechanism and a contributing factor to chronic pain in a subset of individuals. Interventional analgesic trials which are focused on chronic pain should include CPM testing and correlate baseline impairment and subsequent changes in CPM efficiency with analgesic responses (*120*).

Neuroanatomy of DNIC

DNIC in animals appears to be dependent on the spino-bulbo-spinal pathway, which involves convergence of diverse sensory neuron pathways through the caudal medulla. DNIC acts as a "whole body receptive field" nociceptive modulating pathway which explains the reason why heterotrophic stimuli are able to cause antinociception (*28*, *32*, *121–124*). Within the dorsal horn, NK1 afferent neurons activate DNIC through the ventrolateral spinal cord ascending pathway and the final efferent pathway converges on spinal cord wide dynamic range neurons via the dorsolateral funiculus (*125–129*). Wide dynamic range neurons in the deeper laminae of the dorsal horn are largely responsible for temporal summation and wind-up and are dampened by DNIC (*20*, *122*, *130–132*). Nociceptive signals derived from the test site traveling to the dorsal horn are potently inhibited by strong heterotropic stimulus (*123*, *133*, *134*). DNIC in

animal models appears to result in post-synaptic inhibition of wide dynamic neurons. Since glutamate induced neuron firing is selectively blocked, there is no inhibition of non-noxious sensory processing or proprioception (*135*). The efficiency of DNIC can also be visualized by using immunostaining of c-fos protein products of dorsal horn afferent neurons. C.-fos is an immediate-early gene that is upregulated by noxious stimulus. Activation of DNIC reduces neuron c-fos expression induced by standard test pain (*136*, *137*).

The ascending loop of the spino-bulbo-spinal DNIC loop involves the ventrolateral spinal cord while the descending loop involves the dorsal lateral funiculus (*27*, *96*, *121*, *127*, *138*, *139*). The ascending loop ascends through the lateral parabrachial area in the brainstem (*125*, *140*). Activation of the descending pathway is through convergence connections located in the caudal medulla subnucleus reticularis dorsalis (SRD) (*121*, *124*, *126*, *127*, *141–144*). Destruction of the nearby PAG, RVM and locus coeruleus does alter DNIC responses (*126*, *127*, *145–147*). Both the SRD and RVM contain opioid receptors. Blockade of mu receptors in the SRD leads to loss of DNIC while blockade of RVM and PAG mu receptors enhances DNIC (*148–151*). There is an indirect connection between the SRD, RVM and PAG which influences DNIC. DNIC conditioning stimulus decreases RVM "on" cells which are responsible for downward facilitation (*152*). Paradoxically, RVM and PAG mu receptors impair DNIC when activated (*29*, *153–159*). The adverse effects of morphine on DNIC are lost one week after destruction of the PAG and 3 weeks after destroying the RVM in animal models (*153*, *154*).

Cerebral Cortex and DNIC

The SRD receives corticofugal projections from motor cortex, somatosensory cortex and insular cortex. SRD efferents terminate not only in dorsal horn but also thalamic areas that influence sensorimotor cortical regions. Corticofugal projections augment or suppress SRD signaling, depending on the site (*160*). The anterior cingulate gyrus has a functional connection with the SRD. High-frequency tetanic stimulation of the anterior cingulate gyrus or micro-injection of N-methyl-D-aspartate into the anterior cingulate induces facilitation of spinal nociceptive which is blocked by destruction of the SRD (*161*). CPM in humans shifts EEG dipole activity mainly in the P300 (cingulate gyrus) area (*162–165*), suggesting that there is a close inverse interaction between anterior cingulate gyrus activity and CPM efficiency. When visceral pain is used as the test pain, CPM dampens anterior cingulate cortex activity measured by functional MRI (fMRI) (*79*). CPM reduces activity in the primary sensory cortex, anterior cingulate gyrus and amygdala when using the RIII reflex monitored by fMRI. Amitriptyline which increases CPM efficiency blocks activation of perigenual cingulate cortex and reduces pain in irritable bowel syndrome patients (*79*). By EEG based standardized local resolution brain electromagnetic tomography, CPM reduces somatosensory cortex, anterior cingulate gyrus and supplemental motor cortex activity, while increasing activity in the orbitofrontal cortex; activation of the orbitofrontal cortex dampens pain (*47*, *166*). CPM influence in anterior

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cingulate gyrus activity seen with fMRI, magnetoencephalography and EEG is lost in those with osteoarthritis and irritable bowel syndrome; both diseases are associated with impaired CPM (*79*, *167*). Short-term plastic (reversible) changes in anterior cingulate gyrus activity caused by CPM result in reduced emotional and affective responses to pain (*93*, *168*, *169*). Hence, several studies have determined that the prefrontal cortex and cingulate gyrus are important to CPM activity (*6*). CPM dampens the emotional component of pain processing through actions on the cingulate gyrus while reducing pain intensity through dampening wide dynamic range neurons within the dorsal horn.

Opioids, Receptors and CPM / DNIC

Morphine paradoxically dampens nociception through the RVM while impairing CPM while intracerebroventricular morphine reduces intractable pain (*170–173*). The mechanism is related to post-synaptic inhibition or presynaptic disfacilitation of "on cells" within the RVM (*174*). Naloxone reverses the inhibitory effects of morphine on “on” cells in the RVM (*175*, *176*). RVM descending inhibition blocks spinal C-fiber evoked responses (*177–179*).

Paradoxically, opioids are capable of causing pain and hyperalgesia (*180–183*). Increased opioid sensitivity and analgesic tolerance have been demonstrated using short-acting opioids in the experimental setting, in the postoperative setting, in those on methadone maintenance and in those on chronic opioids (*184–188*). Opioid-induced hyperalgesia and tolerance are related to an altered balance between several pronociceptive and antinociceptive processes (*189–195*). Impaired CPM by morphine may be one of the mechanisms

Two parallel downward modulating systems, one through the SRD, the other through the PAG/RVM, contain mu opioid receptors (*196–199*). There appears to be a counterbalance between the two inhibitory systems. Noxious stimulus activates inhibitory neurons in the SRD but activates "on" cells in the RVM (*152*, *200*). Intracerebroventricular morphine activates "off" and blocks “on” cells in the RVM, but blocks DNIC in a dose-dependent, naloxone-reversible fashion (*157*, *159*). Activation of mu receptors in the RVM decreases DNIC / CPM (*29*, *156*, *201–203*). Noxious stimulus increases serotonin, the main neurotransmitter for DNIC, located in the dorsal horn. Spinal cord serotonin release is blocked by morphine (*204*). Destroying or transectioning the dorsolateral funiculus and serotonin receptor blockers reduce DNIC in animals (*205–208*). Destroying the PAG and RVM prevents morphine blockade of DNIC (*116*, *153*, *157*, *159*, *203*). Animals made morphine tolerant lose DNIC and have a markedly increased expression of c-fos neuron staining in deeper laminae of the dorsal horn from test pain compared with animals who are morphine naïve (*209*).

Clinically, morphine reduces CPM in men (*29*). CPM impairment is found in those on low doses of morphine (less than 45 mg per day) to the same extent as high doses morphine (greater than 45 mg per day). CPM efficiency worsens with time on morphine (*29*).

Naloxone reverses the adverse effects of morphine on DNIC in animals and CPM efficiency in healthy humans (*153*, *154*, *202*). Paradoxically, when

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naloxone is given alone it impairs DNIC; the reason appears to be due to binding mu receptors in the SRD (*150*, *156*, *210*). Injection of naloxone into the RVM of animals does not alter DNIC (*150*, *151*). Selective destruction of the PAG reverses morphine related impairment DNIC (*153*, *210*). In humans, CPM is blocked by oral naltrexone only in the low catastrophizing individuals (who presumably have intact CPM and endogenous opioid systems) but not in high catastrophizers (*56*, *57*).

It may be that morphine impaired DNIC is a contributing factor to opioid-induced hyperalgesia and/or tolerance. However, there is no direct evidence for this at the present time. The opposing effects of morphine at the brainstem level (analgesia through the RVM and impaired CPM through the SRD) do illustrate the complexity of opioid responses and the precarious balance between opioid pronociception and antinociception. Not all opioids block CPM. Oxycodone does not impair CPM as measured in healthy volunteers (*211*). Unique opioid ligand-receptor interactions, mu receptor subtypes in the brainstem and selective downstream signaling may be important factors to opioid ligand-DNIC interactions.

Serotonin, Transporters and Receptors

There is an increase in CSF serotonin synthesis and levels in the dorsal horn following noxious stimulus which is necessary for DNIC (*204*, *212–214*). Dorsal horn injections of D-lysergic acid diethylamide and the serotonin antagonists cinanserin and methylsergide reduce inhibitory neurotransmission from the brainstem (*215–220*). Treatment with the serotonin inhibitor p-chlorophenylalanine dramatically impairs DNIC (*204–206*). Intraperitoneal injection of the serotonin precursor 5-HTP strongly potentiates DNIC which is blocked by naloxone (*214*).

Duloxetine, a norepinephrine-serotonin reuptake inhibitor (SNRI) which improves pain associated with fibromyalgia, augments descending inhibition by enhancing both extracellular serotonin and norepinephrine in the brainstem and spinal cord (*221*, *222*). SNRIs and SSRIs are effective in reducing pain associated with fibromyalgia (*223–227*). Fibromyalgia is associated with reduced CPM analgesia, decreased plasma and CSF tryptophan (a precursor to serotonin), serotonin, 5 hydroxyindole acetic acid and altered serotonin transporter expression (*77*, *103*, *228–232*). In the same way, diabetics with painful neuropathy and impaired CPM responde to duloxetine, independent of the effect of duloxetine on mood. The greater the improvement in CPM analgesia with duloxetine, the greater improvement in pain. Those with enhanced temporal summation and reduced CPM had a significant reduction in temporal summation with duloxetine (*43*).

CPM inefficiency is associated with polymorphisms of the promoter site of the serotonin transporter gene. Tandem repeats (*44*) in the promoter site influence gene expression through increased transcription. Reduced number of tandem repeats is associated with reduced transporter expression. In two studies, lower serotonin transporter expression was associated with reduced CPM efficiency

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in normal individuals. CPM efficiency was associated with long allele carriers (*233*, *234*).This may be paradoxically due to activation of sertonin autoreceptors presynaptically leading to downregulation of important sertonin receptors in the CNS necessary for DNIC (*235–241*).

Adrenoceptors, Gamma Aminobutyric Acid (GABA) and N-Methyl-D-Aspartate (NMDA) Receptors

There is conflicting evidence regarding the interactions between alpha adrenoceptor agonists and DNIC. An alpha-2 adrenoceptor blocker, yhomibine, is reported to diminish DNIC (*242*). However, dexmedetomidine, also an alpha-2 adrenoceptor agonist and phenylephrine, an alpha-1 adrenoceptor agonist, blunt DNIC in animals and CPM in humans. This is reversed in animals by a selective alpha-2 adrenoceptor antagonist, atipamezole and phentolamine mesylate, respectively (*243*, *244*). Phenylephrine injected into the RVM blocks DNIC in rats, suggesting that alpha adrenergic agonist blocking activity on DNIC is supraspinal rather than spinal (*245*)

GABAergic medications such as lorazepam do not alter CPM in healthy individuals (*246*). Dopamine does not play a role in CPM (*15*, *99*, *100*, *233*).

Ketamine, a non-competitive antagonist to NMDA receptors, increases temporal summation thresholds (*247*, *248*). Intrathecal NMDA increases firing of convergence neurons in superficial and deep dorsal horn lamina. Heterotropic conditioning stimulus inhibits NMDA-induced firing of convergence neurons (*249*). Ketamine in healthy individuals completely blocks CPM and causes pain facilitation with conditioning stimulus (*250*). Excitatory amino acids do not appear to reduce CPM efficiency but NMDA receptors blockade does impair CPM.

Clinical Implications

There are weak and mostly indirect associations between CPM responses and acute and chronic pain severity. The strongest evidence is found with fibromyalgia and related idiopathic pain disorders. The plasticity of CPM begs the question of whether it is the cause or effect of pain. Correlations of duloxetine pain control with impaired CPM in diabetic neuropathic pain suggests that at least in a subset of individuals with chronic pain CPM is clinically important and should be targeted by medications that are known to improve CPM efficiency in those individuals with impaired CPM. Clinically, one might elect to use duloxetine as the initial analgesic in someone with diabetic neuropathy, pain and impaired CPM, which would avoid opioids (*251*, *252*). The same may be true for other chronic pain syndromes.

Clinically, CPM is impaired by morphine. It is tempting to attribute morphine related hyperalgesia and/or analgesic tolerance to impaired CPM. However, there are several other mechanisms such as increased expression of cholecystokinin in the RVM. Increased release of dynorphin in the spinal cord and up-regulation of NMDA receptors are thought to be causes of opioid-induced hyperalgesia

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(*253–256*). Rotation to oxycodone could potentially reestablish CPM analgesia and reverse morphine hyperalgesia or analgesic tolerance as one rationale for selecting opioids in rotation (*211*). By speculation, it may be that those individuals with impaired CPM who have poor pain control with morphine, would benefit from oxycodone in rotation.

In animal models, low doses of antidepressants which facilitate serotonin neurotransmission have significant analgesic activity when combined with sub-analgesic doses of morphine (*257*). Amitriptyline and venlafaxine are noted to increase morphine analgesia and reduce morphine related analgesic tolerance in rats (*258*). The combination of duloxetine plus morphine synergistically reduces mechanical allodynia in animals with experimentally induced neuropathic pain (*259*). The mechanisms behind the benefits of this combination are unknown. DNIC as a phenotype or CPM as a predictor should be explored in the choices of combination analgesics. SSRIs and SNRIs potentially prevents morphine induced impaired DNIC/CPM and thus improve analgesia.

In a randomized trial, duloxetine reduced postoperative morphine requirements in those individuals undergoing knee replacement (*260*). Impaired CPM has been associated with chronic postoperative surgical pain (*15*). Perioperative venlafaxine reduces postmastectomy acute pain and prevents chronic postmastectomy pain (*261*). The gabapentinoids reduce postoperative acute pain but do not prevent the development of chronic surgical pain. The reason why venlafaxine reduces chronic surgical pain while gabapentin does not is not known, but it is possible that venlafaxine improves CPM which, in turn, prevents chronic pain in those individuals with preoperative impaired CPM. The gabapentinoids do not improve CPM in those with chronic pain and impaired CPM (*262*). SNRIs may improve gabapentinoid analgesia through improvement in DNIC

A subset of individuals with chemotherapy-related peripheral neuropathy has impaired CPM (*88*). Duloxetine is an effective treatment for this group of individuals (*263*, *264*). It would be of interest to know if this subset has impaired CPM and enhanced temporal summation.

In case series, ketamine is reported to reduce neuropathic pain (*265*). The difficulty with case series and single arm trials are placebo responses which can occur in greater than 50% of individuals with chronic pain (*266*). There are unpredictable differences between single arm analgesic studies, case reports, cohort series and randomized trials. The effect size is usually greater in uncontrolled studies then placebo controlled randomized trials (*267*). Ketamine has recently been found to be ineffective in improving morphine related analgesia in advance cancer patients (*268*). One reason for this lack of ability to improve morphine analgesia may be due to ketamine impairment of CPM and, presumably, DNIC

Conclusion

Much of the discussion has been built on indirect associations and assumptions. However, CPM is plastic, measurable, "targeted" by certain drugs

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like duloxetine and can be imaged by EEG and functional MRI. Therefore, CPM can be phenotyped for adaptive design trials of targeted analgesics (*43*).

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Chapter 4

Treatment of Pain and Opioid Abuse

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Treatment of substance abuse is challenging. One such challenge of increasing concern is the condition of co-existing chronic pain and opioid dependence. Abuse of prescription opioids sometimes develops after legitimate dispensing or prescribing of narcotics for the treatment of pain. This suggests a need for further understanding of how these conditions are related. The objectives of this chapter are to 1) provide a brief epidemiological review of medical and nonmedical use of prescription narcotics and 2) describe how opioid abuse has impacted chronic pain management. The benefits and challenges of opioid agonist treatment (methadone and buprenorphine) for opioid dependence are reviewed here. In addition, the utility of methadone and buprenorphine in pain management is explored with a summary of new developments that may aid in deterring the abuse of prescription opioids.

Introduction

The use of opiates dates back centuries when the cultivation of opium occurred in the third millennium B.C. Observation of its useful analgesic properties was noted almost immediately, but it wasn't until 1850 that the active ingredient of opium, morphine, was isolated and developed as a treatment for acute and chronic pain. The addictive properties of morphine also soon became evident in years to follow. Ironically, in efforts to find a more potent and abuse-free opioid, heroin was synthesized in 1898. This was the first of several claims of a novel opioid with less abuse potential (*1*). Over a century later, the search for synthetic opioid compounds led to the development of meperidine, followed shortly thereafter by methadone (*2*). Although its abuse liability is

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relatively high, methadone is commonly used today for the treatment of chronic pain as well as opioid dependence. Several other synthetic opioid formulations, such as fentanyl, oxycodone, and hydromorphone, were developed and essential in medical practice by the 1990's. Primary care physicians began taking on the responsibility of treating pain conditions instead of consulting pain specialists. As a result there was an upsurge in the number of high-potency prescriptions in large doses for treatment of non-cancer pain. In addition, internet access to prescription drugs, and unethical and illegal prescribing practices became contributing variables to the subsequent rise of prescription opioid dependence and overdose deaths (*3*). In an effort to combat this problem, a great deal of energy and investment has been spent in the development of safer, more efficacious, non-addicting opioids (*1*).

Although opioid medications are an accepted element of chronic pain management, the relationship between pain and prescription opioid abuse is poorly understood (*4*). This chapter reviews common treatments for opioid dependence, with an emphasis on the prescription opioids methadone, buprenorphine and buprenorphine/naloxone. Opioid abuse within the context of pain management will be discussed here as well with a summary of new developments that may aid in deterring the ongoing abuse of prescription opioids.

Epidemiology

Nonmedical Use of Prescription Pain Relievers

The National Survey on Drug Use and Health (NSDUH) defines misuse as "the use of prescription drugs that were not prescribed for the respondent or use of these drugs only for the experience or feeling they cause." The 2011 survey revealed that rates for initiation of nonmedical pain reliever use were ranked second only to initiation rates of marijuana use. Since 2002, there have been reports of nearly 2 million or more new users of nonmedical pain relievers each year. Of these new users, over 500,000 initiate use without ever having used another illicit drug. The new and continuing users of prescription pain relievers have contributed to substantial increases in problems associated with use, such as increased rates of emergency room visits and admission to substance abuse treatment programs (*5*). The number of emergency department visits involving nonmedical use of narcotic pain relievers increased from 145,000 in 2004 to 306,000 in 2008 (*5*). Although the number of people receiving specialty treatment for drug dependence overall was essentially unchanged between 2002 and 2011, the number of persons receiving specialty substance use treatment within the past year for misuse of pain relievers more than doubled (from 360,000 to 726,000) in this same time period. The 2011 Center for Behavioral Health Statistics and Quality report revealed similar trends in admissions to publicly funded substance abuse treatment programs for primary non-heroin opioid problems (*5*).

In addition, consequences of prescription opioid use in the adolescent and young adult population have raised a significant degree of concern. The rate of prescription opioid dependence for persons aged 12 or older increased steadily between 2002 and 2011 (from 0.4 to 0.7 percent of the population) (*5*). The

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Centers for Disease Control and Prevention (CDC) reported that the poisoning death rate among teens aged 15–19 years nearly doubled from 2000 to 2009, from 1.7 to 3.3 per 100,000, in part because of an increase in prescription opioid overdoses (*6*). The increase in death rate due to prescription opioid use amongst all ages is astounding. Unfortunately, it is occurring despite numerous warnings and recommendations over the past decade for voluntary education of providers about more cautious use of opioid pain relievers (*7*).

A high dose of opioids is not clearly defined, however data suggest a 3-fold increase in the risk of opioid-related mortality is associated with 200 mg or more of morphine (or equivalent) and a significant but attenuated increase with intermediate doses of opioids (50-99 mg morphine equivalents) (*8*). Prescription opioids now account for more overdose deaths than heroin and cocaine combined (*9*). In fact, the number of deaths secondary to prescription drug overdose now exceeds the number of motor vehicle accident deaths in the United States, which was previously the leading cause of injury death. A prominent contributor to these prescription drug-related deaths and ED visits is the use of opioid pain relievers.

Prescription Opioid Abuse in Chronic, Non-Malignant Pain

The morbidity and mortality associated with prescription drug abuse clearly has become an important public health concern. Part of the reason for the increased use of prescription pain relievers is that in the last few decades, the treatment of chronic pain has expanded in the primary care setting (*10*). Population-based studies reveal that more than 75 million Americans (about 25% of the entire population) have chronic or recurrent pain. Of these, 40% report the pain as having moderate to severe impact on their lives. Chronic pain is a frequent cause of disability with an estimated cost greater than $61 billion annually (*11*). We can expect that the prevalence of chronic pain conditions will only increase with the advancing age of our population (*12*).

While therapy for cancer pain has improved dramatically over the past decade, treatment of nonmalignant chronic pain remains a challenge for many practitioners and patients (*13*). Narcotic use for non-malignant chronic pain conditions commonly treated in primary care is controversial and less well studied (*14*). Therefore, difficulties of inadequate pain relief persist despite an array of medications available to treat non-malignant pain. In fact, recent evidence suggests that opioids are responsible for another problem that may limit their usefulness: opioid-induced hyperalgesia, which is a paradoxical response that occurs when a patient receiving opioid treatment for pain may actually become more sensitive to certain painful stimuli (*15–18*). No currently approved drugs are available to treat this hypersensitivity, and it remains an area of much-needed research.

The relationship between pain and prescription opioid abuse is poorly understood. Determining whether a patient is seeking additional opioid medications in order to alleviate pain or to abuse the drugs can be difficult (*4*). Many primary care providers have had little specific training in pain medicine and addiction, and are unsure about how to safely prescribe opioids (*19*). Primary care providers' fear of contributing to opiate addiction is a frequently mentioned barrier

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to narcotic use in the management of chronic pain. Therefore, prescriptions for opioids are often withheld by physicians for fear of diversion, neuropsychological impairment, development of drug addiction, and overdose. On the contrary, physicians may also feel helpless in treating the most distressed patient with pain problems. So rather than carefully selecting patients with a low-risk for opioid addiction, physicians form a pattern of prescribing patients with the highest risk for poor outcomes high doses of opioids. This is a phenomenon referred to as "adverse selection" (*20*). Many specialty pain clinics have reported rates of prescription narcotic abuse in chronic pain patients ranging from 3% to 20% or higher (*21*). Reid and colleagues (2002) concluded, "A significant minority (24% from VA and 31% from primary care clinic) of patients had documentation of prescription opioid abuse behavior." Predictors of prescription drug abuse behavior were a lifetime history of a substance use disorder and younger age. In addition, the high prevalence of psychiatric comorbidity in those who misuse or abuse prescription drugs contributes to the complexity of treating chronic pain with opioids (*10*).

It is not surprising that chronic pain patients with histories of substance abuse are at higher risk for prescription drug abuse behaviors. Inadequate treatment of pain may be responsible for the higher rates of these behaviors, but an important issue that is not adequately addressed is whether pain patients with substance abuse histories gain any pain relief or functional improvement from narcotics. Given the complex relationships we know exist between psychiatric disorders and chronic pain syndromes, understanding how prescription drug abuse is related would be instructive.

The presence of multiple aberrant behaviors, such as using more than the prescribed amount of opioids, frequent requests for early refills, seeking prescriptions from multiple providers, or the recurrence of any of these behaviors may suggest the need for consultation with pain management physicians or addiction specialists. Clinicians should also consider temporary or permanent tapering of opioid doses, and possibly discontinuation if more serious behaviors are evident (i.e. diversion or intravenous use of oral formulations). Psychiatric referrals or psychological support with individual counseling (i.e. cognitive behavioral therapy) may be helpful for some individuals, which highlights the need to screen for depression, anxiety and other psychiatric disorders at the beginning of chronic opioid treatment (*4*). For those patients identified with an opioid addiction, structured opioid agonist therapy with buprenorphine or methadone at a licensed program may be beneficial to help treat pain and addiction.

Methadone

Methadone is a synthetic opioid and is one of the most effective therapies for opioid dependence; it is also a useful treatment for chronic pain. Oral methadone is available as a solid tablet, a rapidly dissolving wafer (diskette), and a premixed liquid, all of which are essentially bioequivalent. Each of the formulations is 80

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to 95 percent bioavailable (compared with only 30 percent for oral morphine) and readily absorbed (*22*).

Methadone's pharmacological effects are primarily mediated by the activation of μ opioid receptors centrally and in the periphery. This activity produces the effects common to all μ opioid agonists: analgesia, euphoria, constipation, sedation, respiratory depression, nausea, and miosis. Cytochrome P450 (CYP) enzymes metabolize methadone, as well as buprenorphine, primarily through the CYP3A4 pathway (*22*).

Methadone has a number of unique pharmacologic properties. It has a slow onset and long duration of action. Peak plasma levels of methadone occur 2-6 hours after administration. The half-life ranges from 13-50 hours and achievement of steady-state plasma levels may take 5-10 days, making it an appropriate choice for opioid therapy of pain and addiction. Methadone is also an antagonist of the glutamate receptor N-methyl-D-aspartate (NMDA) and inhibitor of serotonin/norepinephrine reuptake, which may increase its effectiveness in the treatment of neuropathic pain compared with other opioids (*19*, *23*).

Methadone in Treatment of Opioid Dependence

Methadone has been a mainstay of opioid addiction treatment for detoxification or as a maintenance medication in the United States. Maintenance treatment with methadone for addiction may only be provided in specially licensed clinics that are allowed to treat only a limited number of patients. According to Cunningham and colleagues (2007), it was estimated that treatment slots for methadone maintenance were available to only 20% of Americans with opioid addiction (*24*). Unfortunately, methadone is not available in some U.S. states (*25*) and communities often resist allowing methadone clinics to open or expand. In addition, the regulatory complexity limits expansion of this treatment model (*26*).

Methadone Challenges in Opioid Abusing Populations

Methadone is a useful therapy that is effective for treatment of opioid addiction, but it can also be a dangerous street drug if used improperly. While prescription pain relievers, such as oxycodone and hydrocodone, have contributed to an increase in hospitalizations due to drug poisoning and in overdose deaths in the last decade, methadone has also received much of the spotlight (*27*). Of the total number of opioid prescriptions dispensed by pharmacies in 2011, methadone represented only 5%. However, one-third of opioid related deaths nationwide implicated methadone (*28*). The term "methadone-associated mortality" broadly encompasses fatalities in which methadone was detected during postmortem analysis or was otherwise implicated in a death. Defining methadone's role in such deaths is an unsettled area, complicated by inconsistencies in methods of determining and reporting causes of death, the presence of other central nervous system (CNS) drugs, and the absence of information about the decedent's pre-mortem physical or mental condition and level of opioid tolerance (*29*).

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Several risk factors for methadone-related mortality have been identified. First is the "poison cocktail." This is the concomitant use of multiple psychotropic drugs such as benzodiazepines, other opioids, and/or alcohol (*30*, *31*). When used alone, many of these substances are relatively moderate respiratory depressants; however, when combined with methadone, their additive or synergistic effects can be lethal (*32*). In methadone clinics, take-home medication is provided to stable patients. This may contribute to the diversion of methadone and its nonmedical use. If the patient does not ingest take-home doses as directed, respiration can be affected.

The cardiopulmonary effect of methadone is another factor that contributes to the dangers of methadone use. The peak respiratory depressant effects of methadone typically occur later and persist longer than its other peak effects, particularly during the initial dosing period. This phenomenon occurs because methadone's elimination half-life is longer than its duration of action (4 to 8 hours). The half-life of methadone ranges from 15-60 hours, which means that it could take up to 12 days to reach a steady state level. Opioid-tolerant individuals transitioning from high doses of other opioids to methadone should not exceed 30 to 40 mg per day (*4*). As with most other opioids, the primary toxic effect of excessive methadone is respiratory depression and hypoxia, sometimes accompanied by pulmonary edema and/or aspiration pneumonia (*33*, *34*). In some patients, there is an elevated risk for QT interval prolongation, which can lead to the potentially fatal heart rhythm known as Torsades de Pointes. In fact, there has been an increase in methadone-associated deaths that may be related to cardiac arrhythmias (*35*, *36*).

Methadone in Treatment of Chronic Pain

In 1976, restrictions for prescribing methadone were lifted and physicians with appropriate Drug Enforcement Agency registration were allowed to prescribe methadone for analgesia. No relationship has been established between methadone plasma concentration and analgesic effect. Therefore, methadone doses for treatment of chronic pain should be titrated to clinical effect rather than a drug level (*4*). Treatment of chronic pain with methadone is effective, but pharmacologic and pharmacokinetic properties of methadone present special challenges of which providers should be aware.

Methadone Challenges in Pain Populations

Methadone is increasingly prescribed as an analgesic because it is a generic, inexpensive drug that can provide long-lasting pain relief. However, in recent years, problems associated with methadone use have been increasingly reported, which may be due in part to the fact that prescriptions for methadone have increased. The amount of all formulations of methadone (liquid, tablet, or dispersible tablet) distributed or delivered by manufacturers rose dramatically from 2000 to early 2007, with increases ranging from 9 to 22 percent annually. Tablets distributed with a prescription through pharmacies had the largest

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increase of all formulations of methadone, suggesting the indication of increased use of methadone was for treatment of chronic pain (*37*). In fact, the number of methadone tablet prescriptions dispensed increased by nearly 700 percent between 1998 and 2006 (*38*). The Research Abuse, Diversion and Addiction-Related Surveillance System (RADARS System) retrieves valuable prescribing information from sources such as the poison control centers, opioid treatment programs and law enforcement. According to this system, the poison control center reported that the highest fatal poisoning rate for all people filling prescriptions was amongst those who were prescribed methadone. Of these reported cases, 25% of the deaths were secondary to the tablet form of methadone (*39*). Since substance abuse programs typically provide methadone by liquid suspension, and not by tablet form, this suggests that methadone was not dispensed from an opioid treatment program. Instead, it may have been prescribed through a specialty clinic for management of chronic pain (*40*).

Prescriptions for methadone should be handled with care and expertise because unfortunately, methadone can cause fatalities among individuals who have not developed any tolerance to opiates. Poor clinical practice during the start of methadone treatment (induction dosing phase) is a commonly encountered problem. The largest proportion of methadone-associated deaths has occurred during the induction phase, usually when treatment personnel overestimate a patient's degree of tolerance for opioids (*39*). Also, the pharmacokinetic and pharmacodynamics properties of methadone discussed earlier pose a challenge to the most experienced physician. Tissue accumulation of methadone can occur resulting in a variable time period for methadone to reach full potency. Despite increasing awareness of this problem in the medical community, inadequate or erroneous induction dosing and monitoring by physicians continues to warrant concern, primarily when prescribing methadone for pain.

Buprenorphine and Buprenorphine/Naloxone

An injectable formulation of buprenorphine (Buprenex®) was approved for analgesic use in the United States in 1985 as a Schedule V medication and was rescheduled in 2002 to a Schedule III after sublingual formulations of buprenorphine (Subutex®) and buprenorphine/naloxone (Suboxone®) were approved for office-based treatment of opioid dependence (*41*). In 2010, an extended-release transdermal formulation of buprenorphine (Butrans®) was approved by the FDA for the management of moderate to severe chronic pain. Buprenorphine is a synthetic opioid that is being increasingly used for substitution therapy of opioid dependence, as well as chronic pain. Buprenorphine's mechanism of action, like morphine and methadone, is mediated by the activation of opioid receptors, principally of the μ subtype (Table 1). Buprenorphine acts as a partial agonist at μ-opioid receptors and as an antagonist at k-opioid receptors. This unique pharmacology makes buprenorphine useful as an analgesic while also theoretically providing some abuse deterrence. Buprenorphine has certain features that make its use possible in physician office settings, which may

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alter current strategies for maintenance and detoxification for opioid-addicted individuals.

Table 1. Comparison between Methadone and Buprenorphine or Buprenorphine/Naloxone

	Methadone	*Buprenorphine/ Buprenorphine+Naloxone*
Pharmacology at μ receptor	Full agonist	Partial agonist
Formulation	Oral suspension or tablet	Sublingual (SL) tablet available for buprenorphine only. Buprenorphine+ naloxone available only as SL film
Location of treatment	Licensed opioid treatment program	Physician's office or opioid treatment program with a special registration code issued by the DEA
Half life	24-36 hours	36-48 hours
Abuse potential	High	Lower than methadone
Effectiveness	More effective for severe opioid dependence	Effective for mild to moderate opioid dependence
Withdrawal syndrome	Mild	Moderate to severe
Cost	Inexpensive	Moderately expensive
Use in pregnancy	Category C: Standard of care	Category C: Buprenorphine +naloxone not recommended, rather switch to buprenorphine alone or methadone
Protective factors	None	Ceiling effect limits overdose risk

Buprenorphine and Buprenorphine/Naloxone in Treatment of Opioid Dependence

In the United States, the buprenorphine/naloxone combination (Suboxone®) film is prescribed preferentially over buprenorphine alone (Subutex®) tablet for the treatment of opioid dependence. However, if the patient is an opioid dependent female who is pregnant or planning to conceive, Subutex is preferred over Suboxone, given the potential risks of naloxone to the fetus. As Schedule III

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substances, Subutex and Suboxone are considered to have a potential for abuse less than substances such as methadone and oxycodone, which are Schedule II. The buprenorphine/naloxone combination is available as a sublingual film containing buprenorphine and naloxone in a 4:1 dose ratio, respectively, and is available in four doses, 2:0.5 mg, 4:1 mg, 8:2 mg, and 12:3 mg.

There are several benefits to using buprenorphine to treat opioid dependence. In contrast to methadone, buprenorphine may be prescribed in a physician's office, and medication ingestion can occur outside of a clinical setting, which is an important consideration for patients who are still working (Table 1). When treatment is initiated in a physician's office, the co-existing physical and mental health issues that so often accompany opioid dependence can also be addressed.

The exact mechanism by which buprenorphine blocks the effect of exogenously administered opioids is unknown, but it could do so via cross-tolerance, its partial agonist effects, or a combination of the two. Because of its low intrinsic activity and high affinity, buprenorphine may precipitate opioid withdrawal, thereby reducing its abuse liability in individuals who are physically dependent on short-acting opioids (*42–44*). Also, because buprenorphine is a partial agonist, it produces less-than-maximal effects for many endpoints, including respiratory depression. Thus, buprenorphine has low toxicity, even at high doses, which provides a greater margin of safety and decreases the danger of overdose in comparison to full agonists, such as methadone (Table 1) (*45*). Finally, buprenorphine's slow dissociation from μ-opioid receptors results not only in a long duration of action, but also diminishes symptoms and signs of withdrawal upon cessation, permitting accelerated tapering schedules. A recent study compared the effect of a 7-day buprenorphine taper with a 28-day buprenorphine taper on treatment outcome of prescription opioid dependent patients maintained on buprenorphine. Surprisingly, the longer taper was not associated with better outcomes and the shorter 7-day taper did not result in greater withdrawal symptoms or cravings. In fact, the 7-day taper group reported fewer concomitant medications (i.e. clonidine, benzodiazepines) used to address withdrawal symptoms than the 28-day taper group (*46*).

Buprenorphine may be preferred over methadone for certain individuals with particular medical concerns. For example, buprenorphine causes less QT prolongation than methadone, which is especially preferable for patients with a cardiac history (*47*). Also, Rapeli and colleagues (2007) reported that buprenorphine-naloxone treatment is preferable to methadone treatment for preserving cognitive function in early treatment—an important benefit for prescription opioid dependent individuals who are employed (*26*, *48*).

Buprenorphine is clinically effective and well accepted by patients. The office-based treatment provides an increased availability of medication-assisted treatment for opioid addiction and creates earlier access for patients who have more recently started abusing opioids. The NIDA Clinical Trials Network Field Experience reported high completion rates of short-term opioid detoxification with buprenorphine treatment. Although minimal problems of diversion or adverse clinical events with buprenorphine treatment have been reported in the U.S., data from other countries suggest that problems associated with buprenorphine use do exist (*49*).

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Buprenorphine abuse is more common in European and Asian nations in comparison to the U.S. (*50*), in part because it has been available in these countries for a longer period of time. Buprenorphine abuse by injection was first recorded in the mid-1980s and remains the most commonly reported route of administration for misuse of the medication. Initially, it was thought that because of its mixed agonist-antagonist properties, the abuse potential of buprenorphine would be less than that of full opioid agonists (*51*). However, both epidemiological data and data collected in laboratory settings have shown that buprenorphine does have significant abuse potential in certain situations (*52–56*).

A laboratory study conducted in buprenorphine-maintained individuals demonstrated that the abuse liability of intravenously administered buprenorphine alone was comparable to that of heroin, but the abuse liability of buprenorphine/naloxone was lower than that of buprenorphine alone and heroin (*57*). In non-opioid-dependent individuals, intravenous buprenorphine/naloxone produced significantly lower ratings of drug liking and good effects compared to buprenorphine alone, although drug self-administration was comparable for the two formulations (*52*). Among intranasal drug abusers, positive subjective ratings and street value of intranasal buprenorphine were higher than buprenorphine/naloxone, but these differences were not statistically significant (*58*). However, the data did suggest that the bioavailability of intranasal naloxone may be sufficient to precipitate withdrawal in opioid-dependent individuals, which would support an overall lower abuse liability profile of the combination product among opioid abusers (*58*).

Furthermore, Alho and colleagues (2007) conducted a study with surveys distributed to attendees of a needle exchange program in Finland (*59*). Survey items inquired about experience with IV buprenorphine alone and with the combination buprenorphine/naloxone product. Sixty-eight percent of those who returned the survey had tried IV buprenorphine/naloxone and 66% of those who tried it, took it again or even regularly. This suggests that combining naloxone with buprenorphine does not necessarily block all the opioid agonist effects when used IV, which is consistent with the laboratory data described above (*55*, *57*, *58*). However, 80% reported that they had had a "bad" experience with the combination product, while less than 20% reported it "similar" to experiences with IV buprenorphine. These participants were willing to pay a significantly higher price for buprenorphine than for the combination product. The study published by Comer and colleagues in 2010 suggested that in buprenorphine-dependent individuals, the naloxone component did not precipitate withdrawal symptoms but instead blunted the euphoric effects of buprenorphine. When participants were maintained on the higher 8 mg and 24 mg sublingual buprenorphine doses, self-administration of IV buprenorphine/naloxone was lower than when participants were maintained on 2 mg sublingual buprenorphine. This study provides important data suggesting that the abuse potential of buprenorphine/naloxone is subject to each individual's recent opioid exposure and that a higher buprenorphine maintenance dose is an important factor in curtailing misuse (*57*).

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Buprenorphine has been reported to have an analgesic potency 25 to 50 times greater than morphine (*60*). Although sublingual buprenorphine is not available in North America as an approved medication for treatment of chronic pain, the Drug Enforcement Administration (DEA) has acknowledged the legality of an off-label use to treat pain with up to a dosage of 2 mg buprenorphine or buprenorphine/naloxone sublingual preparation (*61*). Different mechanisms have been suggested for opioid-induced analgesia and antihyperalgesia, with some studies suggesting that pure μ-opioid receptor agonists may contribute to the induction of hyperalgesia. Thus, in a patient being treated with such an agent, increasing pain intensity could be caused by either the development of tolerance or opioid-induced hyperalgesia, posing a diagnostic dilemma. By contrast, buprenorphine has a pronounced antihyperalgesic effect, which may be because of its κ-receptor antagonism (*62*).

Although SL buprenorphine is not approved for the treatment of pain, some evidence suggests that it can be of benefit to patients who suffer from chronic pain with or without opioid abuse. Daitch and colleagues (2012) measured the level of analgesia for chronic pain patients who converted from opioid agonist drugs to SL buprenorphine for 2 months (*18*). Once patients were converted to buprenorphine and established on an effective dose, there was no escalation in use or dose of the medication and patients rarely ran out of medications early. Patients generally felt less sedated and noted improved cognition compared to their prior regimen (*63*). This benefit, in addition to a long half-life and excellent safety profile, is of clinical relevance to many chronic pain patients.

Many patients suffering from pain, malignant and non-malignant, are older and at increased risk of adverse events when treated with narcotic analgesics. Opioid use in the elderly population should therefore include a good safety and tolerability profile. For instance, the half-life of active drug and metabolites is increased by all opioids, except buprenorphine, in elderly patients with renal dysfunction. This does not mean that all other opioids are contraindicated for use in the elderly, however more intense monitoring of drug dosing is required. Age is also related to a decline in the immune system, which increases risk of infection. In addition, men on methadone maintenance have been shown to have a higher prevalence of erectile dysfunction in relation to hypogonadism and depression in comparison to buprenorphine maintenance (*64*). Though the effects of opioids in the elderly population is not fully understood, it is important to provide adequate analgesia, while sparing the patient of further complications.

New Developments for Deterring Abuse of Opioid Drugs

The Food and Drug Administration (FDA), DEA, Office of the National Drug Control Policy, National Institute on Drug Abuse (NIDA), Substance Abuse Mental Health Services Administration (SAMSHA), and other organizations have made serious efforts to address the prescription opioid abuse crisis. The National All Schedules Prescription Electronic Reporting Act (NASPER) is a law signed by Congress in 2005 aimed at preventing doctor shopping, or the act of seeking

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multiple prescriptions of controlled substances by various providers. The FDA now requires opioid makers to propose a Risk Evaluation and Mitigation Strategy (REMS) for individual extended and long-acting opioid medications. These REMS use new safety measures to reduce the risks and improve the safety of opioids while continuing to provide access to these medications for pain patients by expanding state-based prescription drug monitoring programs. Prescription drug monitoring programs were developed to provide focus on education, monitoring, and proper disposal of prescription drugs. It is also important that public service organizations continue to educate parents and the public about warning signs of drug abuse by their children. Better education in medical schools of pain management and substance abuse is necessary to begin the process of formulating good practice guidelines to help physicians prescribe opioids safely and effectively. In addition, physicians who prescribe chronic opioid therapy for nonmalignant chronic pain are more vulnerable to investigation or disciplinary action when they fail to comply with legal regulations and standards of care.

In addition to government regulation, there is much attention placed on abuse-deterrent formulations (ADFs). ADFs are safe and effective opioid medications that are theoretically less likely to be abused. Several products with ADFs are under development and pending approval by the FDA. These products vary in the methods by which they deter abuse, yet are important steps toward prevention and detection of prescription opioid abuse. Listed below is a brief description of some of the approaches to creating ADFs. Also see Table 2 for product listing.

Alternate Method of Delivery

In addition to the combination tablet and film, another method for deterring the abuse of buprenorphine is to alter the route of delivery (i.e. implant or patch). Probuphine®, is a form of buprenorphine which utilizes sustained release technology over a 6 month period in the form of a hard-to-extract subdermal implant. Larger trials are required before this product can be utilized on a widespread basis. Transdermal buprenorphine patch (BuTrans®) is an FDA approved formulation and could be utilized during acute detoxification. Recent studies have shown that transdermal buprenorphine is safe, well-tolerated, and clinically effective for heroin detoxification, suggesting that a 7-day application of transdermal buprenorphine may be an effective mode of opioid detoxification (*65*, *66*).

Agonist-Antagonist Drug Combinations

Agonist-antagonist drug combinations were developed to limit the potential for crushing and dissolving an opioid product for intranasal or intravenous use. One such example is Embeda, a morphine sulfate and naltrexone combination capsule consisting of morphine pellets containing a sequestered core of naltrexone. When taken orally, the morphine provides analgesic relief while the naltrexone remains sequestered with no significant pharmacological effect. However, if the tablet is crushed, naltrexone is released and exerts its opioid antagonist effects, which may cause symptoms of withdrawal in opioid-dependent patients (*67*).

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Table 2. Abuse Deterrent Formulations

On the market	*Manufacturer*	*Technology*	*Methods not amenable to abuse*	*Mechanism to deter abuse*	*Approval status*
Morphine/ Naltrexone (Embeda) (*67*)	Pfizer, King Pharmaceuticals	Agonist-Antagonist Combination	Crushing, dissolving for IV or IN use	Morphine wrapped around a core of naltrexone. Taken orally, naltrexone is not available, but when tablet is tampered naltrexone may cause opioid withdrawal.	Approved 2009, but withdrawn by manufacturer
Oxymorphone (Opana) (*75*)	Endo Pharmaceuticals	INTAC Technology	Crushing	Preserves the ER characteristics of the medication while imparting crush-resistant properties.	Schedule II
OROS Hydromorphone (Exalgo) (*76*)	Mallinckrodt	Osmotic extended-Release Oral delivery System (OROS)	Crushing, extraction	Consists of an osmotically active bilayer core enclosed in a semipermeable tablet shell membrane, eliminated in feces. Allows hydromorphone release over a 24-hour period. Tamper deterrent for crushing and extraction for injection.	Approved 2010

Continued on next page.

Table 2. (Continued). Abuse Deterrent Formulations

On the market	*Manufacturer*	*Technology*	*Methods not amenable to abuse*	*Mechanism to deter abuse*	*Approval status*
Oxycodone Controlled Release (OxyContin) (*77*)	Purdue Pharma	N/A This product is not marketed as an abuse-deterrent drug.	Diminished ease of cutting, breaking, chewing, crushing, and dissolving	No studies indicate true deterrent qualities. Package insert states diminished ease of cutting, breaking, chewing, crushing, and dissolving this formulation.	Approved 2010
Oxycodone HCl, without niacin (Oxecta, formerly known as Acurox) (*68*)	Pfizer	Aversion Technology (Patent by Acura Pharm)	Crushing, dissolution	Formulation originally contained niacin that would cause flushing, itching, sweating, chills and headache 15 minutes after excess consumption and resolves 75- 90 minutes later. Removal of niacin was required for FDA (Oxecta). There is a higher incidence of nasopharyngeal and facial adverse events. Decreased ability to dissolve or use IN, but no evidence that there is a decreased abuse potential.	Niacin free formulation approved in 2011
Under Development	***Manufacturer***	***Technology***	***Methods not amenable to abuse***	***Mechanism to deter abuse***	***Approval status***
Oxycodone HCl/ Acetaminophen (Acuracet) (*78*)	Acura Pharmaceuticals, Inc.	Aversion Technology	Should reduce IN and IV use	Should reduce nasal snorting and IV injection.	Formation and Stability phase
Hydrocodone Bitartrate/ Acetaminophen (Vycavert) (*79*)	Acura Pharmaceuticals, Inc.	Aversion Technology	Should reduce IN and IV use	Goal is to reduce nasal snorting and injectable abuse	Formation and Stability phase

Under Development	*Manufacturer*	*Technology*	*Methods not amenable to abuse*	*Mechanism to deter abuse*	*Approval status*
Oxycodone/ Naltrexone (Oxytrex) (*80*)	Pain Therapeutics, Inc.	Oxytrex Science and Technology	Reduces the development of tolerance and dependence	Studies show reduced dependence on opioids with pain relief comparable to non-ADF	Phase 3 clinical trials complete
COL-003 (*81*)	Collegium Pharmaceutical	DETERx Technology	Chewing, crushing, dissolution, heat resistant	Small beads within a capsule, when tampered are resistant to crushing, dissolving, and heat exposure.	Phase 3 clinical trials proposed
NKTR-181 (*82*)	Nektar Therapeutics	Small-Molecule Delivery	N/A	Small molecular polymer conjugates allows for slow delivery to CNS, reducing sedation and respiratory depression	Accepted into FDA's fast-track development program
PF329 (*74*)	PharmacoFore, Inc.	Bio-Activated Molecular Delivery and Multi-Pill Abuse Resistance Technology	Crushing, chewing, IV	Prevents active drug from being released until it has been exposed to the intestine where opioid molecules are cleaved off by the enzyme trypsin. Not susceptible to extraction, crushing, chewing, and injecting.	Phase I proof-of-concept
Extended-Release Oxycodone (Remoxy) (*83*)	Durect Corp	Oradur Technology	Breaking, chewing, IN, and thermal extraction for IV use	ER formulation in a high viscosity, hard-gelatin (water insoluble) matrix capsule. Deters breaking, chewing, snorting, and thermal extraction for injection.	Declined 2011

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Aversive Agents

Another strategy for reducing the abuse of opioid medications approved for treating pain has been to add an aversive component to the medication. For example, a product containing oxycodone and niacin (Acurox) was under development by King Pharmaceuticals (*68*). It was hoped that excessive use of this medication would be prevented because at supratherapeutic doses, the niacin in the product would cause an unpleasant reaction (flushing, itching, chills, headache). However, the FDA did not approve this medication because of the increased risks of adverse events among pain patients without opioid abuse problems.

Prodrugs

Yet another approach has been to develop medications that must be converted from a non-opioid parent compound into an active opioid metabolite. An example of this type of medication is tramadol. The parent form of this medication blocks the reuptake of serotonin and norepinephrine, while its metabolite, (+)*o*-desmethyltramadol, acts at mu opioid receptors. Even though its active metabolite is an opioid compound, the DEA has not scheduled tramadol as a controlled substance because its abuse liability is quite low (*69*–*72*). The delay needed to convert the medication to an active opioid is believed to be at least partly responsible for its low abuse liability. Most drug users prefer a rapid and intense high that tramadol does not provide.

Another technology that also capitalizes on the lower abuse liability of compounds that have a delayed onset of effects prevents abuse at the molecular level. When a compound using the Bio-Activated Molecular Delivery and Multi-Pill Abuse Resistance Technology (Bio-MD) enters the small intestine, an amino acid is cleaved off by the digestive enzyme trypsin. The formulation is inactive in the blood if it has not passed through the small intestine first and prevents the medication from being converted into the active drug if it enters the systemic circulation alone, such as through injection. Multi-Pill Abuse-Resistance (MPAR) works in conjunction with the Bio-MD technologies. Combining the Bio-MD™ and MPAR™ technologies removes the incentive to abuse by consuming multiple pills, and protects against oral overdose (*73*, *74*).

Mechanical Barrier Technologies

Most recently, new opioid products have used formulations that provide a mechanical barrier to abuse. For example, a new formulation of OxyContin was recently approved by the FDA for treating pain. This formulation deters abuse of the medication because it is difficult to crush and turns into a gelatinous substance when mixed with fluids, deterring both intranasal and intravenous abuse of the medication. Other opioid products currently on the market that use similar strategies are Nucynta, an extended-release formulation of tapentadol (mu opioid agonist and norepinephrine reuptake inhibitor), and Opana, an extended-release

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formulation of oxymorphone (mu opioid agonist) (*75*). Both of these formulations are intended to deter intravenous and intranasal abuse of the medication.

Conclusion

Narcotic analgesic use continues to be on the rise with the rate of misuse increasing as well. Treatment with methadone or buprenorphine has specific advantages and disadvantages and when used properly either agent can help patients avoid illicit opioid use while improving mental health, pain conditions and quality of life. An emphasis remains in creating innovative drugs for the appropriate treatment of pain while developing an aggressive, comprehensive approach that will help minimize prescription opioid abuse. ADFs are a relatively new but potentially promising component of this strategy. The development of new abuse-deterrent opioids remains an important goal for future research. As these formulations are approved for use, future studies will be imperative to better evaluate the success of these novel medications.

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Chapter 5

Treatment of Opioid Dependence

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The past few decades have witnessed an increasing prevalence of opioid dependence/opioid use disorder. Optimal management requires pharmacotherapy. Three medications are available for treatment of this disorder, methadone, buprenorphine, and naltrexone. Each of these medications has its own unique characteristics, which are described in detail in this chapter as are the concepts underlying the appropriate prescribing of the medications. Behavioral interventions often add to the benefits of the medications. Patients with opioid dependence/use disorder also need attention for co-occurring other substance, psychiatric, and medical disorders to achieve desired treatment outcomes.

Introduction

This chapter will touch concisely on the definition of and epidemiology of opioid dependence to elucidate the scope of the problem. It will then shift to the primary focus of the chapter, the pharmacologic treatment of opioid dependence. Finally, the chapter will briefly mention behavioral interventions for opioid dependence which typically work best in concert with pharmacologic interventions and will also note the other substance use disorders, psychiatric disorders, and medical disorders than commonly co-occur with opioid dependence that need attention during treatment.

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Definition of Opioid Dependence and Opioid Use Disorder

In the United States opioid dependence is defined via criteria established in the American Psychiatric Association Diagnostic and Statistical Manual, DSM-IV (*1*). A new version of this manual (DSM-5) is scheduled for release in 2013 so the precise criteria as well as the name of the disorder will change somewhat. This chapter relies mainly on the newer criteria set. However, one of the aims of both criteria sets is to distinguish between physiologic and non-physiologic criteria and make an important distinction between simple physiologic dependence and the syndrome of opioid dependence which requires maladaptive behavior. The two physiologic criteria are tolerance (need for markedly increased amounts of opioids to achieve desired effect or markedly diminished effect with use of the same amount of opioids) and withdrawal (which includes the typical clinical opioid withdrawal signs and symptoms: dysphoric mood, nausea, vomiting, muscle aches, lacrimation, rhinorrhea, pupillary dilation, piloerection, sweating, diarrhea, yawning, fever, insomnia). A patient appropriately treated for pain with opioid analgesics over an extended period might be expected to develop some tolerance and also to manifest withdrawal if the opioid dosage were decreased. However, if that individual did not have maladaptive behavior, despite showing evidence of "physiologic dependence," the individual would not qualify for the diagnosis of the syndrome that in DSM-IV was also labeled "opioid dependence." In the DSM-5 this syndrome will be called "opioid use disorder" to help emphasize the distinction between physiologic dependence and addiction.

To qualify for a diagnosis of opioid use disorder an individual must have at least 2 of 11 possible criteria over a 12 month period leading to clinically significant impairment or distress, and, if the person is taking opioid medication under medical supervision, tolerance and withdrawal do not count towards the total of 2 or more. The additional 9 behavioral criteria are

1) recurrent opioid use resulting in a failure to fulfill major role obligations,
2) recurrent opioid use in situations in which it is physically hazardous,
3) continued opioid use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of opioids,
4) opioids often taken in larger amounts or over a longer period than was intended,
5) a persistent desire or unsuccessful efforts to cut down or control opioid use,
6) a great deal of time spent in activities necessary to obtain opioids, use opioids, or recover from the effects of opioids,
7) important social, occupational, or recreational activities given up or reduced because of opioid use,
8) opioid use continued despite knowledge of having a persistent or recurrent physical or psychological problem likely to have been caused or exacerbated by opioids,
9) craving or a strong desire or urge to use opioids.

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Mild opioid use disorder is denoted by presence of 2 or 3 criteria, moderate opioid use disorder by presence of 4-6 criteria, and severe opioid use disorder by presence of 7-11 criteria.

The diagnosis of opioid use disorder is generally established by a clinical interview. Physical examination can aid in the diagnosis via signs of opioid intoxication or withdrawal or via evidence of multiple injection sites. Urine toxicology testing showing the presence of non-prescribed opioids can also aid in the diagnosis.

Epidemiology of Opioid Dependence/Opioid Use Disorder

Humans have used and misused opium throughout recorded history. In recent years the widespread availability of heroin and the increasing availability of prescription opioids diverted from pain treatment have fueled an epidemic of opioid dependence. In the U.S. in 2010, an estimated 200,000 individuals used heroin in the past month, whereas an estimated 2 million had non-medical use of prescription pain medication, a number largely unchanged over the prior 8 years (*2*). Worldwide 2009 saw an estimated 15-39 million problem users of opioids (*3*). In the U.S. in 2009, treatment admissions for heroin use represented 14% of nearly 2 million admissions for a substance use disorder; admissions for prescription opioids comprised 7% of the overall 2 million admissions up from 1% in 1999 (*4*). This widespread use and misuse of opioids has led to a secondary epidemic of poisoning deaths. Overall, in 2008 deaths from drug related poisonings equaled deaths from motor vehicle accidents (*5*). Opioid analgesics caused at least 14,000 of these poisoning deaths in 2008 (*5*). Opioid users, particularly injection heroin users, have a high risk for contracting infectious diseases, notably human immunodeficiency virus (HIV) and Hepatitis C virus (*3*, *6*). Because of these infections and other medical and psychiatric complications individuals with opioid use disorder also incur extensive and expensive health care utilization (*3*, *7*). In addition, because opioids are typically obtained and used illicitly, individuals with opioid use disorder have high rates of criminal justice involvement. Good evidence suggests that treatment for opioid use disorder reduces rates of overdose (*8*), HIV risk behavior and serconversion (*9*), health care utilization (*7*), and crime (*10*).

Pharmacotherapy for Opioid Dependence/Opioid Use Disorder

Pharmacotherapy forms the foundation of treatment for opioid use disorder with behavioral interventions serving as an essential adjunct. Attempts to manage patients with opioid dependence/use disorder without medications typically fail with 80-90% relapse rates (*11*, *12*). Three medications have FDA approval for the treatment of opioid dependence: methadone, a full μ-opioid agonist; buprenorphine, a μ-opioid partial agonist and κ-opioid antagonist; and naltrexone, an opioid antagonist. Details on the use of each of these medications follow.

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Methadone for Opioid Dependence/Opioid Use Disorder

In the U.S. methadone for opioid dependence/use disorder can only be dispensed from federally licensed clinics and cannot be prescribed through physicians' practices. Some other countries (e.g. Canada, United Kingdom) do allow physicians to prescribe methadone from their practices. The federally licensed clinics in the U.S. are tightly regulated. Patients must have documentation of current opioid dependence/use disorder of at least one year's duration as a requirement for admission. This requirement may be waived by a program physician for patients recently released from incarceration, previously treated within the past two years in a licensed clinic, or for pregnant patients. Initially patients must have their methadone doses dispensed under daily observation. Eventually some take-home doses for self-administration are permitted with the number specifically dictated by the regulations based upon continuous time in treatment and patient stability. The regulations require that adequate medical, counseling, vocational, educational, and other assessment and treatment services be provided.

Methadone Pharmacology

Methadone has a unique and complicated pharmacology. Good oral bioavailability, gradual onset, and generally long half-life contribute to its efficacy, but the long half-life also may lead to medication build up and unintended toxicity. Methadone also has numerous possible drug-drug interactions and effects on cardiac conduction. These safety issues caused the FDA to place a black box warning in the product label concerning respiratory depression and QT interval prolongation on the electrocardiogram (ECG).

Marketed methadone consists of a racemic mixture of two stereoisomers, *levo(l)*-methadone and *dextro (D)*-methadone. The *l*-methadone enantiomer provides the majority of pharmacologic activity, although the *d*-methadone has antitussive action and may contribute to side effects. Oral methadone is supplied as a solid tablet, a rapidly dissolving wafer, and a premixed liquid, all of which are basically bioequivalent.

Methadone Pharmacokinetics

Absorption occurs rapidly after oral ingestion of methadone (*13*). Methadone has an average bioavailability of about 80% but inter-individual variation ranging from 41-95% (*14*). Initial effects appear within 30 minutes, but peak effects and peak plasma levels are achieved on average about 4 hours after ingestion, with a range of 1-6 hours (*15*). Methadone displays an average terminal half-life of 22 hours, with a range of 5 to 130 hours (*16*). Most absorbed methadone leaves the circulation and enters tissue stores in liver, kidneys, lungs, and brain. Tissue

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stores return back into the circulation usually when serum levels decrease but also potentially at unanticipated times and at unexpectedly rapid rates. Methadone remaining in the blood is 60-90% bound to plasma proteins, primarily α1-acid glycoproteins. The amount of free methadone available to tissues can alter with the amount of protein available for binding. For example, levels of α1-acid glycoproteins increase during stress, which would elevate the amount of bound methadone and decrease free methadone (*14*).

Methadone metabolism is complex and not yet entirely elucidated. Most available data indicate that its metabolism is mainly catalyzed by the liver enzyme CYP 450 3A4 (*17*). There is accruing evidence that other enzymes including CYP2B6, CYP2D6, CYP1A2, CYP2C9, and CYP2C19 contribute as well (*16*, *18*). Recently questions have been raised about the primary role of 3A4 (*19*), but the key point is that that these enzymes exhibit wide inter-individual variation in activity based mainly upon genetics but also upon environmental factors. Methadone also has the capacity to induce its own metabolism so that serum levels and effects may decline over time, particularly during the first month of treatment (*16*).

Methadone has no active metabolites. The major metabolite is 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), although several other minor metabolites occur (*16*). Though elimination varies between individuals, the main route for both parent drug and EDDP is renal with some eliminated in the feces. As with other aspects of the pharmacokinetics of methadone, inter-individual variation also occurs in clearance rates. In addition, more acid urine leads to faster elimination (*16*). An important message for clinicians is to grasp the great variability in methadone absorption, metabolism, storage, and elimination both across individuals and within a given individual over time.

Methadone Pharmcodynamics

Methadone exerts its primary actions as an agonist at the μ-opioid receptor, but, unlike most other opioids, it also antagonizes the N-methyl, D-aspartate (NMDA) receptor (*20*) and blocks the serotonin and norepinephrine transporters (*21*). Methadone also blocks one of the cardiac potassium channel, hERG, which, as mentioned, can result in a prolonged QT interval on the ECG (*22*). Prior to the development of tolerance, methadone has typical μ-opioid agonist effects including miosis, analgesia, sedation, possible euphoria, decrease in gut motility, release of histamine, and respiratory depression. Methadone serves as a substrate of the transport protein, p-glycoprotein (*23*). P-glycoprotein activity varies both by genetic predisposition and environmental effects such as the presence of other drugs. P-glycoprotein activity could affect absorption of methadone from the gut but also, importantly, could have a substantial impact on the relationship between plasma and brain levels, reducing brain levels when it is more actively transporting methadone out of the central nervous system. The present findings are somewhat mixed as to whether polymorphisms in the gene coding for p-glycoprotein actually have a clinical impact on methadone treatment (*24*, *25*).

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Clinical Use of Methadone

The unique methadone pharmacology elucidated above makes clinical use of methadone challenging. Since every patient has an individualized response to any given dose of methadone, each patient needs specific personalized attention to the monitoring and management of the methadone dose. Dose conversion calculators are notoriously inaccurate for switching patients from other opioids to methadone and should not be relied upon. Because of methadone's long half-life, a steady state on a given dose is not attained for several days so the ultimate response to a given dose cannot be immediately determined. Too rapid dose escalation can lead to unanticipated medication accumulation causing serious side effects including respiratory depression, respiratory arrest, and death. These fatalities occur particularly among individuals who do have any tolerance to opioids, and the greatest risk period for fatal intoxication occurs during the first weeks of treatment and during periods of dose adjustments (*26*).

One of methadone's potentially dangerous characteristics, its long half-life, also makes it a highly effective pharmacologic intervention for opioid dependence/use disorder. For the vast majority of patients a once daily oral dose averts opioid withdrawal symptoms, which are a strong driver for ongoing illicit opioid use. Pre-clinical evidence indicates that methadone antagonism of excitatory NMDA receptors could decrease opioid tolerance, largely eliminating the need for a constantly escalating dose to obtain the same effect (*27*). Methadone blockade of serotonin and norepinephrine transporters, mimicking the action of many antidepressant, could have mood elevating effects.

Methadone Induction

The induction period subsumes the interval from the initial dose until the time a stable dose is achieved, usually a period of two to four weeks. Prior to inducing a patient onto methadone, the health care provider should conduct a medical history and physical examination including information on past and recent illicit opioid and other substance use as well as a record of all the patient's current medications. Knowledge of the patient's experience with tolerance and withdrawal helps to determine the initial induction dose. The federally required (syphilis serology) and additional indicated laboratory tests should be obtained along with a urine specimen for toxicology analysis to confirm the patient's recent substance use history. Informed consent should be provided to assure that the patient understands the risks of methadone treatment particularly the fact that physiologic dependence will occur. The patient should sign a consent document that also conveys all the expectations for methadone treatment.

Before administration of the first dose, the clinician managing the patient should establish that the patient does not display clinical evidence of sedation or intoxication. The initial dose of methadone can range from 5 to 30 mg. Thirty milligrams is the maximum first dose allowed by federal regulations.

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Determination of the initial dose hinges on a number of patient specific factors including any history of prior response to methadone and prior methadone dose, type, amount, and frequency of recent opioid use, recent use of other substances such as sedatives or ethanol which could have additive or synergistic effects with methadone on level of consciousness or respiratory drive, and current medical conditions or concomitant prescribed medications which could affect the pharmacokinetics or pharmacodynamics of methadone. The patient's age must also be considered since older individuals generally metabolize methadone less rapidly. If any uncertainty arises as to the appropriate initial dose, it makes sense to choose a lower dose to circumvent possible risks of intoxication or overdose. For patients not exposed to any opioids for three or more days prior to induction, 5 to 10 mg represent the most appropriate initial dose range. For patients with extensive recent use of heroin, the maximum allowable initial dose of 30 mg typically successfully decreases any withdrawal symptoms.

Ideally the patient should receive additional assessment two to four hours following the initial methadone dose. If the patient shows no observable evidence of withdrawal or intoxication and reports feeling comfortable, the appropriate first day dose has been achieved. If signs or symptoms of withdrawal persist, the clinician may administer additional doses of methadone to a maximum of 40 mg total for day one. In the highly unusual situation when a dose higher than 40 mg for day one is considered, it must be clearly documented that a dose higher than 40 mg was essential to manage opioid withdrawal. In the equally rare instance when a patient exhibits sedation or intoxication two to four hours after the initial dose, the patient should at the very least stay in the clinic for observation until the effects have resolved, or if necessary, emergency measures such as naloxone administration (to reverse intoxication) and airway preservation should be instituted.

The patient subsequently returns to the clinic daily for evaluation of signs and symptoms of withdrawal or intoxication and observed medication administration. Commonly the initial dose does not entirely relieve withdrawal symptoms over a full 24-hour period. Nevertheless, with the 22-hour average half-life of methadone and the fact that it takes four to five half-lives to achieve a steady state, it will require four to five days on a specific dose to determine the ultimate effect of that dose. Even though the patient may experience some withdrawal symptoms during the 24-hour dosing interval, the safest and most conservative strategy calls for upward titration of methadone dosage in 5 to 10 mg increments every four to five days. Following this schedule, dosages of 60-80 mg per day can be reached within four weeks of initiation.

Achieving a Stable Dose of Methadone

The goals for the induction period and the longer term are to stabilize the patient on a methadone dose that 1) eliminates opioid withdrawal symptoms throughout the 24-hours following the administration of the dose; 2) abolishes cravings or urges to use other opioids; 3) establishes adequate tolerance to

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preclude euphoria caused by use of illicit opioids; 4) eradicates use of illicit opioids as demonstrated by self-report and urine toxicology testing; and 5) minimizes side effects so that the patient does not experience any intoxication and can function normally. All these goals cannot always be achieved by doses that are safely attained during the induction period. Thus, continuing methadone dose increases in increments of 5 to 10 mg every five to seven days should be pursued until these goals are achieved. After the daily dose exceeds 40 mg, 10 mg increments usually are quite safe and appropriate. Finding the optimum dose for each individual patient often involves a clinical balancing act since doses needed to establish sufficient tolerance and discourage illicit opioid can lead to some side effects. Clinical trial evidence showed that methadone doses of 80-100 mg per day have significant advantages over lower doses in reducing illicit opioid use and retaining patients in treatment (*28*). For most patients a stable dose will range from 80-120 mg per day. However, in view of inter-individual differences, some patients stabilize on lower doses and some need higher doses. Within 2 to 3 months of starting methadone maintenance, most patients will stabilize on a daily dose that necessitates little change over a protracted period.

Nevertheless, given the plethora of pharmacological, environmental, and physiological dynamics that can occur over time to affect methadone's activity, including its capacity to induce its own metabolism, patients may develop symptoms or signs of instability after an interval on a stable dose. The instability may become apparent through patient self-report of withdrawal symptoms, side effects, or illicit opioid use, or illicit opioid use could be detected by urine screening. In such scenarios after re-evaluating the patient, the clinician should address any contributing factors (such as recently diagnosed medical conditions or institution of concomitant medications that could be interacting with methadone), and consider changes in the daily methadone dose. The dose can again be increased in 5-10 mg increments every five to seven days until the criteria for stability are once more met.

Patients may also miss methadone doses because of failure to attend the clinic. If more than one consecutive day is missed, the patient should receive a medical evaluation, and the methadone dose may need to be temporarily reduced if a loss of tolerance is suspected. The methadone dose can then be re-titrated upward in a fashion analogous to induction to return ultimately to the stable dose.

Methadone Serum Levels

Stabilizing most patients on methadone will be accomplished with close clinical monitoring and dose adjustments as clinically indicated. However, some patients may struggle to gain stability and may continue to experience withdrawal symptoms, opioid craving or even ongoing illicit opioid use. Some of these patients might be ultra rapid metabolizers of methadone for whom obtaining serum methadone levels may pinpoint the problem. Precise therapeutic serum concentrations have not been definitively established, but the literature suggests that minimum therapeutic trough levels would range from 100-400 ng/ml (*29*). Peak serum levels seem to vary widely among stable patients in

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methadone treatment. It appears that the rate of decline from peak to trough, as opposed to concentrations at discrete time points, operates as the best predictor of withdrawal symptoms and instability (*30*). Since ascertaining the actual rate of decline requires multiple samples over a 24 hour period, a sampling regimen not possible in a clinical context, as a rough approximation, the ratio of peak to trough concentrations may provide a proxy for the rate of decline. A peak to trough ratio of greater than 2:1 has been proposed as an indicator of ultra rapid metabolism and hence inadequate coverage throughout the 24-hour dosing interval leading to clinical instability (*31*). Ultra rapid metabolizers respond best to a split-dosing regimen rather than to an increase in daily dose. Thus, proper evaluation of serum methadone concentrations entails obtaining a trough level 24 hours after the last dose and a peak level about 3 hours after the last dose. Consideration of the discrete levels, the ratio between them, and the clinical context may guide rational methadone dosing in these rare instances.

Methadone Drug Interactions

Table 1 conveys potential drug-drug interactions involving methadone. Additive or synergistic effects can occur between methadone and other opioids or sedatives that also dampen respiratory drive leading to toxicity or overdose.

In view of the numerous potential pathways for methadone metabolism, multiple drug-drug interactions are theoretically possible. Inhibitors of the enzymes that catalyze methadone metabolism could cause elevated methadone serum levels and increased opioid effects. Generally, these types of predicted interactions have not led to problematic adverse events. For example, a human lab study showed that fluconazole (a CYP 3A4, 2C9, and 2C19 inhibitor) significantly increased methadone serum levels but did not result in any clinically observable effects (*32*). Nevertheless, a single case report describes co-administration of fluconazole leading to methadone toxicity (*33*). Likewise, one case report noted that concomitant administration of fluvoxamine (an inhibitor of CYP enzymes 3A4, 1A2, 2C9, and 2C19) caused methadone toxicity (*34*).

Clinically significant drug-drug interactions do occur with co-administration of drugs that induce the enzymes catalyzing the metabolism of methadone. In this situation a probable decrease in methadone serum levels triggers opioid withdrawal. Drugs known to cause this effect include the anticonvulsants, phenytoin and carbamazepine; the antibiotic, rifampin; and antiretroviral medications, lopinavir, efavirenz, and nevirapine (*35*). Ideally, these medications should be avoided in methadone treated patients, but if their use is necessary, fairly substantial increases in the methadone dose usually are needed to manage the emerging withdrawal symptoms. Other antiretroviral medications may slightly affect methadone pharmacokinetics but do not seem to cause clinically observable withdrawal.

As noted above, methadone has a black box warning for QT interval prolongation. Since a multitude of other medications also prolong the QT interval, co-administration of methadone with these other medications could have additive effects elevating risk for QT prolongation.

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Table 1. Potential Methadone Drug-Drug Interactions

Class or Specific Drug	*Interaction*	*Putative Mechanism*	*Notes*
Antiretrovirals			
Efavirenz, Lopinavir, Nevirapine	Reduction in serum methadone levels	Induction of CYP 450 enzymes	Clinically significant opioid withdrawal symptoms likely
Abacavir, Etravirine, Nelfinavir, Ritonavir, Squinavir, Tipranavir	May reduce serum methadone levels	Induction of CYP 450 enzymes	Clinically pertinent opioid withdrawal symptoms usually not seen with these agents
Didanosine, Stavudine	Reduction in didanosine, stavudine plasma concentration	Decreased bioavailability	Possible decreased efficacy of didanosine, stavudine
Zidovudine	Increase in zidovudine plasma concentration	Unknown	Risk of zidovudine toxicity
Delavirdine	Increased methadone serum concentration	Inhibition of CYP 450 enzymes	No clinically meaningful adverse events observed
Antidepressants			
Tricyclics: amitriptyline, clomipramine, desipramine, doxepin, imipramine, nortriptyline, protriptyline, trimipramine	Increases risk for constipation and sedation. Increases risk for QT prolongation and arrythmia	Anticholinergic effects. Blockade of hERG channel.	Clinical experience with combination indicates it is generally safe with careful clinical monitoring.
Serotonin reuptake inhibitors: citalopram, escitalopram, fluvoxamine, fluoxetine, paroxetine, sertraline	May increase serum methadone levels. Increased risk for serotonin syndrome	Inhibition of CYP enzymes. Blockade of serotonin transporter.	Clinical experience with combination indicates it is generally safe with careful clinical monitoring.

Continued on next page.

Table 1. (Continued). Potential Methadone Drug-Drug Interactions

Class or Specific Drug	*Interaction*	*Putative Mechanism*	*Notes*
Monoamine oxidase inhibitors: Isocarboxazid, phenelzine, selegiline, tranylcypromine	Increased risk for serotonin syndrome.	Inhibition of serotonin metabolism.	Use with extreme caution and careful clinical monitoring.
Serotonin/norepinephrine reuptake inhibitors: Duloxetine, desvenlafaxine, venlafaxine	Increased risk for serotonin syndrome. Increases risk for QT prolongation and arrhythmia (venlafaxine)	Blockade of serotonin transporter. Blockade of hERG channel (venlafaxine).	Clinical experience with combination indicates it is generally safe with careful clinical monitoring.
Antibiotics			
Ciprofloxacin, clarithromycin, erythromycin, azithromycin	May increase methadone serum levels. Increases risk for QT prolongation and arrhythmia	Inhibition of CYP enzymes. Blockade of hERG channel	One case report of sedation (ciprofloxacin). Clinical monitoring required.
Rifampin	Reduction serum methadone levels	Induction of CYP enzymes	Severe opioid withdrawal can occur. Will need increased methadone dose.
Antifungals			
Ketoconazole, fluconazole voriconizole	May increase methadone serum levels.	Inhibition of CYP enzymes	Little evidence for important clinical effects
Anticonsulsants			
Carbamazepine, phenytoin	Reduction in serum methadone levels	Induction of CYP enzymes	Severe opioid withdrawal can occur. Will need increased methadone dose.
Antiarrthymics			
Procainamide, quinidine	Increases risk for QT prolongation and arrhythmia	Blockade of hERG channel	Careful clinical monitoring required

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Table 1. (Continued). Potential Methadone Drug-Drug Interactions

Class or Specific Drug	*Interaction*	*Putative Mechanism*	*Notes*
amiodarone	May increase methadone serum levels. Increases risk for QT prolongation and arrhythmia	Inhibition of CYP enzymes. Blockade of hERG channel	Careful clinical monitoring required
Benzodiazepines	Additive CNS and respiratory depressant effects	Increased GABA activity	Careful clinical monitoring required
Barbiturates	Additive CNS and respiratory depressant effects	Increased GABA activity	Careful clinical monitoring required
Cimetidine	May increase methadone serum levels.	Inhibition of CYP enzymes	No evidence major clinical effect
Naltrexone	Precipitated opioid withdrawal	Displaces methadone from μ-opioid receptors	contraindicated

Methadone Cardiac Effects

Some evidence indicates that a corrected QT interval longer than 500 msec increases the risk for a serious cardiac ventricular arrhythmia, torsades de pointes (*36*). Although such events have been documented to occur in individuals being treated with methadone, it has almost always been reported to occur in the context of other risk factors in addition to methadone treatment (*37*, *38*) and likely occurs very rarely (*39*, *40*). Currently some controversy exists about the appropriate way to address the concern about methadone treatment leading to torsades, with some recommending routine ECG screening for all patients on methadone and others encouraging an ECG only in the presence of other risk factors (*40*, *41*). At this juncture it seems reasonable to consider obtaining ECGs on methadone treated patients who have known structural heart disease or who have a history of syncope or a family history of sudden cardiac death, since there can be genetic predisposition to a prolonged QT interval (*42*). If patients on methadone have a corrected QT interval above 500 msec, consideration should be given to discontinuing other medications that also prolong the QT interval, to stopping illicit cocaine use, correcting electrolyte imbalances, and reducing the methadone dose if clinically feasible. Another potential strategy is to switch the patient from methadone to buprenorphine, although such a switch is often clinically

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very challenging to manage because of the risk and likelihood of precipitated withdrawal.

Take Home Status

Regulations permit take-home doses of methadone for stable patients. Patients can eventually be granted a maximum of a one month supply of take-home doses after remaining in continuous treatment for 2 years or longer. Patients find take-home doses highly desirable so that take-home privileges can be successfully deployed as a contingency management strategy. Patients who conform to program requirements including abstinence from illicit drug use receive the reward of additional take-home doses. Patients who do not conform and/or submit positive urine specimens get negatively reinforced by rapid removal of take-home doses. This approach contributes to a reduction in illicit drug use (*43*). Methadone program physicians must take into account the patient's ability to store, self-administer, and transport take-home doses safely. Individuals other than the patient, particularly children, are susceptible to accidental overdose from take home medication. Federal criteria for take home privileges include: 1) Absence of recent abuse of drugs (opioid or non-narcotic) including alcohol; 2) Regularity of clinic attendance; 3) Absence of serious behavioral problems at clinic; 4) Absence of known recent criminal activity, e.g., drug dealing; 5) Stability of the patient's home environment and social relationships; 6) Length of time in comprehensive maintenance treatment; 7) Assurance that take-home medication can be safely stored within the patient's home; 8) Determination that the rehabilitative benefit to the patient derived from decreasing frequency of clinic attendance outweighs the potential risk of diversion.

Diversion Risk Reduction

Methadone programs must balance diversion risk against the potential benefits to the patient in receiving take home doses such as less travel time and stress and more time for productive activities. If a urine specimen tests negative for methadone or metabolite, additional investigation is needed to determine if a reasonable explanation or indication of urine tampering or methadone diversion exist. Although diversion of take-home doses of methadone to individuals for whom it is not prescribed certainly occurs, few data have been gathered to indicate the frequency of such events. A study of methadone medical maintenance (see below) in which subjects received either 5 or 6 days versus 27 days of take-home medication used random monthly medication call backs by which subjects are notified to return to the clinic within 24 hours with all outstanding medication (*44*). Using this method, diversion can be inferred when patients cannot produce for inspection all expected outstanding medication. The study showed that over a 12 month period of treatment 0.9% to 5.0% of subjects brought an incorrect

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amount of medication at the time of call back, and 1.7% to 5.6% failed to return for the call back. An Australian survey of patients receiving methadone maintenance at community pharmacies, found that 12.6% admitted to ever having diverted or trying to divert their methadone, and 2.2% admitted to diverting or trying to divert methadone in the past 12 months (*45*). Because some diversion of methadone does occur, programs must implement diversion reduction plans. Careful application of criteria for take-home eligibility and removal of take-home privileges quickly with evidence of instability as enumerated above constitute the foundations of diversion reduction. Instituting a random call back system also is feasible and creates an additional safeguard. As conveyed by King et al. programs can randomly phone a few patients each week and ask them to return within 24 hours with all methadone expected to be in their possession (*44*). Sometimes diversion will be detected by the call backs, and take-home privileges can be removed from patients who fail the call backs. The call backs may also create deterrence in that patients who might consider diverting methadone refrain from engaging in such behavior because of trepidation about failing a call back and losing take-home privileges.

Medical Maintenance

In the U.S. experimental models of methadone treatment show that patients who have already stabilized in a licensed methadone program can transfer their methadone care to a physician practicing in an office-based setting, otherwise known as medical maintenance. These patients usually come in to pick up their methadone weekly to monthly, see the physician, provide a urine specimen, and receive take home doses until the next appointment. Both uncontrolled trials and randomized controlled trials demonstrate that the majority of already stabilized patients succeed in medical maintenance treatment and that patients randomly assigned to medical maintenance have equivalent outcomes to clinic-based patients (*44*). In the U.S. medical maintenance requires an exception to the methadone treatment regulations so that very few medical maintenance practices have actually been created, but similar models are used routine in other countries such as Canada and the United Kingdom (*46*).

Interim Methadone Maintenance

In some areas of the U.S. methadone treatment services are not readily and immediately accessible to all individuals who desire such treatment. Interim methadone maintenance provides medication-only treatment as an alternative to having individuals who want methadone treatment wait with no treatment until entrance to full methadone treatment including ancillary services is available. Interim methadone provides methadone induction and then a daily, stable observed dose of methadone with no take home doses and no other services except emergency counseling. Although full methadone treatment shows superior outcomes to medication-only treatment, interim methadone compared

to a wait-list control in at least three randomized controlled trials demonstrated reduced illicit drug use and higher rates of subsequent entry into comprehensive methadone treatment (*47*).

Buprenorphine for Opioid Dependence/Opioid Use Disorder

In the U.S., as in most other countries, buprenorphine for opioid dependence/use disorder, in contrast to methadone, can be prescribed by appropriately qualified physicians in any medical setting. The patient can then go to a pharmacy and pick up the medication for self-administration as the patient would for any other prescribed medication. Physicians can qualify by obtaining a waiver and a special Drug Enforcement Agency (DEA) number by passing addiction specialty examinations given by the American Society of Addiction Medicine or by the American Board of Psychiatry and Neurology or by completing 8 hours of training afforded by several different medical specialty organizations. During the first year after obtaining the waiver, physicians are limited to a total of 30 patients for whom they can prescribe buprenorphine at any given time. After the first year physicians can request to increase the total number to 100. Although most physicians prescribe no more than 30 days of buprenorphine at any one time, as a DEA schedule III medication, the amount of buprenorphine that can be prescribed is only subject to the DEA regulations for schedule III which allows for a maximum of 5 refills or 6 months, whichever comes first. Buprenorphine can also be administered and dispensed similarly to methadone in federally licensed clinics without the physician having a waiver as long as all federal regulations for these clinics are adhered to.

Buprenorphine Pharmacology

Buprenophine also has a unique and complex pharmacology, but, in contrast to methadone, one of its outstanding characteristics is its safety profile. Buprenophine has extremely poor oral bioavailability, so in the formulations currently FDA approved for treatment of opioid dependence/opioid use disorder, it is taken by the sublingual route. As with methadone, its gradual onset, and generally long half-life contribute to its efficacy, but as a partial μ-opioid agonist, rather than a full agonist, it has a ceiling effect on its activity such that at some point increasing doses to do not lead to increasing activity, and, thus, the risk of respiratory depression and overdose is miniscule (*48*, *49*). Buprenorphine has many fewer clinically meaningful drug-drug interactions than does methadone, and buprenorphine appears to have lesser effects on cardiac conduction (*50*).

Buprenorphine/Naloxone

At the present time marketed buprenorphine for opioid dependence/opioid use disorder comes in 3 sublingual formulations: 1) buprenorphine sublingual tablets; 2) buprenorphine/naloxone sublingual tablets; and 3) buprenorphine/naloxone

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sublingual film. The buprenorphine/naloxone formulation is intended to deter parenteral misuse of the medication. Naloxone, a μ-opioid antagonist, has minimal sublingual bioavailability, and, when the medication is taken by the sublingual route, insufficient naloxone is absorbed to have any clinically apparent effect. However, since naloxone has very good parenteral bioavailability, an attempt to crush and inject the buprenorphine/naloxone tablet results in the simultaneous administration of both a partial agonist and an antagonist. The naloxone will blunt any of the parenteral effects of buprenorphine and could also potentially precipitate opioid withdrawal if full agonist opioids are present (*51*). Buprenorphine/naloxone is the predominant formulation prescribed in the U.S., which has thereby seen few problems with parental misuse of buprenorphine. The buprenorphine only formulation is recommended primarily during pregnancy to prevent the potential harm of even trace amounts of naloxone reaching the fetus and for patients who have a documented allergy to naloxone.

The tablet formulations come in 2 dosage strengths. The buprenorphine only tablets are supplied as 2 mg and 8 mg tablets. The buprenorphine/naloxone combination is supplied as 2mg (buprenorphine)/0.5 mg (naloxone) and 8mg (buprenorphine)/2mg (naloxone) tablets. (The current manufacturer of the combination tablets intends to discontinue their production in 2013. It is not clear if or when a generic combination tablet might become available. For the foreseeable future the only combination product available will be the film.) The film comes in 3 dosage strengths, 2mg (buprenorphine)/0.5 mg (naloxone), 8mg (buprenorphine)/2mg (naloxone), and 12mg (buprenorphine)/3mg (naloxone).

An experimental formulation of buprenorphine as a subcutaneous implant which releases active medication over a 6 month interval, while still undergoing evaluation, appears safe and efficacious (*52*).

Buprenorphine Pharmacokinetics

Absorption occurs rapidly after sublingual ingestion of buprenorphine (*53*). Sublingual bioavailability shows large inter-individual variability but is generally around 35% for the xtablet (*53–55*). There is evidence that for unknown reasons the bioavailability of the buprenorphine/naloxone tablet formulation is slightly higher than the buprenorphine only formulation (*56*). Initial effects appear within 30 minutes with peak effects and peak plasma levels reached on average about 1 hour after ingestion (*53*, *56*, *57*). Buprenorphine has an estimated average terminal half-life of 32 hours (*53*), although there is wide variation across studies and individuals (*58*). Buprenorphine is 96% bound to plasma proteins, primarily to α- and β-globulin fractions (*53*).

Buprenorphine undergoes both glucuronidation and N-dealkylation. Most available data indicate that N-dealkylation is catalyzed by the liver enzyme CYP 450 3A4 (*53*). The product of N-dealkylation is an active metabolite, nor-buprenorphine (*53*, *57*). The main route of elimination for both parent drug and metabolites is fecal with lesser amounts excreted by the kidneys (*53*). Although peripheral exposure to norbuprenorphine is roughly equivalent to that

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for buprenorphine, central nervous system exposure is presumed to be less for norbuprenorphine, which, as a more polar compound, fails to penetrate the blood brain barrier as easily as buprenorphine (*53*).

Buprenorphine Pharmcodynamics

Buprenorphine serves as a partial agonist with high affinity and slow dissociation at the μ-opioid receptor and also acts as an antagonist at the κ-opioid receptor (*59*, *60*). It also has agonist properties at the nociceptin/orphanin FQ (NOP) receptor (formerly known as ORL1 receptor) (*61*). Prior to the development of tolerance, buprenorphine has typical clinically observable μ-opioid agonist effects including miosis, analgesia, sedation, possible euphoria, decrease in gut motility, and respiratory depression with a ceiling on the latter effect (*59*).

Clinical Use of Buprenorphine

Since the buprenorphine/naloxone formulation and the buprenorphine only formulation are close to interchangeable in their effects, the following discussion, for ease of reading, will refer to the buprenorphine doses in the formulation, with the assumption that in most cases the buprenorphine/naloxone formulation will be prescribed. The challenges in starting a patient on buprenorphine are quite different than those of starting methadone. With its excellent safety profile, buprenorphine poses virtually no risk from rapid dose escalation. However, as a partial μ-opioid agonist with high affinity for the receptor, it does pose the risk of causing precipitated opioid withdrawal if administered when a full agonist occupies the receptors because it can displace the full agonist and abruptly reduce the activation of the receptors (*62*). As with methadone, buprenorphine's long half life permits once daily dosing, and for most patients a once daily sublingual dose averts opioid withdrawal symptoms. However, since buprenorphine in the office based setting is not administered under observation, some patients may prefer to divide their daily dose and take smaller doses two to four times per day.

Buprenorphine Induction

The induction period subsumes the interval from the initial dose until the time a stable dose is achieved, usually a matter of a few days. Prior to inducing a patient onto buprenorphine, the physician should conduct a medical history and physical examination including information on past and recent illicit opioid and other substance use as well as a record of all the patient's current medications. Any indicated laboratory tests should be obtained along with a urine specimen for toxicology analysis to confirm the patient's recent substance use history. Informed

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consent should be provided to assure that the patient understands the risks of buprenorphine treatment particularly the fact that physiologic dependence will occur. The patient should sign a treatment agreement that also conveys out all the expectations for buprenorphine treatment.

To avoid the risk of precipitated opioid withdrawal, induction on buprenorphine requires the patient to abstain from other short acting opioids ideally for a period of about 24 hours and enter a state of moderate opioid withdrawal prior to the administration of the first dose of buprenorphine. If the patient has recently taken methadone, it may take 48 to 72 hours after the last methadone dosage for moderate opioid withdrawal to commence. When the patient presents for induction, the physician must verify the presence of objective signs of opioid withdrawal such as lacrimation, rhinorrhea, yawning, sneezing, coughing, piloerection, restlessness, or tremor. If desired the physician can use an instrument to measure and quantify opioid withdrawal such as the Clinical Opiate Withdrawal Scale (*63*). At the point when objective signs of withdrawal are observed, the induction can begin with a low buprenorphine dosage of 2 mg or 4 mg. This first administration of medication is likely to alleviate most of the withdrawal signs and symptoms within 30-60 minutes. Once the withdrawal shows this improvement, it is safe to administer additional doses until any residual withdrawal symptoms are eliminated, usually within one or two days at doses of 8 to 16 mg per day. At that point induction is completed.

Managing Precipitated Withdrawal

In the rare instance when buprenorphine is administered prior to the development of sufficient opioid withdrawal, precipitated withdrawal may occur. Precipitated withdrawal will be manifest by the abrupt appearance of much more severe withdrawal symptoms. Because buprenorphine is occupying the μ-opioid receptors, full agonist opioids will not relieve this precipitated withdrawal. Thus, the physician has essentially two management options in the face of precipitated withdrawal. One is to stop the induction and treat the withdrawal symptomatically using clonidine or lofexidine (latter not approved in the U.S.) for autonomic nervous system signs and symptoms, benzodiazepines for muscle cramping and agitation, and anti-emetics and antidiarrheals for gastrointestinal signs and symptoms. When the precipitated withdrawal is resolved, the buprenorphine induction can be attempted again. Option two involves pressing ahead with the buprenorphine induction with the idea that the withdrawal will resolve over the next 24 hours, and the patient will then be inducted onto buprenorphine. This option could also include use of the medications mentioned above for symptomatic management.

Achieving a Stable Dose of Buprenorphine

Once induction is completed, the physician can proceed to stabilization which involves additional possible dose adjustments over days or weeks until the

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optimal dose is attained. No federal regulations specify how often physicians see patients on buprenorphine. Typically, physicians will see patients within a few days of the initial induction day, then weekly for a few weeks until good stability is achieved, then monthly. Similarly to stabilization on methadone the goals of buprenorphine stabilization are to: 1) eliminate opioid withdrawal symptoms throughout the 24-hours following the administration of the dose; 2) abolish cravings or urges to use other opioids; 3) establish receptor occupancy to preclude euphoria caused by use of illicit opioids; 4) eradicates use of illicit opioids as demonstrated by self-report and urine toxicology testing; and 5) minimizes side effects so that the patient does not experience any intoxication and can function normally. As with methadone, determining the optimum dose for each individual patient often involves a balancing act since doses needed to establish sufficient receptor occupancy and discourage illicit opioid can lead to some side effects. Doses can be increased in 2 mg to 8 mg increments. The optimal dose can range from a minimum of 2 mg per day to a maximum of 32 mg per day, although most patients stabilize on a dose between 12 and 24 mg per day. As with methadone, even after stability is achieved, adjustments up or down over time may be needed. For many patients after a period of stability, the dose can be gradually reduced without compromising the stability achieved. Patients who have a lapse in adherence to buprenorphine would need to repeat the induction process if they have used other opioids during that interval.

Buprenorphine Drug Interactions

Although buprenorphine metabolism is catalyzed by the same enzyme system as methadone metabolism, to date combining medications that induce or inhibit the CYP 450 3A4 system with buprenorphine has not caused any clinically meaningful adverse effects (*35*). The explanation proposed for this phenomenon is that, given its strong affinity for the μ-opioid receptor, changes in plasma levels of buprenorphine do not rapidly affect buprenorphine occupancy of the receptor. As with methadone, additive or synergistic effects can occur between buprenorphine and sedatives that also dampen respiratory drive leading to toxicity or overdose. Some rare cases of fatal overdoses have been reported from the combination of benzodiazepines and buprenorphine (*64*), but the combination is by no means contraindicated as long as the benzodiazepine doses are moderate, and the patient is under careful clinical supervision. Obviously, the combination of buprenorphine with other opioids can be problematic. As mentioned, if buprenorphine is initially given when other opioids are on board, it can precipitate withdrawal. If other opioids are given after buprenorphine maintenance is established, it is not likely to be harmful, but the effects of the other opioids are likely to be greatly diminished as a consequence of buprenorphine already occupying the majority of μ-opioid receptors. The combination of buprenorphine and the opioid antagonist naltrexone for clinical treatment of opioid dependence/opioid use disorder and for cocaine addiction has been studied experimentally (*65*, *66*) but should be used clinically only with utmost care at the present time.

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Managing Side Effects of Methadone and Buprenorphine

Methadone and buprenorphine both can produce many of the side effects typical of opioid medications. Table 2 lists potential side effects. If troublesome side effects are present, and the patient has stopped illicit opioid use and does not have withdrawal symptoms, many side effects can be managed by incremental dose reductions of methadone 5-10 mg or of buprenorphine 2-4 mg every 5 to 7 days until side effects are resolved, tolerable, or until withdrawal symptoms occur. If dose reductions do not seem possible because the patient has continued illicit opioid use or still has withdrawal symptoms, other interventions can be attempted to manage some of the commonly occurring side effects.

Constipation is one of the most frequent and bothersome side effects in patients on methadone or buprenorphine, though it is usually less of a problem with the latter medication. Constipation can be handled by encouraging patients to consume more water, eating a diet higher in fiber content, and partaking in moderate exercise such as walking. If these life style changes prove insufficient, psyllium or other bulk-forming laxatives can be used but should be used cautiously unless adequate fluid intake is maintained. Other patients respond to emollient laxatives such as docusate, or to stimulant laxatives (bisacodyl) or osmotic laxatives (lactulose). Although constipation usually is only a nuisance, it can obviously progress to impaction and small bowel obstruction, so early intervention to address it is warranted.

Both methadone and buprenorphine can cause nausea. If persistent, nausea will often resolve with a dosage reduction if possible or else usually responds well to anti-emetics.

Edema can also be an unpleasant side effect of methadone (*67*) that is seen much less often with buprenorphine. The mechanism by which methadone causes edema remains unidentified. Edema usually does not improve with sodium restriction. It sometimes improves with a decrease in methadone dosage if the patient is stable enough to tolerate a decrease. If severe edema does not respond to these measures, diuretics, such as furosemide, often have benefit. If a diuretic is prescribed, potassium levels should be checked to ensure that potassium depletion has not occurred.

Both medications can cause hormonal alterations related to sexual functioning (*68*). These issues have been much better studied with methadone, though some work has been done with men on buprenorphine (*68*). The effects seem to occur more frequently with methadone. These medications, like other opioids, act at the hypothalamus altering the release of gonadotropin releasing hormone, leading to a reduction in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and subsequent suppression of testosterone levels or estrogen levels. In men reports of side effects include orgasmic and erectile dysfunction and decreased sexual desire. The reductions in FSH and LH normalize after several years of methadone treatment despite a persistent decrease in testosterone in some individuals (*69*). Sexual dysfunction may respond to a methadone or buprenorphine dosage reduction. If that is not possible or does not help, erectile function often improves with use of phosphodiesterase type 5 inhibitors (presuming contraindications for this class of medications such as

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cardiac conditions are not present). Testosterone replacement may improve sexual functioning among methadone or buprenorphine treated men with low serum testosterone levels. For women on methadone, depressed libido and oligomenorrhea or amenorrhea have been reported; conversely, in some cases regular menses resume after stabilizing in treatment. Irregular menses may lead some women to assume erroneously that they cannot become pregnant or are pregnant when they are not. For female patients with low libido or menstrual irregularities, medical work-up should include a discussion of the possibility of becoming pregnant without regular menses and about the use of birth control. Referral to an endocrinologist or gynecologist can identify or rule out other medical conditions causing amenorrhea or oligomenorrhea.

A hormonal effect of methadone that persists in some individuals after years of treatment but not yet studied in regard to buprenorphine is altered prolactin release. Rather than the normal diurnal variation, prolactin release becomes reactive to the peak level of methadone (*69*). Problems related to hyperprolactinemia include galactorrhea, menstrual disturbance, erectile dysfunction or long-term loss of bone mineral density. In one study many methadone maintained patients evaluated had low bone mineral densities and low vitamin D levels (*70*, *71*).

Table 2. Potential Methadone or Buprenorphine Side Effects

Body System	***Side Effects***
Body as a Whole	Asthenia (weakness), Edema, Headache
Cardiovascular	Arrhythmias, Bigeminal rhythms, Bradycardia, Cardiomyopathy, ECG abnormalities, Extrasystoles, Flushing, Heart failure, Hypotension, Palpitations, Phlebitis, QT interval prolongation, Syncope, T-wave inversion, Tachycardia, Torsade de pointes, Ventricular fibrillation, Ventricular tachycardia
Digestive	Abdominal pain, Anorexia, Biliary tract spasm, Constipation, Nausea, Dry mouth, Glossitis
Metabolic and Nutritional	Hypokalemia, Hypomagnesemia, Weight gain
Nervous	Agitation, Confusion, Disorientation, Dysphoria, Euphoria, Insomnia, Seizures
Respiratory	Pulmonary edema, Respiratory depression
Skin and Appendages	Pruritis, Urticaria, Other skin rashes, and rarely, Hemorrhagic urticaria, Diaphoresis
Special Senses	Hallucinations, Visual disturbances
Urogenital	Amenorrhea, Antidiuretic effect, Reduced libido, Erectile dysfunction, Urinary retention or hesitancy

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Excessive sweating is another common complaint among patients on methadone (*72*) that may also occur with buprenorphine. As with other side effects, lowering the methadone dose if possible is the initial intervention to try. In patients treated with additional drugs that induce sweating such as cholinesterase inhibitors, selective serotonin reuptake inhibitors or tricyclic antidepressants, using an alternative to one of these medications can decrease the severity of sweating. Methadone and other opiates induce release of histamine by degranulation of mast cells, which is implicated in the side effects of sweating and itching. By stabilizing mast cells antihistamines may alleviate sweating in methadone patients.

Headache is a side effect more common in buprenorphine treated patients than in methadone treated patients. The headache is usually mild and may spontaneously resolve. If not, it may respond to a dosage decrease or to non-steroidal anti-inflammatory medication or to acetaminophen. If none of these actions work, and the headache is intolerable, the patient might consider a switch to methadone.

Retrospective data and case reports suggested that buprenorphine had the potential to cause transaminitis (*73*) or drug-induced hepatitis (*74*), but a recent large randomized clinical trial did not show any difference between methadone and buprenorphine in rates of elevated liver transaminases and indicated that viral hepatitis, rather than the medications, was mainly responsible for transamines elevations during treatment with these medications (*75*).

Medically Supervised Opioid Withdrawal

Medically supervised withdrawal or tapering from methadone or buprenorphine may be performed for several reasons. Usually it is best avoided because of high rates of relapse back to opioid dependence/opioid use disorder (*12*, *76*). Justifications for medically supervised withdrawal include: patients who want methadone but do not qualify for maintenance because they have had less than a one year history of opioid dependence/use disorder, patients who must enter a controlled environment such as incarceration where methadone or buprenorphine are not available, administrative tapers for patients who do not comply with program or office policies, and voluntary tapers for patients who evince a personal desire to be off opioid maintenance therapy. All the available evidence indicates that for methadone gradual tapers promote better outcomes (*76*), whereas for buprenorphine rapid tapers pose no disadvantage and may even work slightly better (*77*).

Patients who want methadone but do not qualify for maintenance because of less than a one-year history of opioid dependence/opioid use disorder may be appropriate for medically supervised withdrawal from methadone. One common approach is to use a 180-day schedule to perform induction and stabilization, similar to what would be done for a maintenance patient, and continue a stable dose to the 120-day mark. At that juncture the dose can be tapered over 60 days

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with the rate of taper slowing in the latter part of this interval. Shorter tapers can be conducted in similar fashion.

For patients on methadone receiving an administrative discharge from a federally licensed program for abrogating program policies, the usual custom is to perform a taper over 21 days. Although this time frame almost certainly obviates success, it does give the patient an opportunity to make alternative plans while assuring that the patient will leave the program within a few weeks.

For patients on methadone desiring a voluntary taper, a number of signs of stability should be evident before commencing a taper, including an ample period without illicit drug use, a stable living situation, stable relationships, a reliable source of income, and absence of unstable medical or psychiatric conditions. The rate of taper should be adjusted to the patient's needs and expectations. These types of tapers may take many months or even years depending upon the starting dose. A typical rate would be a decrease of 5 to 10 mg from the daily dose every one to two weeks. A taper proceeding successfully should make the patient feel better by reducing methadone side effects. If the patient begins to feel worse, the taper is going too rapidly. One reasonable approach is to permit the patient to halt the taper or regain the previous higher dose upon request if the patient experiences any instability. The taper should certainly be stopped if any signs of instability such as a positive urine specimen or failure to comply with program rules occur. As the daily dosage drops below the 40 to 60 mg per day range, the rate of taper typically should be decreased. Patients who successfully complete a taper should be encouraged to continue counseling and to make a decision to resume methadone if relapse seems imminent. Some patients may opt to initiate opioid antagonist therapy with naltrexone (see below), once they no longer have physiologic dependence, to protect themselves from relapse.

If desired, a taper from buprenorphine can be conducted in a similar slow fashion by reducing the daily dosage in increments of 2 or 4 mg over an extended period, though as mentioned above, no advantage seems to accrue to prolonging the taper. In this scenario many patients have exhibited considerable difficulty relinquishing buprenorphine after tapering down to 2 or 4 mg per day. In a comparison of a 7 day taper to a 28 day taper after 4 weeks of stabilization, the rates of successful completion without illicit opioid use immediately after the taper were superior for the 7 day taper, and the long term outcomes were poor and equivalent for both schedules (*77*). Buprenorphine is also frequently used for short term medically supervised withdrawal to transition patients from a state of physiologic dependence on illicit opioids to abstinence. This procedure has very limited success. Schedules using 5 and 14 day tapers have been described (*78*, *79*).

A long used and alternative method for tapering patients off opioids involves the use of α_2-adrenergic agonists such as clonidine or, where available, lofexidine (*80*, *81*). These medications bind to pre-synaptic α receptors in the brain and decrease the output of norepinephrine, thereby decreasing sympathetic nervous system activation during opioid withdrawal. For clonidine a maximum of 1.2 mg and for lofexidine a maximum of 3.2 mg per 24 hours in divided doses can be given. The dosage is usually tapered over 5 to 14 days (*78*, *79*, *81*). In some patients dosing is limited by development of hypotension. Adjunctive medications

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for opioid withdrawal symptoms not totally mediated by the sympathetic nervous system are usually also needed such as benzodiazepines for insomnia and muscle cramps, anti-emetics for nausea, and anti-diarrheals for diarrhea.

Efficacy of Methadone and Buprenorphine Maintenance

A plethora of studies have investigated methadone and buprenorphine treatment over nearly 50 years. Evaluating these treatments through the most rigorous approach, double-blind, placebo-controlled randomized trials, creates some challenges. Opioid withdrawal does not demonstrate much response to placebo so maintaining a blind can prove problematic. Two studies with methadone and one with buprenorphine did overcome this concern and randomly assigned subjects in double-blind fashion to active medication maintenance versus a control condition of a blinded medication taper followed by placebo. The two studies involving methadone are included in a meta-analysis which surveyed the literature through 2001 and found six trials with a total of 954 subjects published between 1969 and 1993 that compared methadone maintenance to some sort of control condition. The analysis showed that subjects receiving methadone maintenance were three times as likely as control subjects to remain in treatment and one-third as likely to have used heroin (*82*). The study with buprenorphine randomly assigned 40 subjects to a year's treatment with 16 mg per day or to a 6 day taper followed by placebo. All subjects received intensive behavioral interventions. Seventy-five per cent of buprenorphine treated subjects remained in treatment for one year versus none of the placebo treated subjects. Seventy-five per cent of urine specimens collected from the buprenorphine treated subjects were negative for illicit drugs. Four of the placebo treated subjects died during the year versus none of the buprenorphine treated subjects (*83*). A similar controlled, though not blinded, trial, randomly assigned 34 heroin addicts either to receive methadone treatment or to a control group which offered psychosocial treatment only. The control group had a mortality rate of 11.8% within two years compared to 0% in the methadone group (*84*). Reduced mortality rates are also observed for opioid dependent individuals in methadone treatment compared to those out of methadone treatment (*7*).

Another meta-analysis of methadone treatment included 24 studies conducted between 1964 and 1994 and found that methadone maintenance had a large effect on drug-related crime, a small to moderate effect on drug and property related crime, and a small effect on non-drug related crime (*10*). Most of the studies included in this analysis compared criminal behavior prior to entering methadone treatment to such behavior during methadone treatment. A more recent study which randomly assigned subjects to interim methadone treatment versus referral to community-based methadone treatment, found that the referral group was much less likely to enter comprehensive methadone maintenance and received significantly more illegal income at follow-up than did the interim maintenance group (*47*). Numerous observational studies also show that methadone treatment reduces HIV risk behavior, and two observational studies indicate that it reduces HIV seroconversion (*9*, *10*).

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The effects of buprenorphine on crime and HIV risk behavior have not been as well studied. A multitude of randomized clinical trials have directly compared buprenorphine treatment to methadone treatment on the outcomes of illicit opioid use and treatment retention. A meta-analysis looked at 24 of these studies and found the treatments equally efficacious at reducing illicit opioid use but found methadone slightly better at retaining patients in treatment (*85*).

There is experimental evidence that for some patients who fail methadone maintenance, heroin maintenance might have better efficacy (*86*), but it requires patients to attend the clinic several times per day to inject heroin and is unlikely to come into widespread application.

Naltrexone (Opioid Antagonist) Treatment for Opioid Dependence/Opioid Use Disorder

For patients who are highly motivated and who either do not want or fail opioid maintenance treatment, and who are willing to undergo opioid withdrawal, antagonist pharmacotherapy with naltrexone offers another option. Patients must have all other opioids completely out of their system before starting naltrexone to avoid the risk of precipitated opioid withdrawal. The state of being opioid free is typically determined via a naloxone challenge test as described below. From a purely theoretical standpoint naltrexone is the ideal pharmacotherapy. Naltrexone occupies the μ-opioid receptor and blocks it. If the patient uses an opioid while on naltrexone, the opioid will therefore have no effect (*87*).

Naltrexone Pharmacology

Naltrexone comes in two formulations, 50 mg oral tablets or 360 mg extended release intramuscular injection. The tablets have been FDA approved for treatment of opioid dependence since 1984. The extended release injection received FDA approval for treatment of opioid dependence in 2010 after it was demonstrated in a double-blind, placebo controlled trial in Russia to reduce illicit opioid use and enhance treatment retention (*88*). In this formulation naltrexone microspheres are encapsulated in a biodegradable polylactide-coglycolid polymer that slowly degrades and releases naltrexone into the surrounding tissue following deep intramuscular injection (*89*).

Experimental formulations of naltrexone as a subcutaneous implant which release active medication over a 2 month or longer interval, while still undergoing evaluation, appear safe and efficacious (*90*, *91*).

Naltrexone Pharmacokinetics

Absorption occurs rapidly and completely after oral ingestion of Naltrexone with 80%-95% of the oral dose undergoing first pass hepatic metabolism (*89*, *92*). Because naltrexone acts as an antagonist, initial subjective or objective effects are scant in the opioid free individual. Peak plasma levels reached on average about

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1 hour after ingestion (*89*, *92*). Oral naltrexone has an estimated average terminal half-life of 4 hours (*89*, *92*). Protein binding is estimated at 20% (*92*).

Absorption also occurs fairly rapidly with the long acting injectable formulation. Naltrexone located at or near the surface of the microspheres is quickly released, giving an initial peak in plasma concentrations 1 to 2 hours after administration (*89*). Concentrations begin to decline 12 hours following administration but increase again 1 day after administration as naltrexone embedded deeper in the microspheres is released, showing a second and higher peak about 2 days after administration (*89*). At about day 14 after administration, plasma naltrexone concentrations decline gradually (*89*). Concentrations are measurable for longer than 35 days (*89*). After sequential dosing the average half-life of naltrexone from the long acting injection is approximately 5 days (*89*).

The metabolism of naltrexone is not catalyzed by CYP 450 enzymes but by aldo-keto reductase enzymes AKR1C1, AKR1C2, and AKR1C4, previously designated as dihydrodiol dehydrogenase enzymes (DD1, 2, and 4) (*93*). Naltrexone undergoes reduction via these enzymes to the active metabolite 6-β-naltrexol. Both parent and metabolite can also undergo glucuronidation (*94*). 2-Hydroxy-3-O-methyl-6-β-naltrexol is a minor metabolite found in trace amounts. The main route of elimination for both parent drug and metabolites is renal with much lesser amounts in the feces (*95*). After oral dosing 6-β-naltrexol levels peak at one hour, and the half-life is about 13 hours (*89*). After the long acting injection, 6-β-naltrexol levels peak at 3 days, and after repeated dosing the half life is about 5 days (*89*). Ratios of plasma levels of metabolite and parent drug are quite different between oral dosing and injection because of decreased first pass metabolism with the injection. For oral dosing the ratio of 6-β-naltrexol to naltrexone is 10:1, but for injection it is 1:1 (*89*). The extended release injection of 380 mg has an area under the curve of naltrexone exposure over 28 days 4 times the area under the curve for the oral form given at 50 mg per day for 28 days (*89*).

Naltrexone Pharmacodynamics

Although naltrexone is believed to function as a non-specific opioid antagonist and have some capacity to block δ- and κ-opioid receptors (*96*, *97*), it exerts its clinical effects primarily by acting as an antagonist at the μ-opioid receptor (*98*). 6-β-naltrexol has weaker antagonist effects than the parent drug (*99*).

Clinical Use of Naltrexone

In order to begin naltrexone, the patient must be completely withdrawn from opioids and free of signs and symptoms of opioid withdrawal. This process typically takes from 3-6 days for short-acting opioids and up to 10 days for methadone. As with buprenorphine, if any opioids remain on the receptor at the time of naltrexone administration, it will precipitate severe opioid withdrawal. Therefore, a procedure called a naloxone challenge test is performed prior to

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administration of naltrexone for opioid dependence/opioid use disorder (*87*). Because of the relatively long half-lives of naltexone and its active metabolite, any withdrawal precipitated by naltrexone would last many hours. Naloxone has a short half-life. Any precipitated withdrawal caused by naloxone would last only a couple of hours. Once the physician is satisfied that the patient is fully withdrawn from opioids and opioid free, and baseline vital signs are obtained, naloxone is administered parenterally (subcutaneous, intramuscular, or intravenous) to a total dose of 0.8 mg. The patient is observed for emerging symptoms or signs of opioid withdrawal or elevations in heart rate or blood pressure. If any indication of even mild withdrawal is observed, the induction onto naltrexone is postponed at least 24 hours, and the naloxone challenge is repeated. If none is observed, naltrexone, can then be administered orally in a dosage of 25 to 50 mg (one-half to one tablet). If the oral medication is well tolerated over one hour, the extended release injection can be administered if desired. If precipitated withdrawal occurs from either naloxone or naltrexone, it should be managed similarly to precipitated withdrawal from buprenorphine, that is with an α_2 agonist and ancillary medications to reduce symptoms.

The usual oral naltrexone dose is 50 mg daily. It is also possible to use a 3 day per week schedule of 100 mg on Mondays and Wednesdays and 150 mg on Fridays. However, now that the extended release form is available, if concerns exist about medication adherence, it probably makes sense to use the extended release preparation. Since the extended release preparation maintains therapeutic blood levels for more than 30 days, it can be given as a deep intramuscular gluteal injection of 380 mg every 28 or 30 days using opposite sides of the buttocks for every other injection. Once the patient is stabilized on naltrexone, the dose is simply maintained unless side effects supervene.

Naltrexone Drug Interactions

Because naltrexone metabolism does not depend upon CYP 450 system, it does not affect the metabolism of other medications, and the only important interactions are with opioids. Clearly, naltrexone will block the effects of other opioids. This interaction presents a potential challenge if a patient on naltrexone unexpectedly needs treatment with opioid analgesics, for example, after serious physical trauma or an emergent medical or surgical condition such as acute pancreatitis or appendicitis. In such an event the patient must be admitted to the hospital for careful monitoring and treated with high intravenous doses of a potent opioid such as fentanyl, hydromorphone, or morphine until the blockade is overcome. In this scenario there is the theoretical potential of an opioid overdose with respiratory depression so that hospital staff needs to be prepared to rescue the patient with intubation and mechanical ventilation. Patients at risk to use large quantities of illicit opioids intravenously also need to be warned of this theoretical risk of overdose. In addition, patient need to e warned of the risk after stopping naltrexone. Since opioid tolerance dramatically decreases during the time patients take naltrexone, a high risk for opioid overdose is present after the medication is discontinued (*100*).

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Naltrexone Side Effects

Common side effects of naltrexone include nausea, diarrhea, dizziness, headache, and insomnia. Typically these annoying but not dangerous side effects appear early in treatment and tend to dissipate so that often patients can be coached through them. If necessary, ancillary medications, such as anti-emetics, can be prescribed. On the potentially more serious side, Naltrexone has a boxed warning for hepatic injury, but in practice no serious or lethal hepatic toxicity has been observed. Nevertheless, it is standard practice to obtain liver function tests prior to and during treatment. Should liver transaminases show a marked upward trend (5-10 times the upper limit of normal) in the absence of other potential etiologies, consideration should be given to discontinuing naltrexone. Depression and suicidal ideation have also been reported. These psychiatric adverse events should be handled as they would for any other psychiatric patient with antidepressants and/or psychotherapy for depression and potential hospitalization for suicidal ideation. If naltrexone is deemed causative, it clearly should be discontinued. The extended release preparation has the additional potential side effect of injection site reactions. Mild injection site reactions can usually be managed with palliative measures like hot compresses and over-the-counter analgesics. In rare severe cases antibiotics or minor surgical intervention might be necessary.

Efficacy of Naltrexone for Opioid Dependence/Opioid Use Disorder

Despite its seemingly ideal pharmacological characteristics, oral naltrexone has not proved to be a widely used treatment because the need to taper off opioids imposes a barrier to patients getting on it, and even when patients succeed in starting naltrexone, the early dropout rate is high, and medication adherence may be less than ideal. A meta-analysis of 10 randomized, placebo-controlled trials of oral naltrexone for opioid dependence/opioid use disorder with 696 participants was conducted. When studies that compared naltrexone versus placebo combined with psychosocial treatment were pooled with studies comparing naltrexone and placebo without psychosocial treatment, naltrexone did have a slight statistically significant edge over placebo in reducing illicit opioid use when participants remained in treatment but no advantage in terms of retention in treatment (*101*). A separate meta-analysis of 15 randomized, controlled trials including 1071 participants came to a roughly analogous conclusion noting that retention moderated illicit opioid use and that participants with high retention who received naltrexone showed reduced opioid use (*102*). Studies in that meta-analysis which used contingency management with naltrexone had better results (*102*). It does appear that oral naltrexone performs well in clinical situations that involve external sanctions. For example, a study of federal probationers or parolees who could be returned to incarceration for drug use randomly assigned participants to naltrexone or no medication in open label fashion. Retention rates at 6 months were 52% for naltrexone-treated participants vs. 33% for participants with no medications, and rates of illicit opioid use were 8% versus 30% respectively (*103*). A study of oral naltrexone in Russia, where methadone and buprenorphine are not available and where participants tend to live with their family of origin

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and hence are under external motivation from parents, randomized 52 participants to naltrexone versus placebo in double blind fashion (*104*). Naltrexone showed superiority in outcomes of both retention and relapse prevention. The few placebo controlled randomized trials done so far with extended release naltrexone suggest that the active medication improves both treatment retention and illicit opioid use (*88*, *105*).

Behavioral Interventions for Opioid Dependence/Opioid Use Disorder

Studies of various intensities of psychosocial services in licensed methadone programs indicate that patients who receive minimal psychosocial services do not fare as well as those who receive moderate or high levels of services (*106–108*). However, the lower cost-effectiveness of more intensive services may nullify any slight advantage they hold over moderate services (*108*, *109*). Two studies of patients on buprenorphine demonstrated similar results with no added benefit from additional counseling added to routine counseling (*12*, *110*). In fact, in one of these studies, patients who received only standard medical management from their physicians did just as well as patients who also got additional counseling (*12*). At this time no evidence supports superiority of any specific type of behavioral intervention for patients with opioid dependence/use disorder, but a moderate amount of some type of behavioral intervention certainly improves outcomes. However, contingency management in which patients may receive a voucher with monetary value for providing a urine specimen negative for illicit drugs definitely decreases rates of illicit drug use during methadone and naltrexone treatment over and above rates achieved by routine counseling (*111*, *112*).

Disorders Co-Occurring with and Needing Clinical Attention in Opioid Dependence/Opioid Use Disorder

Such disorders include other substance use disorders, psychiatric disorders, and medical disorders.

Other Substance Use Disorders

Patients with opioid dependence/opioid use disorder also have high rates of misuse of other substances. Since problematic use of other substances can undermine stability in treatment of opioid dependence/opioid use disorder, misuse of other substances frequently necessitates additional active interventions.

Alcohol

Among patients seeking treatment for opioid dependence/use disorder, rates of alcohol use disorder range from 24% for a current to 50% for a lifetime diagnosis (*113*, *114*). Excessive alcohol use causes serious medical and psychiatric

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morbidity, and, of particular concern, worsens the prognosis in chronic Hepatitis C. Alcohol can act additively with methadone and buprenorphine to suppress central nervous system activity and respiratory drive, increasing the risk of overdose. Alcohol also can induce the activity of cytochrome P-450 enzymes (*115*), thereby inducing the metabolism of methadone and potentially destabilizing the patient. Although naltrexone has efficacy for alcohol use disorder (*116*) in addition to opioid dependence/opioid use disorder, methadone and buprenorphine specifically target only the latter. Naltrexone might have some advantages as a treatment modality when co-occurring alcohol and opioid dependence/use disorder are identified prior to treatment entry. Alcohol use disorder when recognized during treatment requires active intervention. The mainstay of treatment should include one or more behavioral interventions including contingency management, motivational interviewing, relapse prevention, and 12-step facilitation with referral to Alcoholics Anonymous. Pharmacological interventions for alcohol dependence can also be considered. Naltrexone is obviously contraindicated in patients on methadone or buprenorphine. However, monitored disulfiram is used frequently, and clinical case reports suggest some benefit (*117*), though a randomized controlled trial found no difference in outcomes between disulfiram and placebo treatments (*118*). Acamprosate has not been studied in patients with opioid dependence/use disorder.

Benzodiazepines

Benzodiazepine misuse also commonly occurs in patients with opioid dependence/use disorder. The prevalence of current benzodiazepine misuse in methadone treated patients has been estimated between 24.9% and 50.6% (*119*). As occurs with alcohol, the respiratory depressant effects of benzodiazepines on top of methadone or buprenorphine are additive. If the benzodiazepines are prescribed, sometimes an alternative agent may be used for insomnia or anxiety. In the case of benzodiazepine misuse, a taper will be necessary since abrupt cessation of benzodiazepines can cause a medically significant withdrawal syndrome. The taper could be done slowly on an outpatient basis or more rapidly via admission to an inpatient unit. Outpatient tapers often prove difficult because patients become symptomatic and continue use of illicit benzodiazepines. Experimental methods still undergoing study involve the substitution of anticonvulsants, which act through the gama-aminobutyric acid (GABA) system (such as pregabalin, gabapentin, topiramate, or valproic acid) for benzodiazepines (*120*).

Cocaine and Amphetamines

A diagnostic study of 716 patients who recently entered methadone treatment showed a lifetime rate of cocaine dependence of 64.7% and a current rate of 40.7% (*114*). Behavioral treatments, particularly contingency management and cognitive-behavioral therapy, effectively reduce cocaine use among methadone patients (*121*). To date no specific pharmacotherapy has

unequivocally demonstrated efficacy for cocaine dependence. Naltrexone was shown to reduce amphetamine use compared to placebo in individuals without opioid dependence/use disorder (*122*), but in a recent trial in individuals with co-occurring opioid dependence and methamphetamine dependence, while compared to placebo, a naltrexone implant significantly reduced illicit opioid use, it showed insignificant differences compared to placebo in changing methamphetamine use (*91*).

Cannabis

Cannabis use among methadone patients is common but has not been well studied among patients on buprenorphine or naltrexone. Among methadone maintenance patients, in contrast to the deleterious effects of alcohol, benzodiazepines, or cocaine use on methadone patients, cannabis use itself does not have a measurable negative effect on typical methadone treatment outcomes such as treatment retention, illicit opioid use, or employment (*123*). Possibly some patients with opioid dependence/use disorder with co-occurring cannabis dependence experience the low motivation and cognitive disruption seen with the latter disorder and could benefit from cannabis abstinence.

Co-Occuring Psychiatric Disorders

Patients with opioid dependence/opioid use disorder have higher rates of some co-occurring, non-substance related, psychiatric disorders than rates seen in the general population. Major depression, bipolar disorder, and anxiety disorders are frequently seen. Rates of schizophrenia are not different from what occurs in the general population, about 1%. Rates of personality disorders are also elevated with antisocial personality being the most common (*113*, *114*). Regarding posttraumatic stress disorder, rates were determined to be 20% for women and 11% for men among opioid dependent patients in a study of this specific disorder (*124*). Eating disorders and attention deficit hyperactivity disorder occur commonly in opioid dependent individuals (*125*, *126*).

Since high rates of substance-induced psychiatric disorders also occur among opioid dependent individuals, assessment for co-occurring psychiatric disorders often entails a thorough psychiatric interview. Ideally, following a patient until several weeks of abstinence from illicit opioids have elapsed to see if symptoms spontaneously remit may indicate that the disorder is not a primary psychiatric disorder but rather a substance-induced disorder and eliminate the need for specific treatment. Depressive symptoms frequently improve substantially for many patients during their initial weeks of treatment for opioid dependence/opioid use disorder (*127*).

Active treatment focused on the non-substance induced co-occurring psychiatric disorders is encouraged. Treatment for these disorders is similar to that delivered to any psychiatric patient including pharmacotherapy and psychotherapy, although studies among patients on methadone maintenance indicate that in treatment of major depression, tricyclic antidepressants lead to

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statistically significant improvements compared to placebo (*128*, *129*), whereas serotonin reuptake inhibitors were not superior to placebo in this population (*130*, *131*).

Psychotherapy delivered by trained professionals shows added benefit for patients in methadone maintenance who have moderate or high psychiatric severity, but does not contribute any advantage in patients with low psychiatric severity (*132*).

Co-Occurring Medical Disorders

Medical disorders often develop among individuals with opioid dependence/ opioid use disorder as a consequence of the route of drug administration. Injection drug use incurs risk for infectious diseases including pneumonia, tuberculosis, endocarditis, sexually transmitted diseases, soft tissue infections, bone and joint infections, central nervous system infections, Hepatitis B and C, and human immunodeficiency virus (*133–135*) . Drug smoking can result in pulmonary disease. Cocaine and methamphetamine, taken by any route, can also cause myocardial ischemia and/or infarction as well as cardiac arrhythmias, cerebrovascular accidents, seizures, gastroduodenal ulceration, and acute renal failure. Excessive alcohol use has the potential to damage nearly every organ system. Patients will need attention for any of these co-occurring medical disorders.

Both acute and chronic pain can result as a consequence of these co-occurring medical disorders or from traumatic injury. When pain occurs, conservative measures such as non-steroidal anti-inflammatory medications can be attempted, but these patients often experience hyperalgesia and may not show much response to such interventions. Naltrexone may not be a good choice of medication for patients with pain disorders because it would block the effects of administered opioids if they were needed. Buprenorphine has considerable analgesic activity (*136*) and helps to stabilize pain in many patients with evidence of opioid misuse (*137*). For those who continue to suffer from intractable pain, higher doses of buprenorphine up to 32 mg per day given in divided doses can be tried, or a switch to methadone maintenance could be considered. It should be recognized that neither the regular daily dose of methadone nor buprenorphine, which is intended to stabilize the patient and prevent withdrawal symptoms, may have much effect on pain. For patients on methadone, additional opioid analgesics can be prescribed if indicated, but this strategy rarely works with buprenorphine because it has high affinity for the mu-opioid receptor and by occupying the receptor prevents other opioids from having from having much effect. In circumstances of severe acute pain or emergency elective or surgical procedures, naltrexone or buprenorphine may have to be temporarily discontinued and high dose full agonist opioids given under close observation.

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Conclusion

Since opioid dependence/opioid use disorder is increasing in prevalence, clinicians need to be aware of potential treatment options for this condition. Patients with opioid dependence/opioid use disorder should almost always receive pharmacotherapy directed at this condition because outcomes without medication are often dismal. Each of the 3 medications approved for treatment of opioid dependence/opioid use disorder has its own unique characteristics which require considerable knowledge and skill for appropriate prescribing and management. When patients get stabilized on one of these medications, they also may need interventions for co-occurring other substance, psychiatric, and medical disorders to attain optimal treatment outcomes.

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Chapter 6

Buprenorphine in the Treatment of Neuropathic Pain

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This chapter reviews the current preclinical and clinical data for the role of buprenorphine in the treatment of neuropathic pain syndromes. The published findings seem to support hypotheses regarding the rather unique analgesic mechanisms of buprenorphine as compared with pure μ-opioids like morphine and fentanyl. However, the exact mechanism of its analgesic efficacy still remains largely unknown despite recent advances in preclinical pharmacological studies. Such assessments have demonstrated the sustained antihyperalgesic effect of buprenorphine in diverse animal pain models. These findings are supported in a growing number of clinical studies of oral, intrathecal, intravenous and transdermal buprenorphine. This chapter focusses mainly on the clinical experience concerning the transdermal administration of buprenorphine in neuropathic pain, although preclinical aspects are also addressed in order to provide the readers a complete picture of the unique pharmacological properties of this analgesic drug. A mounting evidence indicates the appropriateness of transdermal buprenorphine in the treatment of diverse neuropathic pain conditions which are often less or not responsive to other opioids. Further studies are certainly warranted to identify even better the clinical syndromes that are most sensitive to buprenorphine treatment, and to compare buprenorphine to other opioids in head-to-head trials of acute and chronic neuropathic pain.

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Introduction

The International Association for the study of Pain (IASP) defines neuropathic pain as "Pain initiated or caused by a primary lesion or dysfunction or transitory perturbation in the peripheral or central nervous system" (*1*).

Despite intensive basic and clinical research this therapeutic area remains one of the least satisfactorily covered by current analgesics. Pharmacological treatment of neuropathic pain still lacks a fully validated rationale. In particular this is true with respect to opioids, with evidence indicating that neuropathic pain may be relatively insensitive to typical μ-opioid analgesics such as morphine. With the role of the "classical" μ-agonists in the treatment of neuropathic pain still remaining controversial, this chapter will show the growing evidence in support of a potentially unique role for buprenorphine in the treatment of neuropathic pain.

Neuropathic Pain as a Complex Clinical Identity

According to the current concept of neuropathic pain, both peripheral and central mechanisms contribute to the abnormal, painful sensations that are observed in the clinical setting. However, evidence is rapidly increasing that central sensitization is present in virtually all types of neuropathy with more and differing pathophysiological changes being linked to specific clinical symptoms. This has in a large part been possible through the development of various animal models, mimicking the various clinical syndromes. However, most of the basic science research has been conducted on mechanical types of injury, whereas thermal and toxic have been understudied. This is one of the reasons why clinical studies on neuropathic pain often focus on the response of drug treatments in mechanical hyperalgesia and allodynia. One of the most commonly used classification systems is the phenomenological classification, relating to the type of damage or related pathophysiology that causes the painful neuropathic condition. Painful neuropathies are characterized by spontaneous and/or abnormal stimulus-evoked pain symptoms. Unfortunately, we do not yet have the sophistication to target individual therapeutic agents at individual symptoms, partly due to the lack of corresponding animal models. Therefore the current goal is to reduce the manifestations of neuropathic pain. To a large extent the current basis of pharmacological treatment relies on drugs belonging to 4 defined classes: analgesics (often opioids), tricyclic antidepressants, antiepileptics and membrane stabilisers (*2*). Analgesics are primarily used for the treatment of nociceptive pain conditions subserved by a functionally normal nervous system. There is still no real consensus as to the effect of opioids on pain induced by nerve injury or an abnormal nervous system.

Effectiveness of Opioids in Painful Neuropathies

There is still some controversy over using opioids for managing neuropathic pain syndromes. The debate is largely fuelled by a lack of definitive data. Until recently many scientists and clinicians were convinced that neuropathic pain was rarely or only partially relieved by opioid analgesics. Opioids were reported to

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be ineffective in producing analgesia in one key study (*3*), but effective due to dose escalation in another (*4*). Moreover, any initial subjective pain relief would be lost through early development of tolerance (*4–6*). This last statement had already been challenged using retrospective data suggesting that opioids indeed were effective as long-term treatment for different types of non cancer related pain (*7*). More recently, firm evidence has emerged confirming an analgesic effect of opioids on a variety of neuropathic pain conditions (*8*, *9*). The level of analgesia obtained is however lower than in patients suffering from nociceptive pain (*10*). As a consequence it is now generally acknowledged that neuropathic pain should not be considered as opioid resistant but rather as less sensitive to the systemic administration of opioids (*4*, *10*). This diminished efficacy of opioids could be explained by changes that occur in the opioid mechanisms in neuropathic syndromes. It is now postulated that in most cases this negative impact could be overcome by a dose escalation (*11*).

Basic Considerations on the Unique Profile of Buprenorphine

The pharmacological effects of opioids are derived from their complex interaction with different opioid receptor types found peripherally, centrally and in structures belonging to the descending inhibitory system that modulates pain at the level of the spinal cord (*12*). At a cellular level, the effects of opioids include a decrease in presynaptic transmitter release, hyperpolarisation of postsynaptic elements and disinhibition. However, increasing evidence has become available indicating that the analgesic mechanisms of buprenorphine may be sufficiently different from those of other opioids to the extent that it may be of special use in the management of neuropathic pain in humans.

Buprenorphine is a semi-synthetic opioid that is used to treat opioid addiction in higher dosages (>2 mg), to control moderate acute pain in non-opioid-tolerant individuals in lower dosages (~200 μg), and to control moderate chronic pain in dosages ranging from 20–70 μg/hour. It is available in a variety of formulations: Subutex®, Suboxone® (buprenorphine HCl and naloxone HCl; typically used for opioid addiction), Temgesic® (sublingual tablets for moderate to severe pain), Buprenex® (solutions for injection to treat pain), Transtec®, Norspan® and Butrans® (transdermal preparations used for chronic pain). Buprenorphine hydrochloride was first marketed in the 1980s by Reckitt & Colman as an analgesic, generally available as Temgesic® 0.2 mg sublingual tablets, and as Buprenex® in a 0.3 mg/mL injectable formulation. Buprenorphine is classified both as an orvinol and as a thevinol, which means it can be derived from either oripavine or thebaine (see figure 1 for chemical structure of buprenorphine). It is one of the Bentley compounds discovered by chemist K.W. Bentley, using thebaine as the initial "backbone" structure. Thebaine is one of the main alkaloids in the Iranian poppy (*Papaver bracteatum*). Papaver bracteatum is a sturdy perennial poppy with large deep red flowers up to 8 inches (20 cm) across on stiff stalks up to 4 feet (1.22 metres) high with a prominent black spot near the base of the petals. It is related to the commonly cultivated oriental poppy, *Papaver orientale*. Non-horticultural use of this species is for the production of thebaine,

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which is commercially converted to codeine and semi-synthetic opiates. Papaver bracteatum does not contain morphine or codeine and no other narcotic alkaloids in significant amounts. Oripavine was reported in minute traces but would not exert a relevant activity.

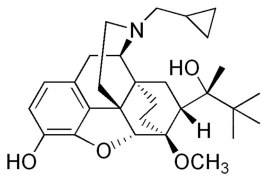

Figure 1. Molecular structure of buprenorphine. Buprenorphine has a molecular weight of 467 and its structure is typically opioid with the inclusion of a C-7 side-chain containing a t-butyl group. This group confers overall lipophilicity on the molecule that has an important influence on its pharmacology, as detailed in the text.

Buprenorphine is not administered orally due to very high first-pass metabolism. Buprenorphine is metabolized by the liver, via CYP3A4 (also CYP2C8 seems to be involved) isozymes of the cytochrome P450 enzyme system, into norbuprenorphine (by *N*-dealkylation). The glucuronidation of buprenorphine is primarily carried out by UGT1A1 and UGT2B7, and that of norbuprenorphine by UGT1A1 and UGT1A3. These glucuronides are then eliminated mainly through excretion into the bile. The elimination half-life of buprenorphine is 20–73 hours (with a mean of 37 hours). Due to the mainly hepatic elimination, there is no risk of accumulation in patients with renal impairment. Buprenorphine's main active metabolite, norbuprenorphine, is a μ-opioid, δ-opioid, and nociceptin receptor full agonist, as well as a κ-opioid receptor partial agonist. Buprenorphine antagonizes its effects.

Buprenorphine has displayed strong efficacy in a number of assays, maintaining some very distinct characteristics compared to other opioids like morphine and fentanyl (*13*). The formalin test in neonatal rats has been postulated as of special merit for evaluating the efficacy of potential analgesics for the treatment of painful neuropathies (*14*, *15*). When comparing the antinociceptive effects of several different opioids, it was observed that buprenorphine was significantly more effective in the neonatal formalin test than after formalin injection in adult rats (*16*); other opioids were all equipotent in both neonatal and adults. This greater antinociception by buprenorphine in neonates suggestes that its analgesic mechanisms may be sufficiently different from that of other opioids, making it of special use in the treatment of neuropathic pain.

Another possible mechanism explaining the beneficial effect of buprenorphine in neuropathic pain was suggested by the discovery that, in rat, low doses of systemically administered buprenorphine (0.3 – 3 μg/kg) block diffuse noxious inhibitory controls (DNIC) (*17*). These inhibitory controls are triggered by heterotopic noxious stimuli and are thought to facilitate the extraction of nociceptive information from neurons activated by noxious stimuli (*18–24*). Blockade of DNIC will lead to a much less pronounced difference between

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incoming noxious input and the 'normal' background neuronal activity which in turn could lead to an inability to discriminate between noxious (painful) and non-noxious (background) neuronal activity. DNIC are also blocked by morphine but only at very high doses (*25*), low doses only resulting in an effective blockade if administered centrally (*26*, *27*). Consequently, function after administration of buprenorphine in neonatal rats could be possibly linked to a supraspinal effect on DNIC (*28–31*).

Intrathecal pertussis toxin (PTX) induced thermal hyperalgesia and allodynia display significant similarities to those produced by neuropathic pain models involving nerve ligation or nerve section (*32*). Through an inactivation of tonic and phasic inhibitory effector systems, PTX administration will likely lead to a predominance of excitatory activity, which in turn could lead to the occurrence of a wind-up phenomenon of the central pathways of nociceptive processing (*32*). This 'wind-up' will result in exaggerated and aberrant responses to both innocuous and noxious stimulation. It has been shown in several animal models that morphine and fentanyl antinociception is blocked in a dose dependent manner by treatment with PTX whereas buprenorphine antinociception is relatively unaffected. In the rat tail flick test, buprenorphine-mediated antinociception was not affected by the prior administration of PTX, whereas morphine-induced antinociception was eliminated (*33*). In phase-2a of the formalin test in adult rats, buprenorphine analgesia is only modified by PTX at the highest dose (above 1 mg/kg, subcutaneous administration) whereas morphine antinociception is significantly inhibited by PTX at low doses (less than 3 mg/kg S.C.) (*33*). These data provide significant proof of the concept that buprenorphine-induced antinociception is transmitted through PTX-insensitive pathways, whereas PTX-sensitive pathways become progressively involved at higher doses of buprenorphine. Buprenorphine is currently the only opioid demonstrating an antinociceptive effect in different types of animal models via PTX-insensitive pathways (*33*), whereas other opioids transduce their analgesic signal via PTX-sensitive inhibitory systems (*32*). Recently, as it has become evident that activation of PTX-sensitive and –insensitive pathways result in divergent effects upon different receptor systems, this special effect of buprenorphine could prove to be of major clinical relevance. Evidence also indicates that buprenorphine, through activation of PTX-sensitive proteins, will lead to activation of a mitogen-activated protein kinase (MAPK) pathway (*34*), a possible important target for the treatment of neuropathic pain.

Buprenorphine displays some specific interaction with the subtype 3 κ opioid receptor (κ_3) (*30*) and there is some circumstantial evidence linking these subtype opioid receptors to neuropathic pain. For example, serotonin specific re-uptake inhibitors (SSRI) potentiate analgesia mediated by the κ_3 receptor subtype whilst having absolutely no effect on the μ-receptors (*31*). Moreover, κ-opioid agonists are potent antinociceptive agents against formalin-induced pain, both in neonates and adults, while having no antinociceptive effect in the tail flick test (*35*).

Numerous studies have demonstrated that agonists of several G-protein-coupled receptors ($G_{i/o}$), such as μ− and δ−opioid receptors and α_2−receptors, open specific K^+ channels in neurons (*36*). $G_{i/o}$ proteins can open two different types of K^+ channels: the K_{ATP} (*37*, *38*) and the GIRK channels (*39*). The involvement of these two types of K^+ channels in opioid-induced antinociception

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has been studied extensively. It was suggested that the opening of K_{ATP} channels plays an important role in the analgesia induced by morphine at supraspinal, spinal and peripheral levels and it was also shown that buprenorphine opens peripheral K_{ATP} channels (*40*). Moreover, buprenorphine itself seems to be very sensitive to the effects of K_{ATP} channel openers and blockers (*41*). In contrast, morphine and methadone analgesia is only modestly enhanced or attenuated by K_{ATP} channel openers and blockers respectively. Fentanyl even demonstrates absolutely no interactions with K_{ATP} agents. Consequently, this suggests that at least two subgroups can be distinguished among μ-opioid receptor agonists, each inducing antinociception through different effector mechanisms. These K_{ATP} channels represent novel opportunities for augmenting opioid analgesia, certainly in pain syndromes where there is altered expression of these ion channels, such as is often the case in neuropathic syndromes (*42*, *43*).

Several authors have described an agonist effect of buprenorphine at the nociceptin (ORL-1) receptor (*44–47*). The role of the opioid-like receptor 1 (ORL-1) and its endogenous ligand nociceptin/orphanin FQ (N/OFQ), in nociception, anxiety and learning remains to be defined. Buprenorphine was identified as a full agonist at the ORL-1 receptor with an IC(50) value of 8.4 +/- 2.8 nM. Fentanyl and 7-benzylidenenaltrexone displayed a weak agonistic activity at the ORL-1 receptor. Compared to nociceptin, buprenorphine exhibits a lower degree of agonism (50-70%) at the nociceptin receptor, leading to antinociception, especially at higher doses, via nociceptin mediated mechanisms. At the same time, activation of supraspinal nociceptin receptors following systemic administration of buprenorphine may well counteract this analgesic effect (*34*). Conversely, sole activation of spinal nociceptin receptors by buprenorphine may lead to an important antinociceptive effect which might explain the strong analgesic action observed after the intrathecal administration of buprenorphine (*48–51*), even although some evidence suggests a supraspinal site of action after neuraxial adminstration (*52*, *53*). Overall, the clinical result of the administration of buprenorphine by whatever route is dose-related analgesia and, therefore, the precise involvement of the nociceptin receptor remains enigmatic.

Preclinical Evidence on Buprenorphine in Experimental Models of Neuropathic Pain

Over the years, a large body of preclinical data on the analgesic effect of buprenorphine has been published. However, most of these studies were performed in animal models of acute pain. It is only recently that a more thorough preclinical assessment of the analgesic efficacy of buprenorphine in animal models micking neuropathic pain conditions has been pursued.

Wang et al. (*54*) observed that buprenorphine produced effective antinociception at doses (0.22 μg/kg) lower than those required to produce antinociception to noxious heat stimuli. These analgesic effects were blocked by naloxone. Cold hyperalgesia is not only relatively resistant to morphine, but is generally considered as a clinical sign of 'chronicity' of a pain syndrome. Therefore the important difference in potency of three orders of magnitude

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between buprenorphine and morphine in this experimental model could be clinically very relevant.

A report on the effect of buprenorphine in animal models of spinal cord and peripheral nerve injury induced pain was published by Kouya et al. (*55*). Photochemically induced spinal cord injury and partial sciatic nerve ischemia were used, of which the former induces mechanical allodynia, representing a model for central pain (*56*). This model has been shown to be particularly resistant to systemic morphine (*56*, *57*) and partial sciatic nerve ischemic injury can be considered as a representation of peripheral nerve injury (*58*). This model of nerve ischemia produces mechanical, heat and cold allodynia, as well as signs of spontaneous pain (*58*). Cumulative doses of buprenorphine effectively alleviated both mechanical and cold hyperalgesia in the sciatic nerve model with significant antinociceptive effect. After photochemical spinal cord lesions, rats developed reductions in vocalization threshold to mechanical stimulation together with an increased response to cold stimulation (hyperalgesia). Again, cumulative doses of buprenorphine significantly decreased the mechanical and thermal hyperalgesia-behaviours in the injured rats. Doses of 0.03 mg/kg significantly increased vocalization threshold to mechanical stimuli and completely normalised sensitivity to cold. The effective (cumulative) doses of buprenorphine in both rat models were identical to those producing moderate analgesia on the hot plate test, an acute pain animal model, but more effective and longer lasting. In both nerve injury induced pain models, even the highest doses of buprenorphine caused no sedation, in contrast to the pronounced sedative effects of morphine in the same animal models. This could be explained by the higher doses of morphine (at least 5 mg/kg) needed to obtain an anti-nociceptive effect thus resulting in pronounced sedation (*56*, *57*, *59*). It is finally important to stress that the antinociceptive effect of buprenorphine in these animal models of nerve injury induced pain was both total and prolonged. These findings provide strong evidence that the analgesic mechanisms for buprenorphine may be different depending on the functional model and, thus, depending on the pathophysiologic mechanism (*33*, *41*). In parallel to the results of the study by Wang (*54*), it seems again that buprenorphine exerts a particularly strong antinociceptive effect on conditions mimicking the clinical symptom of cold hyperalgesia.

Recently Christoph et al. (*60*) studied the analgesic profile of buprenorphine in different rodent models of acute and chronic pain. The animal models included models of somatic, visceral, inflammatory and neuropathic pain conditions. In addition, a broad range of stimulus qualities, such as chemical, thermal and mechanical, were applied in this study as previous studies have shown that buprenorphine exerts a differential antinociceptive effect depending on the type of stimulus involved (chemical or pressure against thermal) (*61*). The antinociceptive effects of buprenorphine were compared to clinically relevant reference molecules (morphine and gabapentin). The neuropathic pain animal models included the sciatic chronic constriction injury (CCI), spinal nerve ligation, the streptozocin model for diabetic polyneuropathy and the vincristine model. Administration of buprenorphine resulted in a strong and dose-dependent alleviation of tactile allodynia in the spinal nerve ligation model. Gabapentin was applied as the reference compound and showed less inhibitory effect on

the postoperative tactile allodynia compared to buprenorphine. In the sciatic chronic constriction model administration of buprenorphine led to an inhibition of cold allodynia in a dose-dependent manner with an ED_{50} value of 0.036 mg/kg iv. In both models administration of doses above the maximal possible effect (MPE) resulted in significantly diminished antinociceptive effect. Buprenorphine caused a dose-dependent inhibition of both mechanical hyperalgesia and cold allodynia in streptozocine and vincristine treated animals. In contrast with the CCI and spinal nerve ligation model, doses beyond the MPE value did not result in appearance of inverted dose-response curves. This result shows that the shape of the dose-response curve of buprenorphine is not dependent upon the type of pain stimulus, since inverted u-shaped curves were obtained with thermal and mechanical stimuli in mononeuropathic pain models but not in polyneuropathic pain models. Finally, this study demonstrates that buprenorphine is fully effective both in mononeuropathic and polyneuropathic pain models in animals.

Another study assessed the role of continuous buprenorfphine delivery in the treatment of diabetic peripheral neuropathy (DPN) (*62*). Herefore the well-established experimental streptozotocine-induced rat model for DPN was used. Implantable osmotic pumps continuously administered buprenorphine at doses of 1.2 and 2.4 μg/kg/h for 3 weeks. After 6 weeks of diabetes, nerve conduction velocity (NCV) and behavioral responses to noxious mechanical and thermal stimuli were assessed. Diabetic rats showed an impairment of NCV, mechanical allodynia, and thermal hypoalgesia. Both doses of buprenorphine significantly reversed the diabetes-induced allodynia up to day 7 of treatment. Buprenorphine did not alter either thermal perception or nerve conduction velocity. These results suggest a possible nociceptive effect of buprenorphine in the management of DPN-associated neuropathic pain.

The specific analgesic and antihyperalgesic properties of buprenorphine in a new human pain model, using painful transcutaneous electrical stimulation, were investigated (*63*). Transcutaneous electrical stimulation (TENS) at high current density induced in human subjects ongoing pain and pinprick hyperalgesia as well as touch-evoked allodynia. Both intravenous as well as sublingual administration of buprenorphine led to an alleviation of hyperalgesic phenomena. Furthermore, this inhibition of hyperalgesia lasted significantly longer than the observed analgesic effect. The authors stated that further studies are needed into the causes of the different time courses of analgesic and antihyperalgesic effects (*63*).

Clinical Perspectives on Buprenorphine in Neuropathies

Opioids are well known to relieve severe, acute, and chronic nociceptive pain (such as somatic and visceral pain), but neuropathic pain is considered to show a relatively poor response to opioids. Buprenorphine seems to block central sensitization (hyperalgesia) that is commonly found with neuropathic pain conditions (*64*). In patients with neuropathic pain and tactile allodynia buprenorphine alleviates the hyperalgesia to a greater extent than pain severity. So, buprenorphine seems to be effective in reducing hypersensitivity in neuropathic

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pain when pure mu agonists fail to produce a response or in individuals who are intolerant to pure mu agonists.

An early report on the analgesic effect of buprenorphine in neuropathic pain was published by Zenz et al. (*65*). Long-term oral opioid therapy in chronic non cancer related pain was studied in 100 patients, 53 of whom suffered from neuropathic pain. Patients having recieved prior opioid treatment without any clear pain-reducing effect were treated with buprenorphine. The initial dose consisted of 0.2mg three times a day, titrated to full pain reduction or a maximum daily dose of 4.8 mg. Although 57 patients were treated with buprenorphine, specific information as to how many neuropathic patients were included was not stated. The authors stated that half the total patients showed good pain relief (decrease by 50% or more in visual analogue scale) and lower daily opioid doses (for buprenorphine, morphine and dihydrocodeine) in patients with neuropathic pain (buprenorphine 1.3mg) than in non-neuropathic pain syndromes (1.6 mg).

The value of buprenorphine in neuropathic pain was confirmed in a publication by Omote and colleagues (*66*). Two patients suffering from post-amputation phantom limb pain were treated by intrathecal administration of buprenorphine and whilst both patients had undergone previous negative trials of epidural bupivacaine, buprenorphine produced complete and long-lasting relief of the symptoms. It was noted that a single intrathecal injection of 0.1 to 0.2 mg of buprenorphine resulted in a complete analgesia lasting for 3 days. In addition, all phantom sensations were completely abolished. The authors also reported a significant increase in temperature of the lower part of the body following buprenorphine, probably produced by sympathetic inhibitory effects or by effects on the spinal thermoregulatory system. This was an interesting finding, since there are conflicting data on the effects of intrathecal opioids on body temperature. Although Rudy and Yaksh have stated that intrathecally administered morphine dose-dependently produces a hyperthermic effect (*67*), others have shown that intrathecal morphine intensifies the hypothermic action of spinal anesthesia in parturients (*68*) and that epidural sufentanil produces hypothermia (*69*).

Glynn et al. (*70*, *71*) reported the intrathecal administration of buprenorphine for painful muscle spasm in paraplegic patients. The response to intrathecal administration of 0.015 to 0.03mg of buprenorphine was investigated. All patients obtained complete relief from their painful muscle spasms for up to 48 hours following a single intrathecal injection, following which significant relief could be maintained with sublingual administration of buprenorphine.

Approximately 50% of patients following thoracic surgery will develop distressing chronic neuropathic pain (*72*, *73*). Benedetti et al. (*74*) investigated the dose-response to buprenorphine of nociceptive and neuropathic postoperative pain in 21 patients following thoracic surgery. The distinction was made between nociceptive postoperative pain (immediately after surgery) and postthoracotomy neuropathic pain (one month after surgery). One month after surgery eight patients complained of shooting and burning pain with paraesthesiae and showed allodynia around the incision, whereas the remaining 13 patients were hypoesthetic, some even showing complete anesthesia. All patients were treated with i.v. buprenorphine in a double-blind randomized design and a reduction of spontaneous pain symptoms, in both allodynic patients as well as those

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displaying hypoesthesia, was reported. Despite lower average pain scores one month after surgery (mean VAS = 8.71 immediately after surgery to mean VAS = 7.24 one month later), the ED_{50} increased after surgery (0.29 postoperatively compared to 0.50 after one month). This might indicate that neuropathic pain responds to buprenorphine but that higher doses of opioid than those which relieve nociceptive pain are necessary. No difference was observed between the ED_{50} of allodynic and hypoaesthetic-anaesthetic patients but treatment of allodynia by buprenorphine resulted in a significant increase in pain thresholds (mean stimulus intensity of 5.75 mA pre-administration versus 11.37 mA after treatment), as measured by electrical stimulation. The findings of this study indcate that neuropathic pain can be adequately treated with opioids, but higher doses may be needed. Responsiveness would appear to be a matter of dosage, as previously asserted by Portenoy (*4*).

Likar and Sittl (*75*) published a report concerning the successful use of the transdermal administration of buprenorphine for the treatment of nerve injury induced pain. Two cases of neuropathic pain and two cases of nociceptive pain with a significant neuropathic component were studied. In each case adequate analgesia was obtained and no problems were experienced in switching from the previous analgesic therapy. Switching from previous opioid therapy to buprenorphine led to dose reductions equivalent to 30%, without any serious adverse events or decreased analgesic efficacy.

Another study examined the efficacy of transdermal buprenorphine specifically in nerve injury induced pain (*74*). This retrospective multicenter study evaluated data from 237 patients. Results showed a significant efficacy of the trandermal buprenorphine patch in relieving neuropathic pain, as measured by significant decreases in VAS. An additional proof of the efficacy of this treatment could be found in the high compliance with treatment. Finally, it was shown that the transdermal buprenorphine had a good safety profile, which even improved over the course of the treatment. Others have presented case reports or small-scale clinical studies reporting effective treatment of nerve injury induced pain syndromes with either sublingual or transdermal buprenorphine administration (*73*, *75*, *76*). Although small in sample size, all of these clinical studies showed additional proof of the good analgesic efficacy of buprenorphine in painful neuropathies.

Short- and intermediate-term analgesic efficacy of buprenorphine TDS in chronic painful neuropathies was examined by Penza et al. (*76*). In this open-label study the safety, tolerability and analgesic efficacy of buprenorphine TDS were investigated. Subjects with visual analogue scale (VAS) score ≥ 5 under stable analgesic treatment were included. The starting dosage of 35 μg/h was increased up to 70.0 μg/h in case of unsatisfactory pain control, as assessed by fortnightly visits. The primary endpoint was the number of patients achieving at least 30% pain relief at day 42 visit. Treatment was considered safe over the study period. Nine patients dropped out for side effects, mostly nausea and daily sleepiness. Buprenorphine TDS was well tolerated in 21 patients. Thirteen patients achieved > 30% of pain relief at the final day 42 visit. Five patients needed to increase the dosage to 52.5 μg/h. Eight patients did not meet the primary outcome, but none allowed increasing the dosage to 70 μg/h, and four patients withdrew consent to

continue the study before day 42 visit because of a 'fear to become addicted,' although 40% had obtained VAS reduction. In this study, which of course needs to be confirmed by a randomized controlled trial, buprenorphine TDS induced clinically meaningful pain relief in about 40% of patients with chronic painful neuropathy.

Interestingly, some case reports have been published describing the application of buprenorphine TDS in the treatment of central neuropathic pain conditions (*77*). Since such central pain syndromes are extremely difficult to treat these reports have some interesting clinical value, although findings should be confirmed in pharmacological studies of higher methodological quality. Results of treatment of central neuropathic pain syndromes with buprenorphine are nevertheless encouraging, suggesting that it might represent a valid alternative to standard approaches (if already available) for central neuropathic pain.

The use of buprenorphine TDS in routing clinical practice was investigated in a large multicenter, prospective, non-comparative, non-interventional post-marketing study (*78*, *79*). Interestingly, 339 investigators in a range of clinical practice settings performed the study. Patients with chronic moderate to severe cancer pain, or chronic severe non-cancer pain that was insufficiently controlled by non-opioids were prescribed buprenorphine TDS 35, 52.5 or 70 μg/h (changed twice weekly). Treatment outcomes and side effects were followed up for 3 months. Additional analgesia, and adjuvant/supportive treatments were allowed at the discretion of the physician. The study enrolled 4030 patients, with a mean age of 62.8 years. The vast majority of patients suffered from cancer-related pain (80.7%). Non-cancer pain was generally musculoskeletal or neuropathic. A starting dose of 35, 52.5 or 70 μg/h was used in 73.4%, 21.5%, and 4.8% of patients, respectively. Buprenorphine dose was increased in 44.7% of patients during the observation, generally from 35 to 52.5 μg/hour. Mean pain intensity (using a 100 mm visual analogue scale) decreased by 73.5% from 62.3 mm at baseline to 16.5 mm after 3 months. Most patients rated pain relief as 'very good' (41.4%) or 'good' (44.5%). Sleep quality also improved. 48.1% of patients needed no additional analgesics during buprenorphine treatment. Most patients (96%) rated the buprenorphine transdermal patch as 'very easy' or 'easy' to change. The most common treatment-related reasons for discontinuation were lack of analgesic effect (3.3% of patients) and adverse drug reactions (0.8%). Most common adverse drug reactions were local skin reactions or vomiting. At study end, it was planned to continue treatment with buprenorphine TDS in 70.1% of patients. In routine clinical practice, buprenorphine TDS was shown to be effective and generally well-tolerated in patients with chronic moderate to severe cancer or non-cancer pain previously insufficiently controlled by non-opioids.

Despite the increasing clinical use of buprenorphine TDS, some questions have persisted about the pharmacological properties of buprenorphine in humans. In October 2008, a consensus group of experts met to review recent research into the pharmacology and clinical use of buprenorphine (*80*). The consensus group agreed that buprenorphine clearly behaves as a full μ-opioid agonist for analgesia in clinical practice, with no ceiling effect, but that there is a ceiling effect for respiratory depression, reducing the likelihood of this potentially fatal adverse event. This is entirely consistent with receptor theory. In addition, the

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effects of buprenorphine can be completely reversed by naloxone. No problems are encountered when switching to and from buprenorphine and other opioids, or in combining them. Buprenorphine exhibits a pronounced antihyperalgesic effect that might indicate potential advantages in the treatment of neuropathic pain. Other beneficial properties are the compound's favorable safety profile, particularly in elderly patients and those with renal impairment, and its lack of effect on sex hormones and the immune system. The expert group agreed that these properties, as well as proven efficacy in severe pain and favorable tolerability, mean that buprenorphine can be considered a safe and effective option for treating diverse chronic pain conditions. These statement and clinical properties have been confirmed in a more recent publication by Davis (*81*).

Future Developments

As stated previously it has been shown that buprenorphine exerts an agonistic activity at the level of the nociceptin receptor. The nociceptin opioid receptor is the most recently discovered member of the family of the opioid receptors (*82*). Due to the subsequent elucidation of its physiological role in both central and peripheral nervous system and in some non-neural tissues, there is a rapidly growing interest in the pharmacological application of substances active on this receptor. Despite the current clinical use of a morphine-based ORL-1/MOP mixed ligand (buprenorphine) as an analgesic and in the treatment of drug addictions, so far just a few clinical trials have been made with selective ORL1 ligands. However, the perspective of their utilization is rapidly growing. Agonists can find applications in the treatment of neuropathic pain, anxiety, cough, drug addition, urinary incontinence, anorexia, congestive heart failure, hypertension; and antagonists for pain, depression, Parkinson's disease, obesity, and as memory enhancers (*83*). Besides peptide ligands, which are still subjected to many pharmacological investigations, many different chemical classes of ORL-1 ligands have been discovered: piperidines, nortropanes, spiropiperidines, 4-amino-quinolines and quinazolines, and others (*84*). The new advances in establishing structure-activity relationships, also with the help of modeling studies, can permit the development of more active and selective molecules.

Despite the high sequence homology between ORL-1 and the opioid receptors, most current opioids lack affinity for the nociceptin receptor (*85*). The affinity and functional profile of opioids possessing activity at the nociceptin receptor was determined using [3H]nociceptin and nociceptin-stimulated [35S]GTPgammaS binding. The mu-opioid receptor-selective agonist lofentanil potently and competitively displaced [3H]nociceptin at rat brain receptors (IC(50) 62 nM). Lofentanil exhibited full agonism for enhancement of [35S]GTPgammaS binding to human recombinant ORL-1 receptors (EC(50) 50 nM). The related piperidines ohmefentanyl and sufentanil and the nonselective opioid receptor agonist etorphine were less potent nociceptin receptor agonists. The kappa(1)+kappa(3)-opioid receptor agonist/mu-opioid receptor antagonist naloxone benzoylhydrazone was a pure antagonist at both rat brain and human ORL-1 receptors. The nonselective opioid receptor partial agonist buprenorphine

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and the nonselective opioid receptor antagonist (-)-quadazocine exhibited pure antagonism at rat brain receptors, but displayed partial agonism at human ORL-1 receptors. Thus, opioids displaying full agonism at the nociceptin receptor are also opioid receptor agonists, whereas opioids that are antagonists or partial agonists at the nociceptin receptor show antagonism or partial agonism at opioid receptors. In addition, the stereo specificity required at opioid receptors appears to be retained at the nociceptin receptor, since (+)-quadazocine is inactive at both receptors. These findings illustrate the structural and functional homology of the opioid recognition site on these two receptor classes and suggest that opioids may provide leads for the design of nonpeptide nociceptin receptor agonists and antagonists lacking affinity for the classical opioid receptors (*83*).

Interestingly, several commonly used opioid drugs, including etorphine and buprenorphine, have been demonstrated to bind to nociceptin receptors, but this binding is relatively insignificant compared to their activity at other opioid receptors. More recently a range of highly selective ligands for ORL-1 have been developed, which show little or no affinity to other opioid receptors and so allow ORL-1 mediated responses to be studied in isolation (see table 1 for overview).

Table 1. In this table an overview is provided of the currently known selective agonists and antagonists targetting the ORL-1 opioid receptors.

Agonists (86–93)	*Antagonists (86, 94–97)*
• Buprenorphine (not selective for ORL1, also partial agonist of μ-opioid and δ-opioid receptors, and competitive antagonist of κ-opioid receptors) • Nociceptin • Norbuprenorphine (not selective for ORL-1, also full agonist at μ-opioid and δ-opioid receptors) • NNC 63-0532 • Ro64-6198 • Ro65-6570 • SCH-221,510 • SR-16435 (mixed mu / nociceptin partial agonist) • MCOPPB (full agonist, CAS# 1028969-49-4)	• JTC-801 • J-113,397 • SB-612,111 • SR-16430

Recently, Grünenthal and Forest Laboratories have entered into a license agreement for the co-development of a novel oral small-molecule analgesic, GRT 6005, and its follow-on compound GRT 6006 (*98–100*). Both compounds were discovered and developed by Grünenthal and represent novel first in class molecules with unique pharmacological and pharmacokinetic profiles that may enhance their effect in certain pain conditions. GRT 6005 and 6006 are novel first in class compounds with potent agonist activity on ORL-1 (opioid receptor like -1) and the well established mu opioid receptor. Preliminary evidence (*93*, *100*, *101*) suggests that targeting ORL-1 receptors may have synergistic effects with mu receptors hence enhancing the therapeutic profile of the compounds in the treatment of pain. The unique pharmacological and pharmacokinetic profile of

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these compounds is particularly suited for the management of moderate to severe chronic pain, including neuropathic pain. GRT 6005 has successfully completed initial proof-of-concept studies in nociceptive and neuropathic pain with further Phase II studies planned prior to initiation of Phase III. The compounds are covered by a composition of matter patent that will expire in November 2023. Several additional follow-up compounds are currently under development or investigation (GRT 6010 and GRT 6012).

Conclusion

Despite the emergence of new therapeutic options, neuropathic pain still remains a scientific and clinical challenge. Although probably less effective than when treating nociceptive pain, opioid analgesics remain an important component of neuropathic pain treatment. Based on the burgeoning preclinical and clinical evidence now becoming available, buprenorphine appears to display a unique molecular, pharmacological and clinical profile that makes this compound optimal for the treatment of the diverse and specific clincial aspects of neuropathic pain.

The introduction of the easy-to-use transdermal formulation of buprenorphine had an additional positive impact on the application of this unique agent in the treatment of the plethora of chronic neuropathic pain syndromes. However, many questions still remain unanswered and future research should, therefore, focus on two major challenges; firstly, the investigation of the analgesic effectiveness of buprenorphine in large scale, well controlled clinical trials and, secondly, the identification of the clinical neuropathic syndromes that are most sensitive to buprenorphine treatment.

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Chapter 7

Buprenorphine and Related Orvinols

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The orvinols, including buprenorphine, emerged from research carried out by Reckitt and Colman in the 1960s and 1970s. Regulatory approval of buprenorphine for the treatment of opioid addiction has been a major success and has ultimately led, in part, to its analgesic activity now being fully exploited. As buprenorphine's unique clinical profile is documented, efforts continue to rationalise this activity through understanding of its receptor pharmacology. This has included the development of new orvinols with mixed mu and kappa opioid receptor agonist activity and others with efficacy at mu and NOP receptors, the latter of particular interest for the development of new analgesics. Orvinols with mu and kappa antagonist activity, coupled with NOP receptor partial agonism have also been developed and have potential for the treatment of polydrug abuse.

Introduction

Over the years buprenorphine has been the subject of extensive clinical and pre-clinical evaluation and this continues today. Since being brought to market in 1978 as a clinical analgesic administered by injection, changes have been made to its formulation and its use has been extended to the treatment of heroin abuse and dependence (*1*). It is in the drug abuse treatment field that buprenorphine first became successful with substantial exploitation of its analgesic activity occurring more recently. The clinical literature concerning buprenorphine has been reviewed elsewhere (*2–6*) and aspects of this will be covered in detail within this volume by

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a number of the other contributors, but it is instructive to consider the breadth of recent publications on this topic. The efficacy and safety of buprenorphine in comparison to other opioid analgesics, primarily morphine, remains of interest and importance, for example, in relation to acute pain management in the emergency department where it was found to be as effective an analgesic as morphine in adults with acute fractures (*7*). A particularly favourable review on buprenorphine in a variety of pain states is provided by Pergolizzi et al, the article being the result of a Grunenthal GmbH sponsored meeting of experts in 2008 (*8*). In it the authors reiterate the view that buprenorphine is fully effective as an analgesic in clinical practise (i.e. has no ceiling effect for analgesia) but that there is a ceiling effect to the respiratory depressant effects, suggesting that buprenorphine is a safe and effective analgesic. Buprenorphine's use in the treatment of cancer pain has been extensively studied and reviewed (*9*) with buprenorphine's antihyperalgesic effect being of benefit compared to the hyperalgesic effects encountered on long-term use of some opioids. The notion that buprenorphine is in many ways 'different' to other opioid analgesics continues to be borne out by other clinical and pre-clinical studies which together suggest that buprenorphine may have utility in indications where other opioids have failed or only provided limited therapeutic effect. Any antihyperalgesic effect (*8*, *10*) would indicate advantages to using buprenorphine in a variety of situations, including the treatment of neuropathic pain; certainly clinical case studies support the use of buprenorphine when other opioids have failed (*11*, *12*). The utility of buprenorphine in neuropathic pain has been reviewed previously (*13*) and is covered in detail elsewhere within this volume. Other therapeutic areas where the use of buprenorphine for analgesia may be particularly beneficial are in the treatment of patients with advanced P-glycoprotein(+) cancers where, unlike morphine, buprenorphine does not show a decreased analgesic effect (*14*) and also in the treatment of analgesia associated with neuroinflammation (*15*). In relation to the latter, the analgesic effect of morphine can be blocked in the presence of elevated levels of chemokines whereas, in this preclinical study in rats, the antinociceptive actions of buprenorphine were not altered.

Buprenorphine's Complex Pharmacology

The mechanism of action of buprenorphine that gives rise to its unique pharmacology is not fully understood; suffice to say (if rather trite) that it is 'complex.' Various excellent reviews are available in which buprenorphine's basic pharmacological profile is discussed (*16–18*). Buprenorphine is traditionally described as a partial agonist at mu opioid receptors (MOPr) and an antagonist at kappa and delta opioid receptors (KOPr and DOPr) with the MOPr agonist effects being of very long duration. It is the activity at MOPr that led to it being developed as an analgesic and later for the treatment of opioid abuse. Partial agonist character means that in some assays buprenorphine will profile as a MOPr agonist and in others as an antagonist, a predictable example being its ability to antagonise the effects of a higher efficacy MOPr agonist (*19*). In this

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regard of particular note is a study conducted by Friderichs and coworkers (*20*) in which buprenorphine, within the dose range for analgesia, was found to provide an additive effect when combined with standard MOPr analgesics rather than acting as an antagonist. This is consistent with the observation that in patients it is possible to switch between buprenorphine and standard MOPr agonists without loss of efficacy (*21*). It was therefore a somewhat unexpected finding that buprenorphine treatment can increase surface MOPr number (an outcome associated with antagonist activity) and in contrast to agonists such as morphine, etorphine and fentanyl which cause a loss of surface receptors (*22*).

More recently it has been recognised that the KOPr antagonism displayed by buprenorphine may be contributing to the clinical profile. Rothman *et al* (*23*) investigated the ability of buprenorphine in conjunction with naltrexone to prevent relapse in heroin dependent patients who had been detoxified. The rationale for using this combination was based on unmasking the KOPr antagonist activity of buprenorphine. The high relapse rates observed in abstinant opioid-dependent patients has been associated with a protracted abstinence syndrome, including feelings of dysphoria, that has been linked to increased levels of the endogenous KOPr agonist dynorphin resulting from chronic administration of an opiate (*23*). Thus a KOR antagonist might be expected to prevent the dysphoric feelings thus acting as a relapse prevention therapy, while also helping treat any co-existing anxiety and depression that addicts often suffer (*24*, *25*). The positive outcome from the work of Rothman prompted a follow-up study by Gerra *et al* (*26*) in which the naltrexone and buprenorphine combination was assessed for efficacy in comparison to naltrexone alone in the treatment of opioid dependence in patients who also had a history of cocaine use. The findings were impressive, with the combination treatment resulting in significantly higher retention rates, significantly lower positive urine tests for morphine and cocaine metabolites, as well as reduced levels of dysphoria and craving. That the combination might have utility in the treatment of cocaine addiction was particularly interesting due to the current lack of pharmacotherapies. The theoretical basis for these studies is underpinned by a substantial body of pre-clinical evidence that KOPr antagonists could have therapeutic utility in preventing stress-induced, but not drug-induced, relapse to cocaine taking. These include studies showing selective KOPr antagonists block reinstatement of cocaine place preference (*27*, *28*) and block footshock-induced reinstatement of cocaine self-administration behaviour (*29*, *30*). It should be noted however that buprenorphine alone (i.e. not in combination with naltrexone) has been evaluated previously and found to be useful in reducing cocaine use. A sublingual buprenorphine solution (16 mg daily) significantly reduced concomitant cocaine and opioid use in patients dependent on both drugs (*31*), with the results indicating that the observed therapeutic effects on cocaine and opioid use might be independent. This could indicate that the observed effects on cocaine were due, at least in part, to activity at a receptor other than MOP. It should be noted however, that in rhesus monkeys buprenorphine alone significantly reduced cocaine self-administration whereas administering naltrexone before the buprenorphine dose-dependently reduced buprenorphine's effects, suggesting that it is the MOPr component of buprenorphine's profile that is important (*32*).

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As will be very apparent from reading other chapters within this volume, buprenorphine has been found to have affinity for the nociceptin/orphanin FQ receptor (NOPr) that may be sufficient for activity at this receptor system to be pharmacologically relevant. Efficacy at NOPr (*33*, *34*) has been used (*35*) to explain the bell-shaped (or inverted U-shaped) dose response curve often reported for buprenorphine (*36*). Lutfy and Cowan have reviewed the work in this area (*17*). That activation of NOPr opposes buprenorphine's MOPr derived analgesic effect appears to be supported by work showing that NOPr antagonists potentiate the anti-allodynic activity of MOPr agonists (*37*). However, while NOPr activation may decrease MOPr analgesia in rodents, the species used in most studies, this appears not to be the case in primates; in fact coactivation of MOPr and NOPr leads to a synergistic analgesic response in rhesus monkeys (*38*). This finding, which is discussed in more detail in Dr Ko's chapter, has considerable implications for the design of new analgesics as it might be predicted that significant analgesic activity could be obtained through only low level partial activation of MOPr and NOPr.

The finding by Gerra *et al* that a combination of buprenorphine and naltrexone reduced cocaine use in the patient group under study was of significant interest (*26*). However it was not clear if this was a direct effect on cocaine intake or whether it was an indirect effect resulting from reduced heroin use. This question was answered by conducting a controlled study in rats using a combination of buprenorphine and naltrexone that was neither rewarding nor aversive, but still possessed MOPr antagonist properties (*39*). In the conditioned place preference extinction and reinstatement method, a combination of 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone completely blocked drug-primed reinstatement in cocaine-conditioned rats and attenuated drug-primed reinstatement in morphine-conditioned rats, confirming a direct effect on cocaine seeking and supporting the use of a buprenorphine/naltrexone combination in the polydrug abuse setting (*39*). Koob and coworkers have also studied a combination of buprenorphine and naltrexone in rodents and found that use of buprenorphine plus a low dose of naltrexone (a dose that would not be expected to block completely all of buprenorphine's MOPr agonist effects) decreased cocaine self-administration and displayed somewhat reduced dependence liability (*40*). These results appear to be consistent with a lower level MOPr partial agonism than displayed by buprenorphine alone. While these preclinical studies are very promising, the ultimate test is of course a clinical trial directly assessing the combination for the treatment of cocaine dependence. To this end the National Institute on Drug Abuse Clinical Trials Network has initiated a Cocaine Use Reduction with Buprenorphine (CURB) trial to examine the safety and efficacy of sublingual buprenorphine (as Suboxone®) in the presence of extended-release injectable naltrexone (XR-NTX) (*41*). While it may not be possible to tell from this study whether very low level partial agonism at MOPr is required for clinical efficacy, the results will be hugely interesting and informative to those working in this exciting field.

These recent studies, combined with the extensive earlier work on buprenorphine, confirm the promiscuous nature of buprenorphine's binding is likely key to its clinical utility.

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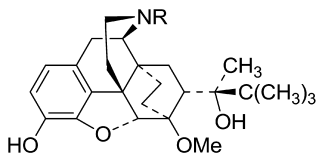
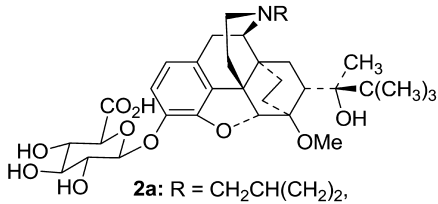
Buprenorphine Metabolites

While buprenorphine's pharmacology is becoming better understood, less well studied is the role of metabolism in its complex profile. There has for some time been recognition that norbuprenorphine (**1b**), an N-dealkylated metabolite of buprenorphine, may contribute to the overall pharmacology as it retains buprenorphine's high affinity for opioid receptors, particularly MOPr; in contrast it has much lower affinity for NOPr (*42*). Efficacy is increased at each receptor resulting in norbuprenorphine having activity in antinociceptive assays (*42–44*) and causing respiratory depression (*16*, *44*). The exact contribution of norbuprenorphine to the overall clinical profile of buprenorphine is difficult to quantify due to the apparent low permeability of norbuprenorphine into the brain (*43*). Further complicating the picture, it is now known that the glucuronides (buprenorphine-3-glucuronide (**2a**) and norbuprenorphine-3-glucuronide (**2b**)) are also found in significant concentrations in plasma. They have sufficiently high affinity for opioid receptors that their presence may contribute to the overall pharmacology (*45*).



1a: R = $CH_2CH(CH_2)_2$, buprenorphine
1b: R = H, norbuprenorphine



2a: R = $CH_2CH(CH_2)_2$, buprenorphine-3-glucuronide
2b: R = H, norbuprenorphine-3-glucuronide

New Delivery Technology

Efforts to extend buprenorphine's use by developing new delivery methods continue to be made. Recent patents include claims for an enhanced transmucosal delivery device (*46*), aerosol delivery (*47*), intranasal delivery (*48*) and implants for sustained release (*49*). Of course the most successful innovation in this regard is the development of transdermal patches, first introduced in 2001. Readers are directed towards excellent reviews from soon after introduction of the patch (*50*) and more recently published (*51*, *52*). Recent studies include open label, post marketing evaluation in patients with chronic moderate to severe cancer pain or chronic severe non-malignant pain (*53*), randomized, double blind studies for chronic back pain (*54*, *55*) and postoperative pain (*56*). Effective analgesia is reported and improved tolerability reported in patients with osteoarthritis pain compared to sublingual tablets (*57*).

A new buprenorphine/naloxone soluble film has been introduced by Reckitt Benckiser, obstensibly to reduce the risk of overdose in children. Strain and colleagues (*58*) showed that both soluble films of buprenorphine alone and of buprenorphine/naloxone could be used as safe and effective methods for opioid induction, though no comparison was made with the current tablet formulations.

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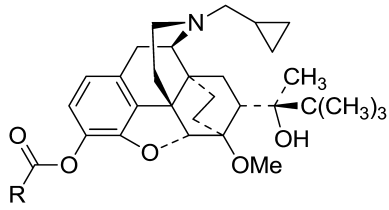
A review of the limited clinical data relating to this new formulation is available and suggests that patients have a preference for the film, possibly due to a lower dissolution time for the film compared to tablets. No data was available to confirm a lower risk of accidental poisoning in children (*59*).

Buprenorphine plus Opioid Antagonist Combinations

The possibilities of combining buprenorphine with opioid antagonists have been extensively studied. This extends beyond the Suboxone (buprenorphine plus naloxone in a 4:1 ratio) combination that was introduced to reduce the likelihood of diversion and abuse of buprenorphine (*60*, *61*). The potential of a buprenorphine/naltrexone combination to prevent relapse to drug taking has been discussed above and perhaps represents the most likely extension to buprenorphine's use. However, most intriguing are the findings that co-administration of ultra-low dose naloxone increased buprenorphine's analgesic activity in healthy volunteers with no increase in adverse effects (*62*). The use of antagonists to enhance the analgesic effect of opioids has been reported in pre-clinical and clinical studies (reviewed in (*61*)) with mechanisms to explain this effect proposed (*63*). However, evidence for a robust response remains elusive and a very recent study using buprenorphine (0.3 mg) with naloxone (0.02 mg) failed to find any evidence of increased analgesia in patients with lingering non-cancer pain (*64*) and it must be concluded that research in this area continues to produce inconsistent findings.

Prodrugs

Derivatives of buprenorphine continue to be prepared with the intention of optimising, or at least improving, physicochemical properties to increase bioavailability via the transdermal and oral routes. Not surprisingly the C3-phenolic group has been the focus of these efforts. Euro-Celtique SA claim C3-O esters (**3**), and a limited number of ethers, of buprenorphine with better skin absorption characteristics, e.g. through increased lipophilicity, though no data is presented to confirm what is ultimately the desired outcome, better delivery of buprenorphine (*65*). Reckitt Benckiser have targeted increased oral bioavailability, claiming esters with a terminal carboxylate (*66*). In particular, the C3-O adipate (**4**) is reported to have higher oral bioavailability and provide higher buprenorphine levels in plasma, in beagle dogs, than achieved with buprenorphine alone.



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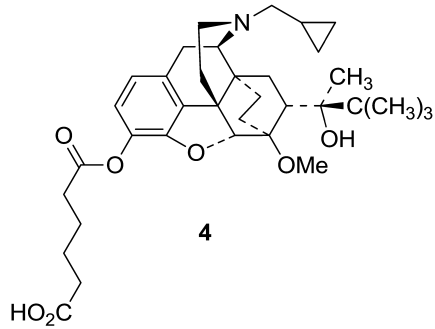

4

Analogues of Buprenorphine

The literature surrounding close analogues of buprenorphine was reviewed in 2004 and emphasised the high affinity non-selective binding profile of the series, their duration of action, and structural features associated with efficacy at MOPr and KOPr (*67*). High affinity to MOPr, KOPr and DOPr is the norm with selectivity arising from differential efficacy at the individual receptors. Since then a number of single compound and multi-compound studies have been reported adding to our understanding of the structure-activity relationships (SAR) within the orvinol family. Following on from their disclosure of thienorphine (**5**) (*68*), a non-selective, high affinity ligand with predominant KOPr partial agonist activity (*69*) Yu et al (*70*) recently published on TH-030418 (**6**), a close analogue of thienorphine but having a 3-thienyl group (2-thienyl in thienorphine) and replacing the N-cyclopropylmethyl group in thienorphine with N-methyl. Standard SAR within the opioids would predict an increase in efficacy, particularly at MOPr, on making this latter change (*67*). TH-030418 is reported as having high affinity at each of the opioid receptors (0.6 - 0.7 nM) and, interestingly, at NOPr (1.6 nM). However no *in vitro* functional activity data is reported and so efficacy of the compound at these receptors is not known. The close structural similarity to phenethyl orvinol (PEO, **7**) (*67*, *71*) and the N-cyclopropylmethyl phenethyl norорvinol (**8**: compound **1k** in Greedy et al (*72*)) would predict substantial partial or full agonism at both MOPr and KOPr. *In vivo* data is presented for TH-030418 showing a potent analgesic action but, surprisingly, no physical or psychological dependence liability. As the authors themselves conclude, further study is needed to confirm these findings in other models. PEO itself has been evaluated as a potential ligand for PET-imaging of opioid receptors with [^{11}C] (*71*) and [^{18}F] versions reported (*73*).

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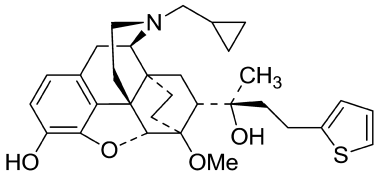
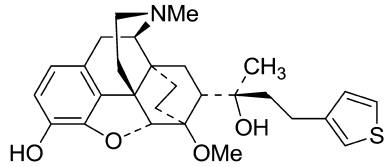
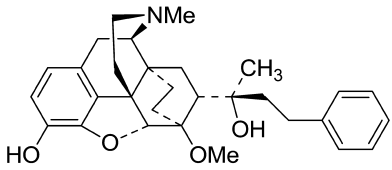


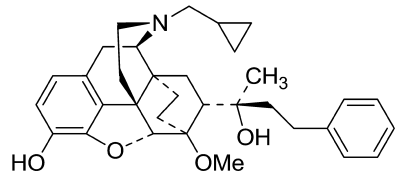
5: thienorphine



6: TH-030418

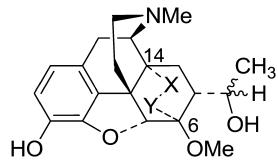


7: PEO, phenethylorvinol

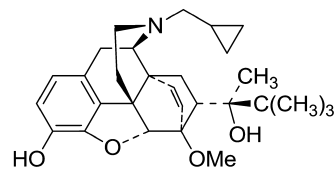


8

The C6-C14 bridge of the orvinols continues to attract some attention with the aim of increasing molecular diversity in this series, potentially offering an alternative site for manipulation of MOPr and KOPr efficacies. In a focused study, introduction of a hydroxyl group to the bridge (**9a**, **9b**) differentially affected efficacy at MOPr and might provide a means of limiting MOPr efficacy in orvinols having a 17N-methyl group (*74*). The influence of the bridge on pharmacological profile was also evaluated by Negri and coworkers (*75*, *76*). They reported on the activity, including antinociceptive activity, of HS-599 (**10**), the etheno-bridged analogue of buprenorphine. In the orvinol series, reduction of the bridge appears to attenuate intrinsic activity, in particular at KOPr (*72*) and so HS-599 would be expected to have somewhat higher efficacy than buprenorphine, particularly at KOPr. This would not readily be observed in the assays used in these studies and therefore it would be interesting to see a side-by-side comparison of buprenorphine and HS-599 in, for example, [^{35}S]GTPγS assays.



9a: X = CHOH, Y = CH_2
9b: X = CH_2, Y = CHOH



10

Interest in understanding, and being able to predict, KOPr activity in the orvinols and close analogues continues to this day. Tang and coworkers (*77*) have looked to develop 3D-quantitative structure activity relationships (QSAR) in order to help develop more selective KOPr agonists from this series. The dependent variables in this study were binding affinity for the KOPr and also

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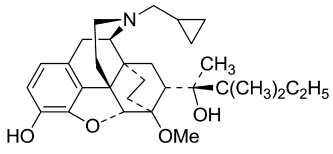
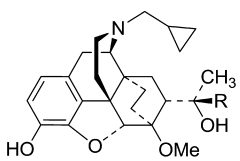
binding selectivity (relative to MOPr). While predictive models could be derived, the inherent high affinity and lack of selectivity in the orvinol series meant that selective QSAR models could not be established. More recently, CoMFA analysis allowed a predictive model for efficacy at KOPr to be generated (*72*) and confirms previous studies (e.g. Husbands and Lewis (*78*)) suggesting that bulk around C20/C21 is detrimental to efficacy at KOPr. The t-butyl group of buprenorphine and the t-pentyl group of BU08028 (**11**) (*79*) are clearly prime examples of this effect.

BU08028 emerged from work aimed at developing a better understanding of the structural requirements for NOPr affinity within the orvinol series (*79*, *80*). As mentioned earlier, the NOPr activity of buprenorphine has been invoked to explain the bell-shaped dose response curve produced by buprenorphine in a variety of behavioural assays (*17*, *35*) and used to explain why buprenorphine at low doses enhances alcohol consumption in rats, but at high doses reduces alcohol intake (*81*). A series of close analogues of buprenorphine were prepared and their affinity and functional activity determined at MOPr, KOPr, DOPr and NOPr. The region occupied by the *t*-butyl moiety of buprenorphine was found to be key to its NOPr activity (as well as activity at MOPr and KOPr). BU08028, differing only in a single extra methylene in this region, had an order of magnitude higher affinity for NOPr and increased efficacy. Other 'R' groups that imparted higher affinity for NOPr than observed with buprenorphine were neopentyl ($CH_2C(CH_3)_3$) (**12a**), dimethylphenethyl ($CH_2C(CH_3)_2Ph$) (**12b**) and bicyclo[2.2.1]heptan-2-ylmethyl (**12c**). The higher NOPr affinity of those groups with a methylene spacer (**12a - c**) is consistent with the high NOPr affinity reported for TH-030418 (*70*), though no comparable data for buprenorphine was reported in this latter manuscript. Of course, as would be predicted by the CoMFA analysis mentioned above, introduction of the methylene also had the effect of increasing efficacy at KOPr. BU08028 was the subject of more extensive investigation in rodents as its profile *in vitro* was almost identical to buprenorphine at MOPr, KOPr and DOPr, but with higher efficacy at NOPr (*82*).



11: BU08028



12a: R = $CH_2C(CH_3)_3$
12b: R = $CH_2C(CH_3)_2C_6H_5$
12c: R = H_2C

The *in vivo* pharmacology of BU08028 in mice is discussed within the chapter 'Pharmacology of Mixed Mu/NOP Ligands;' briefly, BU08028 appeared to have a profile typical of a MOPr agonist, it was a long acting analgesic, exhibited a significant conditioned place preference and tolerance developed to the antinociceptive effect on repeated administration. While there was some increase in antinociceptive activity of BU08028 after pretreatment with an NOPr antagonist, particularly at the higher (3 mg/kg) BU08028 dose, it appears that in mice the MOPr activity predominates. Recently Cremeans *et al* (*38*) reported that a NOPr antagonist neither enhanced or reduced buprenorphine-induced

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antinociception in rhesus monkeys, suggesting that NOPr are not involved in buprenorphine's activity in primates. In contrast, we now have preliminary data (unpublished) showing that the antinociceptive activity of BU08028 can be antagonized by both the MOPr antagonist naltrexone and the NOPr antagonist J-113397 (Figure 1), indicating that in the primate BU08028's analgesic activity is mediated through both NOPr and MOPr as predicted by the *in vitro* data. Since buprenorphine has NOPr activity that appears to be measurable in rodents but not primates, while BU08028 has the opposite, measurable NOPr activity in primates but less distinct in rodents, it appears that very thorough evaluation of this type of mixed activity compound is required with careful interpretation of the data generated.

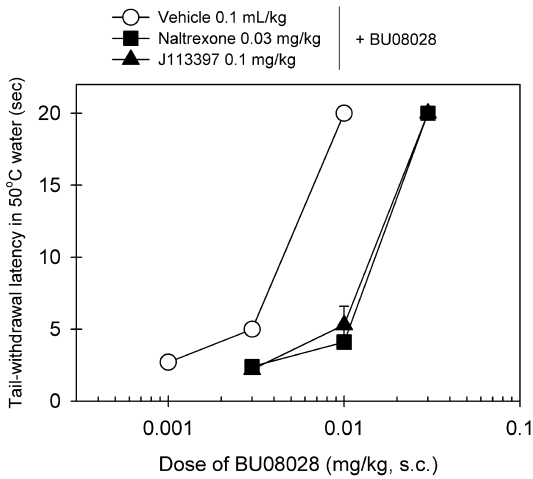

Figure 1. Effects of MOPr and NOPr antagonists on BU08028-induced antinociception in nonhuman primates. The MOPr antagonist naltrexone or NOPr antagonist J-113397 was administered subcutaneously 15 min before determination of the dose-response curve of BU08028. Each data point represents mean +/- SEM (n=3)

In related work it has been found that a phenyl substituent at C20 confers similar antagonist activity at KOPr but instead of the MOPr partial agonism displayed by buprenorphine and BU08028, BU127 (**13**) has virtually no efficacy at MOPr and is a potent antagonist (*83*). Antagonist pA_2 values determined in isolated tissue assays suggest potency at MOPr and KOPr equivalent to, or better, than buprenorphine (Table 1). In addition, BU127 is an NOPr antagonist in the rat *vas deferens* with about half the potency of buprenorphine which is also an antagonist in this assay. In [^{35}S]GTPγS assays BU127 also profiled as an antagonist at MOPr (Ke 0.41 nM), had very low efficacy at KOPr (maximum stimulation 19% of U69,593) with predominant antagonist activity at this receptor (Ke 0.27 nM). At NOPr BU127 displayed around half the efficacy and affinity shown by buprenorphine.

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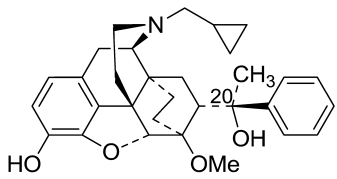
Table 1. Antagonist activity in isolated tissues and agonist activity in [^{35}S]GTPγS assays (at 10 μM)

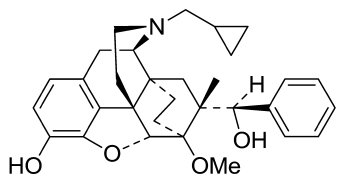
Compound	*pA_2 in isolated tissues*			*% stimulation in [^{35}S]GTPγS*[d]		
	MOPr[a]	*KOPr*[b]	*NOPr*[c]	*MOPr*	*KOPr*	*NOPr*
buprenorphine	9.66	9.24	5.98	20	0	26
BU127	10.3	9.52	5.78	6	19	14
BU10119	10.1	9.85	5.75	2	-2	57

[a] versus DAMGO in rat *vas deferens*, [b] versus U69,593 in mouse *vas deferens*, [c] versus nociceptin in rat *vas deferens*, [d] stimulation at a fully effective dose (10μM) relative to the standard agonists DAMGO, U69,593 and nociceptin.

The discovery of BU127 was used to direct the development of single compound alternatives to the buprenorphine/naltrexone combination discussed earlier, *i.e.* single chemical entities with potential utility in the treatment of polydrug abuse. Out of this programme has emerged BU10119 (Table 1) (*83*). In [^{35}S]GTPγS assays BU10119 (**14**) was a potent antagonist, without any measurable efficacy, at both MOPr and KOPr (Ke's 0.28 nM & 0.09 nM respectively) and a partial agonist at NOPr with twice the efficacy of buprenorphine. Affinity at NOPr was also good (80 nM) relative to buprenorphine (220 nM) [unpublished data].



13: BU127



14: BU10119

McCann (*84*) presents a compelling case for considering the buprenorphine/naltrexone combination in treating polydrug addiction but points out that there are very real, practical problems associated with this approach. In particular, buprenorphine has poor oral bioavailability, but good sublingual availability while naltrexone is the opposite, making administration as a single tablet problematic. In addition, there is evidence that repeated, high doses of naltrexone can be required to completely block buprenorphine's effects over a long time (*85*). To circumvent both these problems a single chemical entity that has potent KOPr/MOPr antagonist activity combined with NOPr agonist activity, to mimic the effects of the buprenorphine/naltrexone combination, could be sought. Indeed in the concluding paragraph of his review, McCann states that "In the long term chemists may discover a buprenorphine analog that acts as an antagonist or much weaker partial agonist at MOPr, yet retains the ORL-1 agonist and KOPr antagonist activities of buprenorphine." It is clear that analogs of buprenorphine such as BU10119, can provide just such a profile and provide hope that an effective treatment agent for polydrug addiction can be developed.

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Conclusion

The interest developing ligands with activity at multiple receptors has reignited research into the orvinol series. Recent advances in our understanding of structure-activity relationships in this series has allowed the rationale design of 'selectively promiscuous' ligands, i.e. ligands with affinity and defined efficacy at each of the opioid and NOP receptors. Compounds with mixed MOPr/NOPr agonist activity are being developed for their analgesic potential while MOPr/KOPr antagonists that also display NOPr agonism have been discovered and have potential in the treatment of polydrug abuse.

Acknowledgments

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Chapter 8

Structure-Activity Relationships of Nociceptin Receptor (NOP) Ligands and the Design of Bifunctional NOP/Mu Opioid Receptor-Targeted Ligands

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The nociceptin receptor NOP, the fourth member of the opioid receptor family, continues to be of significant interest as a target for therapeutic applications in the treatment of drug dependence, anxiety, and pain, to name a few. Its endogenous ligand, a heptadecapeptide nociceptin/orphanin FQ (N/OFQ) has pharmacological actions distinct from the classical opioid peptides, and has been shown to modulate opioid actions in many neurological circuits. Over the past decade, several nonpeptide (small-molecule) and peptide NOP ligands have been reported both by industry as well as academic laboratories, including our own. NOP agonists as well as NOP antagonists have been reported. The recent resolution of the 'antagonist' bound crystal structure of the NOP receptor and our recent report of the active-state homology model of the NOP receptor will facilitate our understanding of the structure-activity relationships (SAR) of NOP antagonists and agonists respectively. This review presents a ligand and structure-based assessment of the SAR for potent NOP binding pharmacophores. This review also presents rational approaches for designing 'bifunctional' NOP/opioid ligands, which have equipotent affinity for the NOP and mu opioid receptors and the desired functional profile of NOP agonist and mu opioid agonist activity. Such bifunctional NOP/mu agonists may have

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utility as non-addicting analgesics and drug abuse treatment with lower propensity of withdrawal-related effects.

Keywords: ORL-1; nociceptin ligands; NOP ligands; NOP/mu; ORL-1 ligands; bifunctional; non-addicting analgesics

The Nociceptin Receptor (NOP) as a Target for Small-Molecule NOP Ligands

Since the discovery in 1994 of the nociceptin receptor as the fourth member of the opioid receptor family, and in 1995, of its endogenous peptide ligand nociceptin/orphanin FQ (N/OFQ), studies on the pharmacology of the NOP-N/OFQ system have demonstrated a significant role of this receptor-peptide system in pain, drug reward, anxiety, feeding and learning/memory. Over the past decade, there has been a significant effort in the discovery and development of 'highly selective' small-molecule NOP receptor agonists, which have been explored as anxiolytics (*1*, *2*), anti-tussives (*3*), drug abuse and relapse treatment (*4–6*) and more recently, for Parkinson's disease and dyskinesia (*7*). NOP receptor antagonists on the other hand, have been investigated for use in chronic pain and also in Parkinson's disease (*8–11*). Several classes of nonpeptide NOP agonists and antagonists have been extensively optimized for their drug-like suitability and removal of toxicity liabilities, as they are progressed into preclinical development for future clinical applications (*12*, *13*). Even peptide-based NOP ligands, stable to peptidases, have been reported and investigated clinically (*14*). Several excellent reviews of peptide and nonpeptide NOP ligands have been published, including recent reviews that compile the various structural classes of nonpeptide and peptide NOP ligands (*15–19*). The recent report of the crystal structure of the NOP receptor bound to an antagonist gives a snapshot of a possible binding modes of nonpeptide antagonist ligands to the NOP receptor (*20*). The structure of the active-state (agonist-induced) NOP receptor, developed by homology modeling and molecular dynamics simulation (*21*), gives further insights into binding of NOP 'agonists' and receptor activation. These structure-based advances in greatly facilitate the structure-activity relationship (SAR) understanding of the binding affinities and functional efficacies of the various classes of NOP ligands. In this chapter, we review the SAR of NOP ligands in the context of the structure of the NOP receptor. These SAR analyses, supported by receptor structure-based comparison, are useful not only to design potent, selective and drug-like NOP ligands but also for designing bifunctional NOP-opioid ligands, as discussed below.

Structural Determinants of NOP Receptor Binding and Activity

The NOP receptor shares 65% overall homology with the other members of the opioid receptor family, and significantly higher homology in the receptor residues within the transmembrane active-site domain (*22*, *23*). Because of this

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close homology, there are inherent challenges in discovering truly selective NOP ligands, particularly small-molecule NOP ligands. Peptidic ligands, on the other hand, tend to have a higher degree of selectivity, because several pharmacophoric epitopes of the peptide ligand can engage the subtle differences in the receptor residues within the binding pocket, resulting in increased selectivity (*16*). Indeed, even though N/OFQ, the endogenous NOP peptide ligand, is very similar to the kappa peptide dynorphin, it has a 1000-fold lower affinity for the kappa opioid receptor and the same is true for dynorphin's affinity for NOP (*24*). Nevertheless, selective small-molecule NOP ligands, both NOP agonists and antagonists, have been discovered and developed. Most NOP ligands reported thus far have been developed by optimizing hits from high-throughput screening of corporate compound libraries e.g. (*25–28*). As such, a large SAR effort is usually required to improve binding affinity and enhance selectivity for the NOP receptor. Factors that are generally guide such SAR efforts and drug design are (i) improving binding affinity for NOP (ii) obtaining selectivity for NOP versus the other opioid receptors, and (iii) obtaining the desired profile of intrinsic activity (agonist or antagonist activity). It is interesting, that with very few exceptions, most NOP ligands contain a piperidine ring core scaffold, and the different classes of NOP ligands mostly differ in the substituents on the piperidine nitrogen and the moieties on the 4-position of the central piperidine scaffold. We had previously proposed that most NOP ligands contain three common pharmacophores which, for ease of the SAR analysis, we label as the *A-moiety*–a heterocyclic or aromatic moiety distal to the piperidine nitrogen (at the 4-piperidine position), the *B-moiety*–a central scaffold (most commonly piperidine), containing a protonatable nitrogen, and the *C-moiety*–a lipophilic substituent on the protonatable nitrogen (see Figure 1) (*29*). As seen in Figure 1, almost all NOP ligands contain this common pharmacophoric pattern. From the SAR analysis presented below, it appears that A- and C-moieties are important determinants of the binding affinity of NOP ligands and selectivity versus the opioid receptors, particularly the mu opioid receptor. The C-moiety further plays an important role in the intrinsic activity of the NOP ligands, as we have shown that subtle one-carbon differences in the C-moiety substituents can convert a NOP agonist into a NOP antagonist, without affecting the binding affinity of the ligand for the receptor (*29*), i.e. subtle differences in the C-moiety substituents are capable of inhibiting activation-associated changes in receptor conformation, resulting in an 'antagonist'.

SAR of Selective NOP Agonists

The structures of some NOP agonists for which binding, selectivity and efficacy data are reported, are shown in Table 1. The first nonpeptide NOP agonists reported in the literature were the triazaspirodecanone series from Roche, obtained by optimizing a high-throughput screening lead (*25*, *30*, *31*). The piperidine substituents (C-moieties) of the two well-characterized NOP agonists from this series (Ro 65-6570 and Ro 64-6198; Figure 1) contained tricyclic aromatic rings. Nonaromatic, lipophilic substituents such as cyclooctyl (**1**) and 4-isopropylcyclohexyl (**2**) afforded a 10-fold increase in binding affinity and

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a 40-fold selectivity versus the mu opioid receptor. Subsequently, a series of hexahydropyrrolopyrroles (A-moiety) containing the cis-4-isopropylcyclohexyl as the lipophilic C-moiety (**3**, Figure 1 and Table 1) were reported as highly selective NOP agonists, having a K_i of 0.49 nM and greater than 1000-fold selectivity over the opioid receptors (*32*). Interestingly, the compounds **2** and **3** differ from each other only in the heterocyclic A-moiety on the piperidine scaffold (Figure 1), yet, **3** is over a 1000-fold selective versus the mu and kappa opioid receptors compared to **2** which is only 40-fold selective. This SAR suggests that the A-moiety plays a role in the selectivity of the NOP ligand, versus the other opioid receptors.

However, in the dihydroindolinone (A-moiety) series of NOP ligands reported from our laboratory, **AT-203**, containing the cis-4-isopropylcyclohexyl as the C-moiety, and **SR16835** (henceforth referred to as **AT-202**) containing the same tricyclic hexahydro-phenalenyl group as in Ro 64-6198 (Figure 1) were only modestly selective (3–8-fold) versus the mu opioid receptor (MOP), but are full agonists at the NOP receptor (Table 1) (*33*). A similar trend is observed with the phenylpiperidine-based (A-moiety) NOP agonists reported by Purdue Pharma. Compound **4**, containing the same cis-4-isopropylcyclohexyl substituent on the piperidine nitrogen (C-moiety) (Figure 1), was only 3-fold selective versus the mu receptor (Table 1) (*34*). This further confirms the SAR that the A-moiety substituent on the piperidine scaffold plays an important role in overall binding affinity and selectivity of the NOP ligands.

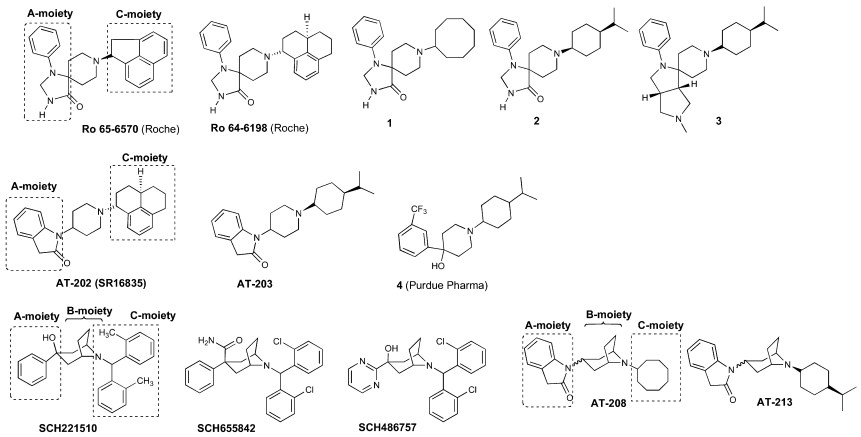
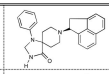
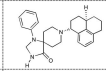
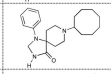
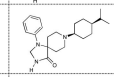
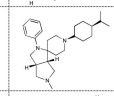
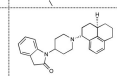
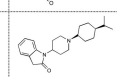
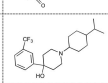
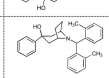
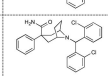
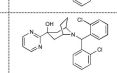
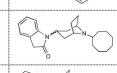
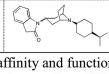


Figure 1. Structures of NOP agonists reported in the literature

Table 1. Binding affinities and functional activity of reported NOP ligands from Figure 1 (NT=not tested)

	Structure	Receptor Binding K_i (nM)[a]				[^{35}S]GTPγS NOP[a]		Reference[a]
		NOP	μ	κ	δ	EC_{50} nM	% Stim	
Ro 65-6570		0.52	5.9	26	250	40	100	*(25)*
Ro 64-6198		0.39	46	89	1380	38	100	*(31)*
1		1.9	13	9.1	>200			*(30)*
2		0.079	3.2	26	242			*(30)*
3		0.49	537	309	2138	65.4 ± 6.3	100	*(32)*
AT-202 (SR16835)		11.4 ± 0.9	79.9 ± 3.9	681.3 ± 62		46 ± 20.5	107± 7.4	*(46)*
AT-203		3.96 ± 1.55	8.0 ± 0.97	149 ± 8.7		26.5 ± 4.3	100± 15	*(33)*
4		12	36	4153	5674			*(34)*
SCH221510		0.3 ± 0.05	65 ± 10	131 ± 33	2854	12 ± 3	100	*(2)*
SCH655842		1.7	38	268	2326	6	100	*(35)*
SCH486757		4.6 ± 0.61	972 ± 40	590 ± 40	14747	79 ± 12	100	*(60)*
AT-208		8.93 ± 0.61	31.14 ± 7.4	148 ± 2.5	NT	52.7 ± 2.9	37 ± 5.85	
AT-213		331 ± 61	410 ± 2.5	NT	NT	NT	NT	

[a] The binding affinity and functional activity values for the industry-reported compounds are from the literature references shown in the References column. These data are from different laboratories, and use different experimental protocols to determine Ki and functional activity. These data are included for discussion purposes only, and as such, cannot be used for direct comparisons of the activity of the different NOP ligands reported by different groups. Data for the AT series of compounds are from our laboratory and reported previously.

Although a majority of the NOP ligands contain a piperidine scaffold (B-moiety) that contains the essential protonatable nitrogen (to provide the anchoring interaction with the Asp130 in the binding pocket), selective NOP agonists containing a tropane-based B-moiety were recently reported by Schering Plough Corporation (*13*, *35–37*). SCH221510, SCH655842 and SCH486757 (Figure 1, Table 1) are selective and potent NOP full agonists and contain a substituted phenyl-based A-moiety, and benzhydryl-based substituents on the tropane nitrogen (C-moiety). Interestingly, compounds containing a tropane-based B-moiety but with the 2-indolinone as the A-moiety and the alicyclic lipophilic cyclooctyl C-moiety (**AT-208**) (Figure 1), synthesized in our laboratory, afforded modest binding affinity for NOP, and only 3-fold selectivity versus MOP (Table

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1). Further, AT-208 has low efficacy and is a partial agonist at the NOP receptor. Surprisingly, the cis-4-isopropylcyclohexyl group on the endocyclic tropane nitrogen (**AT-213**) (Figure 1) had significantly lower affinity for NOP (Table 1), although these same C-moiety and A-moiety (2-indolinone) afforded high binding affinity and full agonist activity on a *piperidine* scaffold (see **AT-203**, Figure 1 and Table 1).

These SAR show that all the three pharmacophoric features of NOP ligands play a role in determining a 'good fit' and hence a good binding affinity for the NOP receptor as well as allowing full receptor activation. This SAR is further elucidated with computational studies of these NOP ligands with the recently derived active-state conformation model of the NOP receptor (*21*) as discussed below.

Computational Structure-Based Analysis of NOP Ligand SAR

Binding of NOP Agonists and the Active-State Conformation of the NOP Receptor

The recent resolution of the crystal structure of the NOP receptor G-protein coupled receptor GPCR) bound to an antagonist (*20*) provides the structure of the 'inactive' state of the NOP receptor. Prior to the availability of the NOP crystal structure, homology models of the inactive-state NOP structure were reported by Topham et al (*23*), Broer et al. (*38*) and Liu et al. (*39*) and used for docking NOP agonists N/OFQ, Ro 64-6198 and a spiropiperidine NOP agonist, respectively. We recently reported a model of the active-state conformation of the NOP receptor, based on the crystal structure of an active-state GPCR opsin (*21*). Comparison of the active conformation of the NOP receptor with the inactive-state crystal structure conformation gives significant information on the activation-associated changes in NOP receptor residues. Docking NOP agonists in the active-state conformation of the NOP receptor gives a clearer picture of the differences amongst the various agonists in terms of their binding and efficacy and how NOP agonists might affect activation-associated residues and transmembrane (TM) helix movements (*21*).

Figure 2A shows the docking of the triazaspirodecanone NOP agonists **Ro 64-6198** (shown in green) and **2** (shown in pink), both of which have some of the highest binding affinities reported for NOP agonists (Table 1). Figure 2B shows the docking of the indolinone NOP agonist **AT-202**, which has the same phenalenyl substituent on the piperidine nitrogen (C-moiety) as the triazaspirodecanone **Ro 64-6198**, but about 10-fold lower affinity. However, both ligands are full agonists at NOP. As seen in Figures 2A and 2B, all three NOP agonists show the characteristic reinforced salt bridge between the protonated piperidine nitrogen and the carboxylate of Asp130 from TM3. The 4-position substituent on the piperidine ring (A-moiety) is oriented towards the extracellular end of the binding pocket. The pendant phenyl ring of the triazaspirodecanones forms a Van der Waals interaction with Ile127 of TM3. The carbonyl of the triazaspirodecanone A-moiety forms a polar interaction with Thr305, which appears to be an important determinant of its high binding affinity, because **AT-202** forms only a weak interaction with Thr305, due to its distance from it.

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Further, the rigidity of the fused phenyl ring of the dihydroindolinone moiety of **AT-202** prevents it from making an optimum Van der Waals interaction with the Ile127, compared to the triazaspirodecanone's pendant phenyl ring. This may also contribute to the lower binding affinity of **AT-202** compared to **Ro 64-6198**.

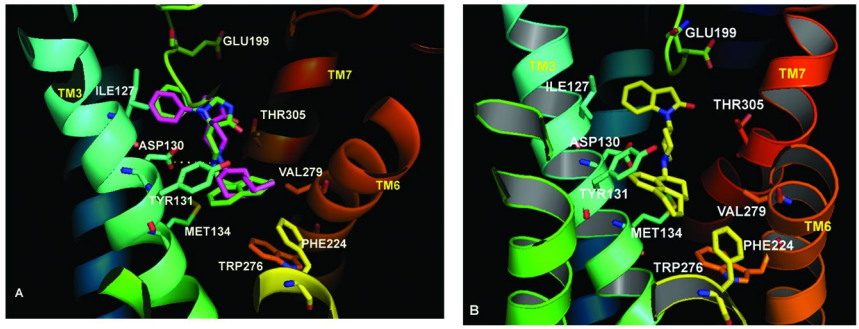

Figure 2. ***(A)*** *Binding pose of triazaspirodecanone* ***Ro 64-6198*** *(green) and* ***2*** *(pink) in the active-state NOP receptor structure transmembrane binding pocket.* ***(B)*** *Binding pose of dihydroindolin-2-one AT-202 (yellow) in the active-state NOP receptor binding pocket. Docking was carried out using Sybyl X1.2 (Tripos). The transmembrane helices and the various binding pocket residues are labeled. (see color insert)*

The piperidine nitrogen substituents (C-moieties) of these ligands sit deep inside the binding pocket, and are surrounded by hydrophobic amino acids such as Tyr131, Met134, Trp276, Phe224, and Val279, shown in Figures 2A and 2B. Notably, Trp276, 'the rotamer toggle switch' in TM6, whose change in orientation is an activation-associated event (see (*21*)), is in the 'active' conformation, in a π-stacking interaction with Phe224, called the 'aromatic lock' (Figure 2A). The binding of these ligands allows the movement of the activation-associated amino acids, leading to 'agonist' activity.

Binding of NOP Antagonists and the X-ray Crystal Structure of the NOP Receptor

The recently solved crystal structure of the NOP receptor GPCR is in complex with a NOP antagonist, **C-24** (Figure 3) (*20*). **C-24**, reported by Banyu (*28*), is a potent and highly selective NOP antagonist belonging to the spiropiperidine class. Its complex with the NOP receptor in its inactive state shows the NOP antagonist bound to the same active site in the TM domain, with the salt bridge between the piperidine nitrogen of **C-24** and the Asp130 of TM3. However, comparing the docked poses of the NOP agonists with the crystal structure pose of the NOP antagonist shows that the NOP agonists and antagonists have different binding modes at the NOP receptor. In the NOP crystal structure bound to the antagonist, the 4-position of the **C-24** piperidine ring, where the spiro-linked benzofuran is attached (A-moiety position of the NOP ligand pharmacophore),

is positioned '*inside*' the binding pocket, oriented towards the intracellular end of the binding pocket. This is in contrast to the orientation of the A-moiety in the NOP agonist docking to the active-state conformation of NOP (see Figures 2A and 2B). Consequently, the piperidine nitrogen substituent (C-moiety in the pharmacophore) of antagonist **C-24** is now oriented towards the extracellular end of the binding pocket. This can be clearly seen in Figure 3, which shows the crystal structure of **C-24** inside the NOP binding pocket. We also docked another NOP antagonist, **SB-612111**, a phenylpiperidine-based NOP ligand, into the NOP crystal structure. Interestingly, this NOP antagonist also docked in a similar orientation as **C-24**, where the substituent on the piperidine 4-position (A-moiety, the dichlorophenyl in **SB-612111**) is oriented towards the intracellular end of the binding pocket, whereas the piperidine nitrogen substituent (C-moiety) is oriented towards the extracellular end. Of further note, is that the Trp276 rotamer toggle switch is notably in the 'off' inactive position, distinct from its position in the agonist-docked NOP active-state conformation (Figures 2A and 2B). Both the antagonists have high binding affinity for the NOP receptor, but do not activate the receptor. It is possible that the relatively large substituents on the piperidine nitrogen in these antagonists, preclude their binding in the 'normal' agonist-like orientation, and therefore, prevent receptor activation. Docking with other known antagonists will also shed further insights into the possibility of differential binding modes of NOP agonists and antagonists. These differences can be further confirmed by site-directed mutagenesis of NOP receptor residues, selected based on structure of the agonist- or antagonist-bound receptor obtained from the crystal structure or homology modeling.

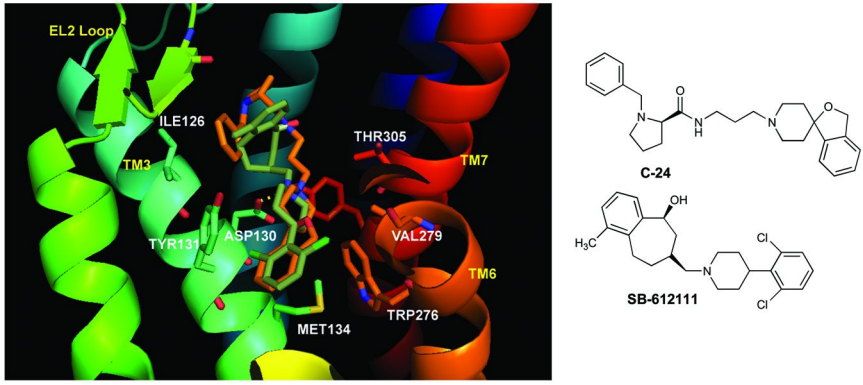

*Figure 3. Binding pose of the NOP antagonist **C-24** (orange) in the X-ray crystal structure of the NOP receptor. Antagonist **SB-612111** (gray) was docked into the NOP receptor crystal structure. The transmembrane helices are shown in different colors (TM3-cyan, TM4-green, TM6-orange, TM7-bright orange, TM7-blue) (see color insert)*

The Design of NOP-Mu 'Bifunctional' Agonists

The concept of 'multi-targeted' ligands/drugs is well accepted and clinically validated as an advantageous therapeutic approach for many disease targets. As an example, dual serotonin and norepinephrine uptake inhibitors, such as venlafaxine, were clinically developed as an improvement over selective serotonin uptake inhibitors. The design of multi-targeted ligands is usually based on a rational approach to modulate complementary pharmacology of two targets in a disease pathway. There are several examples in the literature of the rational design of dual or multi-targeted ligands (see (*40*) for a good review). The field of opioid analgesics is replete with examples of bi- and even trifunctional opioid ligands with varying efficacy profiles at all three classical opioid receptors, in an effort to reduce opioid-related side effects (*41–44*).

Being in the opioid family, both the NOP receptor and its endogenous ligand N/OFQ have been extensively investigated for their effects on opioid-mediated pharmacology (*45*). The detailed pharmacology of the NOP-N/OFQ system and its modulation of opioid-induced reward, tolerance and antinociception is discussed in an accompanying chapter in this series (Toll et al). Since N/OFQ and NOP agonists are known to reduce opioid-induced reward, we hypothesized that dual-targeted, bifunctional NOP agonists/mu opioid receptor (MOP) agonists may provide a novel approach for developing non-addicting analgesics (*46*). Bifunctional NOP agonists/MOP agonists may also have a useful therapeutic profile for treatment of opioid dependence, since NOP agonists have been shown to reduce opioid reward, and MOP partial agonist activity in the same molecule may reduce withdrawal-associated side effects, normally seen when using a MOP antagonist such as naltrexone.

There are several approaches that may be used to conceptualize the design of bifunctional ligands and obtain dual-targeted activity in a 'single chemical entity'. One common strategy extensively investigated in the opioid ligand field is the design of bivalent ligands containing the two pharmacophores separated by a linker. While this approach is suitable for studying receptor dimerization-related pharmacology, it does not produce drug-like compounds suitable for therapeutic development. An ideal strategy would be to have bifunctional activity at both targets within a 'single chemical scaffold'. From a drug design perspective, it is a challenge to obtain and maintain the desired spectrum of activities at two different targets within a single chemical scaffold. However, ligand SAR at the individual receptors and receptor structure-guided SAR can aid optimization efforts. A good starting point is to identify a lead that has a reasonable affinity and activity profile at one of the targets, and then to identify tolerant regions of the ligand pharmacophore, where structural modifications enable binding to the other desired target. For designing NOP/MOP ligands, either NOP ligands or MOP ligands are good starting points.

Very few MOP opioid ligands have affinity for the NOP receptor. Those that do bind to NOP have low binding affinity and efficacy. For example, buprenorphine, a potent mu agonist, has low binding affinity at NOP and low to no agonist activity at NOP in various functional assays (*47–49*). Lofentanil, a

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potent mu agonist, has a Ki of 24 nM at NOP (*50*), although fentanyl, a close analog, has no affinity at MOP (*48*).

Among other opioid ligands, naloxone benzoylhydrazone (NalBzOH), a kappa agonist and mu antagonist, has a binding affinity of 25 nM at NOP and antagonizes the effect of N/OFQ in vitro and in vivo (*51*, *52*). TRK-820, another morphinan-based kappa agonist/mu partial agonist, was also reported to bind to NOP with a Ki of 380 nM and antagonize N/OFQ-mediated cAMP accumulation (*53*). These opioid ligands could serve as good lead compounds for NOP/MOP bifunctional compounds. Buprenorphine analogs, with increased affinity at NOP compared to buprenorphine, have been developed (*54*, *55*).

Our previous work on the discovery of small-molecule NOP ligands has yielded several NOP ligands that have high NOP binding affinity and modest selectivity versus opioid receptors (*29*, *33*, *46*, *56*, *57*). These compounds are good starting leads to 'design in' high affinity for the MOP receptor, and obtain the right profile of functional activity at both receptors. As discussed above, we have developed informative SAR around the main pharmacophoric features of NOP ligands, particularly with respect to opioid selectivity and intrinsic efficacy. From our series of dihydroindolin-2-one NOP ligands (*33*), we identified several NOP ligands, which have high affinity for NOP and MOP, but lower affinity for the delta and kappa opioid receptors (Table 2). As shown in Table 2, **AT-203** and **AT-206** have nanomolar affinity at NOP and a reasonable affinity at MOP. We have explored the SAR of these 'bifunctional' lead compounds, to obtain compounds with different ratios of NOP agonist and MOP agonist activity, in order to determine which profile of NOP/MOP bifunctional agonist activity affords *'non-addicting' anti-nociceptive activity*. This is a work in progress. Our initial SAR is focused on the modification of the piperidine nitrogen substituent (C-moiety of our proposed pharmacophore). Although **AT-206**, containing the cyclooctylmethyl substituent on the piperidine nitrogen, is a NOP antagonist (Table 2), it has low, but measurable partial agonist activity at MOP. Interestingly, conformational restriction and bridging of the cyclooctylmethyl group over the methylene linker, to form a bicyclic bridged cycloalkyl group as in **AT-201**, increased the MOP affinity significantly and retained the high NOP affinity. More importantly however, this converted the NOP antagonist activity in **AT-206**, to a NOP partial agonist activity in **AT-201** (Table 2). **AT-201** (formerly known as **SR16435** (*56*)) has been extensively characterized as a NOP/MOP bifunctional agonist, discussed in another chapter in this series (Toll et al.)

Docking the bifunctional NOP/MOP agonist **AT-201** into the active-state NOP receptor and the active-state homology model of the MOP receptor (Figures 4A and 4B respectively) clearly showed that **AT-201** docked well into both receptors, and that the active sites of the NOP and MOP receptors share a high degree of similarity in the residues that line the binding pocket. The compound interacts with the conserved amino acids in both the receptors, as shown in Figures 4A and 4B, leading to high affinity at both receptors. **AT-201** does not appear to interact with amino acids that are *non-conserved* among NOP and MOP receptors, e.g. Gln280 in TM6 of NOP is *non-conserved* and is His299 in the other three opioid receptors (*58*). Interestingly, this residue has been shown to play a role in excluding opioid binding to the NOP receptor. However, **AT-201**

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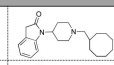
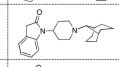
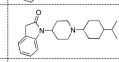
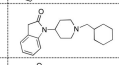
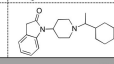
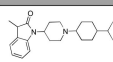
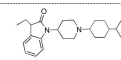
does not interact with Gln280 or His299, when docked with the NOP or MOP receptor (see Figures 4A and 4B), thereby leading to equipotent binding affinity and bifunctional activity at both receptors.

Table 2. Binding affinities and functional activities of bifunctional NOP/mu ligands (NT=not tested)

	Structure	Receptor Binding K_i (nM)			[^{35}S]GTPγS **NOP**		[^{35}S]GTPγS **μ**		[^{35}S]GTPγS **κ**	
		NOP	μ	κ	EC_{50} nM	% Stim	EC_{50}nM	% Stim	EC_{50} nM	% Stim
Modifications of the C-moiety pharmacophore (piperidine N-substituent)										
AT-206 (SR14148)		6.04 ± 0.4	14.4 ± 1.0	229 ± 33	<10K	-	239 ± 43	25.9 ± 6	<10K	
AT-201 (SR16435)		7.49 ± 0.78	2.70 ± 0.05	31.74 ± 4.8	28.7 ± 0.6	45 ± 5	29 ± 10	30 ± 0.2	<10K	
AT-203		3.96 ± 1.55	8.0 ± 0.97	148.7 ± 8.7	26.5 ± 4.3	100± 15	73.5 ± 5	49 ± 0.45	<10K	
AT-209		213 ± 0.95	532.8 ± 42	NT	NT	NT	NT	NT	NT	NT
AT-210		9.98± 2.8	3.44 ± 0.46	43.9 ± 9.2	82.3 ± 16	60± 10	28 ± 3.4	80.5 ± 11	873±293	48 ± 12
Modifications of the A-moiety pharmacophore (the piperidine 4-position heterocyclic ring)										
AT-211		6.53 ± 1.48	3.62 ± 0.38	41.8 ± 2.4	54.9 ± 41	100± 40	119.6	84.3	<10K	
AT-212 (SR16507)		5.22 ± 0.65	1.07 ± 0.17	82.4 ± 16.4	8.5 ± 0.8	95± 12	4.7 ± 1.2	44 ± 5.2	<10K	

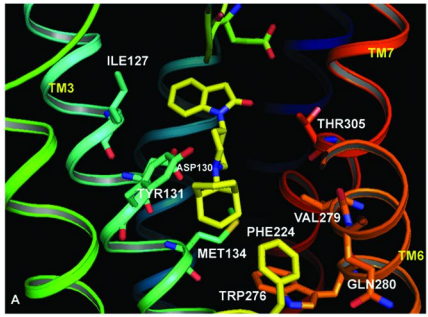


*Figure 4. Binding pose of bifunctional NOP/MOP agonist AT-201 in the **(A)** active-state NOP receptor structure binding pocket, and **(B)** in the active-state homology model of the MOP receptor. Docking was carried out with Surflex Dock in Sybyl X1.2 (Tripos). Note that the nonconserved residues Gln280 (in NOP) and His299 (in MOP) in TM6 do not appear to interact with the ligand. (see color insert)*

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Further SAR exploration of the piperidine nitrogen substituent of **AT-201** has been carried out (Table 2). A cyclohexylmethyl substituent on the piperidine nitrogen, as in **AT-209** (Table 2), surprisingly, led to a significant drop in affinity for both the NOP and the MOP receptor. However, introduction of a methyl group onto the methylene linker of the cyclohexylmethyl group (**as in AT-210**), regains affinity at both receptors, resulting in equal and high binding affinity at both the NOP and MOP receptor. This SAR suggests that the lipophilic substituent (the C-moiety pharmacophore) takes part in important interactions with the hydrophobic amino acids lining the binding pockets, and the size/shape of the substituent may influence the intrinsic activity, particularly at the NOP receptor (*29*).

We have also explored the SAR for the modification of the dihydroindolin-2-one (A-moiety pharmacophore) of the lead compound **AT-203** for bifunctional activity. Introduction of small alkyl substituents on the 3-position of the dihydroindolin-2-one increased MOP affinity and retained the NOP affinity, in **AT-211** and **AT-212**, compared to the lead **AT-203** (Table 2). The resulting bifunctional compounds have high affinity at both receptors, and in case of **AT-212**, have full agonist activity at NOP and partial agonist activity at MOP. Docking **AT-212** into the NOP and MOP receptor active sites (Figure 5) shows that the small alkyl substituents at the 3-indolinone position may interact with a highly conserved tyrosine residue in TM1 (Y58 in NOP and Y77 in MOP). **AT-212** (formerly **SR16507** (*46*)) has also been characterized in vivo in antinociceptive assays, and evaluated for its rewarding properties. This is discussed in an accompanying chapter in this series (Toll et al.)

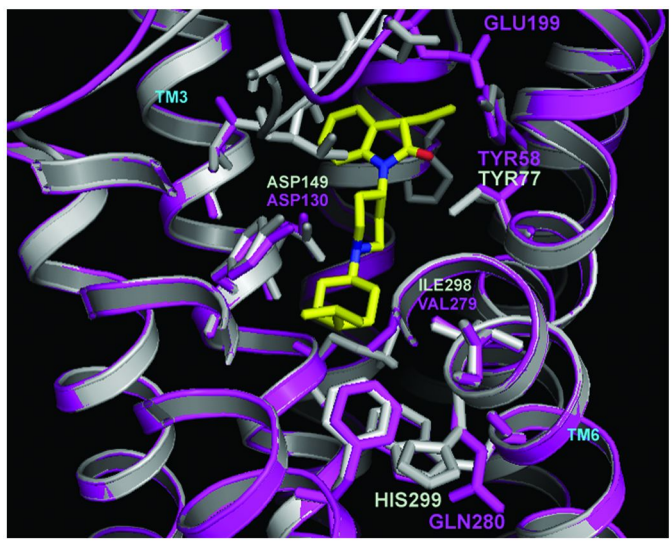

*Figure 5. Overlay of the docked pose of bifunctional NOP/MOP ligand **AT-212** in the NOP receptor (magenta) and MOP receptor (gray) active sites. Numbering of amino acid residues of the respective receptors follows the same color scheme (NOP residues-magenta; MOP residues-gray). The overlaid TM helices are labeled in blue. (see color insert)*

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Our SAR exploration of NOP ligand scaffold and receptor structure docking suggests approaches to obtain bifunctional NOP and MOP activity starting with NOP ligands. The significant homology between the NOP and MOP receptors in the active site domain could be used to design ligands that have high binding affinity at both receptors, as with **AT-201** and **AT-211**. We are continuing to refine our SAR, to obtain varying profiles of NOP and MOP intrinsic activity. The resulting NOP/MOP bifunctional ligands obtained from this SAR will be useful for defining the optimum profile of NOP agonist activity and MOP agonist activity, that will afford 'non-addicting antinociceptives' and/or drug abuse medications.

Conclusions

Several groups have reported large compound libraries with NOP binding affinity. With the availability of the NOP receptor crystal structure and homology models of the active-state receptor, it is now possible to explore structure-based virtual screening approaches to discover novel classes of NOP ligands. However, converting virtual screening hits into useful NOP ligands will require a thorough understanding of the NOP ligand SAR, guided by structure-based lead optimization. Medicinal chemistry and lead optimization, guided by such SAR, are necessary to develop drug-like NOP-targeted compounds to explore various therapeutic applications. Bifunctional NOP/MOP agonists, on the other hand, also hold great promise as potential non-addicting analgesics (*46*, *59*). The SAR approaches discussed here should be useful for designing bifunctional NOP/MOP ligands for therapeutic applications. The availability of the crystal structures of the four opioid receptors will also aid such multi-targeted drug design.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Chapter 9

Mu, Delta and Kappa Opioid Agonist Effects In Novel Assays of Pain-Depressed Behavior

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Pain is associated with stimulation of some behaviors (e.g. reflexive withdrawal responses) but depression of many other behaviors (e.g. feeding, locomotion, positively reinforced operant responding). This chapter reviews effects of mu, delta and kappa opioid agonists in novel assays of pain-depressed behavior, with a particular focus on studies in which intraperitoneal injection of dilute acid served as a noxious stimulus to depress the positively reinforced operant behavior of intracranial self-stimulation (ICSS). Mu agonists such as morphine reliably block most forms of pain-depressed behavior, including acid-induced depression of ICSS. Although delta agonists have not been extensively studied in assays of pain-depressed behavior, the delta agonist SNC80 also blocked acid-induced depression of ICSS. Kappa agonists fail to produce antinociception in assays of pain-depressed behavior, and centrally penetrating kappa agonist such as salvinorin A and U69,593 exacerbate pain-related behavioral depression. The efficacy of mu but not kappa agonists to block pain-related depression of behavior agrees with the clinical finding that mu but not kappa agonists function as effective analgesics in humans. These results suggest that novel assays of pain-depressed behavior may improve predictive validity of preclinical research on development of opioids and other drugs as candidate analgesics.

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Introduction

Preclinical assays of animal behavior have played a key role in research on the neurobiology of pain and development of analgesic drugs (*1*). These preclinical assays share two common elements: (a) a set of independent variables implemented with the intent of producing a pain state, and (b) a dependent measure of behavior interpreted as evidence of that pain state. As one simple example, tail-withdrawal assays of thermal nociception apply a noxious thermal stimulus (e.g. a hot light or hot water) to the tail of a rodent or non-human primate and measure the latency to a tail withdrawal response. In this example, application of the hot stimulus is intended to produce "pain," and tail withdrawal is an unconditioned behavioral response interpreted as evidence of "pain." Drugs or other treatments can then be evaluated for their effects on expression of the pain-related behavior. This simple type of assay has proven especially useful for studies of pharmacology (e.g. for determination of potency, efficacy, time course and receptor mediation of opioid effects); however, results often fail to translate to human studies of pain and analgesia (*1–5*). Shaped in part by efforts to increase the relevance and predictive validity of preclinical research for clinical treatment of pain in humans, these simple assays have evolved in two general ways (*1*). First, with regard to the independent variables, methods have been developed to model inflammatory or neuropathic states that render subjects more sensitive to provocative mechanical or thermal stimuli. Second, with regard to dependent measures, new assays have been developed to assess new classes of pain-related behaviors. This chapter will focus on opioid effects in the latter type of assay.

Many pain-related behaviors can be assigned to two general categories that we have called "pain-stimulated behaviors" and "pain-depressed behaviors" (*6*). Pain-stimulated behavior can be defined as any behavior that increases in rate, frequency or intensity after presentation of a noxious stimulus, and common examples include withdrawal responses from escapable stimuli (such as tail-withdrawal responses from thermal stimuli as described above) or pseudowithdrawal responses from inescapable stimuli (e.g. stretching or flinching responses elicited by injection of noxious chemical stimuli). Antinociception in assays of pain-stimulated behavior are indicated by drug-induced decreases in the target behavior. For example, Figure 1 shows effects of a mu opioid agonist (morphine), delta agonist (SNC80) and kappa agonist (salvinorin A) in an assay of acid-stimulated stretching in rats (*7–9*). In this assay, intraperitoneal injection of dilute lactic acid served as chemical noxious stimulus to elicit a repetitive stretching response, and the number of stretches was counted during a 30 min observation period. All three opioids produced a dose-dependent decrease in acid-stimulated stretching, and these effects were blocked by selective mu, delta or kappa antagonists (data not shown). The antinociceptive effects observed in this assay are similar to antinociceptive effects of mu, delta and kappa opioid agonists in many other assays of pain-stimulated behavior, and such data have often been interpreted as evidence of analgesic effects of mu, delta and kappa agonists. However, exclusive reliance on pain-stimulated behaviors to evaluate effects of opioids or other candidate analgesics is problematic for several reasons (*1*). Perhaps most importantly, drug-induced decreases in pain-stimulated

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behavior can be produced not only by a selective reduction in sensory sensitivity to the noxious stimulus (i.e. true analgesia) but also by nonselective effects such as motor impairment (resulting in "false positive" effects). Notably, kappa agonists have failed to produce safe and/or effective analgesia in human studies despite their frequently observed efficacy in preclinical assays of pain-stimulated behavior.

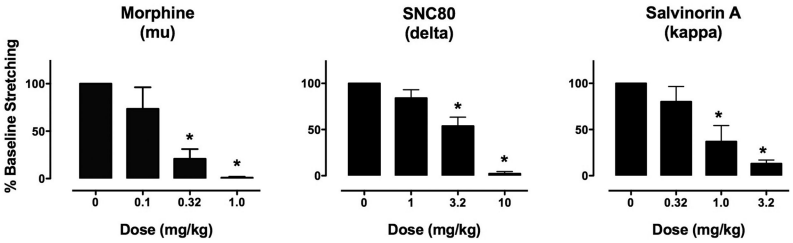

Figure 1. ***Opioid antinociception in an assay of acid-stimulated stretching.*** *Abscissae: Dose in mg/kg, log scale. Ordinates: Percent vehicle control number of stretches observed during the 30 min after intraperitoneal injection of dilute acid (1.8% lactic acid in a volume of 1.0 ml/kg). Each bar shows mean+SEM from 5-6 rats. Asterisks indicate significantly different from "0" dose for each drug. Baseline rates of stretching were 14.0±3.0 for morphine, 18.2±1.9 for SNC80 and 28.4±4.2 for salvinorin A. Data for morphine are unpublished (Altarifi and Negus); data for SNC80 and salvinorin A are adapted from (8, 9).*

By contrast to pain-stimulated behaviors, pain-depressed behaviors can be defined as behaviors that decrease in rate, frequency or intensity after presentation of a noxious stimulus, and common examples include pain-related decreases in feeding, locomotor activity, or rates of positively reinforced operant responding (*6*). Importantly, antinociception in assays of pain-depressed behavior is indicated by an *increase* in the target behavior, and as a result, these assays are not vulnerable to false-positive effects of drugs that produce motor impairment. Assays of pain-depressed behavior may also add value in analgesic drug development for two other reasons. First, the diagnosis of pain in both human and veterinary medicine often relies on measures of pain-depressed behavior (also referred to as "functional impairment"), and restoration of pain depressed behavior is often a goal of treatment (*10–13*). The utility of these measures in clinical contexts suggests that pain-depressed behaviors may also be useful as endpoints in research. Second, pain-related depression of behavior is often accompanied by comorbid depression of mood in humans (*14*, *15*), and preclinical research on pain-depressed behavior may provide insights into the expression, neurobiology and modulation of the affective dimensions of pain.

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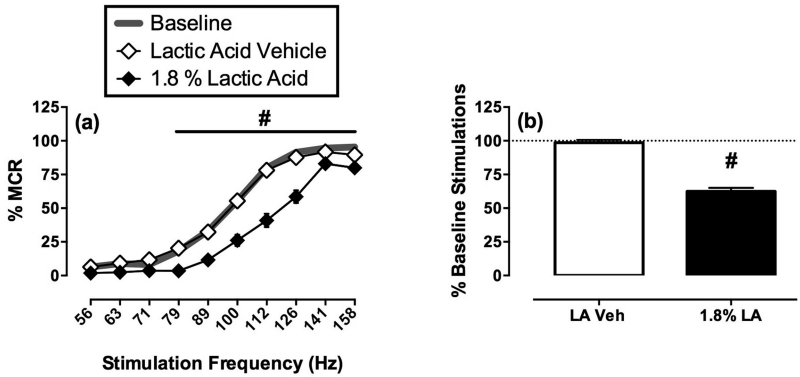

Figure 2. ***Intraperitoneal acid depresses ICSS.*** *Panel a shows acid effects on ICSS frequency-rate curves. Abscissa: frequency of brain stimulation in Hz, log scale. Ordinate: rate of ICSS expressed as percent maximum control rate (%MCR, a normalized measure of ICSS rate, see references for further details). Number sign (#) indicates frequencies at which ICSS was lower after acid than after saline treatment as determined by two-way ANOVA followed by a Holm-Sidak post hoc test. Panel b shows the same data in summary form. Abscissa: treatment with lactic acid vehicle (LA Veh) or 1.8% lactic acid (1.8% LA). Ordinate: percentage of baseline number of stimulations per component. Number sign (#) indicates significantly different from LA Veh as determined by paired t-test. All data show mean±SEM from 34 rats. Data adapted from (8).*

Assays of pain-depressed behavior can measure pain-related decreases in any of a variety of behaviors, although to be experimentally useful, baseline rates of the target behavior should be *relatively high* (to permit detection of pain-related decreases), *stable* (to permit differentiation of effects produced by noxious stimuli and/or drugs from natural variability), and *quantifiable* (to permit precise and objective measurement) (*6*). Operant conditioning provides one strategy for generating high, stable and quantifiable rates of baseline behavior that can be used to assess effects of drugs or other manipulations, and Figure 2 shows an example of operant behavior that can be depressed by a noxious stimulus (*8*). In this assay of intracranial self-stimulation (ICSS), rats were implanted with intracranial electrodes targeting the medial forebrain bundle, and lever-press responding was maintained under a fixed-ratio 1 schedule of brain stimulation. The ability of brain stimulation to function as a reinforcing stimulus was discovered more than 50 years ago (*16*), and ICSS maintained by stimulation of the medial forebrain bundle is mediated by activation of excitatory inputs to mesolimbic dopamine neurons that originate in the ventral tegmental area (*17*, *18*). In the procedure used here, daily sessions consisted of multiple components, and the magnitude of the brain stimulation reinforcer was manipulated across components by manipulating the frequency of stimulation in a descending series of 10 steps from 158 Hz to 56 Hz in 0.05 log unit increments. On test days, response rates maintained across this range of frequencies was evaluated before (baseline) and

after intraperitoneal administration of either vehicle (water) or a chemical noxious stimulus (dilute lactic acid, the same noxious stimulus used above for studies of acid-stimulated stretching). The left panel of figure 2 (Fig. 2a) shows that, under baseline conditions, increasing frequencies of brain stimulation maintained increasing rates of ICSS, and as with many types of operant responding, these frequency-dependent rates of ICSS can be maintained at stable levels for months. Treatment with acid vehicle had no effect on the ICSS frequency-rate curve, but treatment with the dilute acid noxious stimulus depressed ICSS at frequencies of 79 Hz and higher. The right panel of figure 2 (Fig. 2b) summarizes this finding by showing the total number of stimulations across all frequencies as a percent of the baseline number of stimulations across all frequencies, and acid significantly decreased this measure of total ICSS. Overall, then, acid-induced depression of ICSS provides one example of pain- depressed behavior, and analgesics would be expected to block acid-induced depression of ICSS.

Effects of Mu Agonists on Pain-Depressed Behavior

The mu agonist morphine dose-dependently blocks acid-induced depression of ICSS (*7*, *19*). Figure 3 shows the effect of one experiment with a dose of 1.0 mg/kg morphine. The left panel (Fig. 3a) compares the effects of morphine or its vehicle in the absence of the noxious stimulus (lactic acid vehicle), and this dose of morphine had no effect on the ICSS frequency-rate curve under these conditions. The center panel (Fig. 3b) compares the effects of morphine and its vehicle administered as pretreatment to the acid noxious stimulus. After morphine vehicle, acid depressed ICSS as described above and produced a rightward shift in the ICSS frequency-rate curve (compare open diamonds in 3a and closed diamonds in 3b). Pretreatment with morphine blocked this acid-induced depression of ICSS and prevented the acid-induced rightward shift in the ICSS frequency-rate curve. The right panel (Fig. 3c) shows in summary form that 1.0 mg/kg morphine had no effect on ICSS in the absence of the noxious stimulus but blocked acid-induced depression of ICSS.

Three additional points related to this experiment warrant mention. First, acid-induced depression of ICSS is also dose-dependently blocked by other mu agonist analgesics including methadone, hydrocodone and buprenorphine (*20*). Second, as shown in Table 1, morphine has been shown to block many other examples of pain-depressed behavior produced by several other types of pain manipulation. Together, these findings suggest considerable generality in the ability of mu agonists to block pain-related depression of behavior. Finally, the study shown in Figure 3 illustrates the value of including control experiments that examine effects of test drugs administered alone in the absence of noxious stimulation. In this case, the test dose of morphine did not alter ICSS when administered alone, suggesting that morphine effects on acid-depressed ICSS could be more confidently attributed to antinociception and not to nonselective effects of morphine on the target behavior. However, mu agonists and other test drugs often do affect expression of the target behavior, and these effects in the absence of pain can assist with interpretation of drug effects in the presence

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of pain. As one example, the effects of morphine in Table 1 were often shown to display an inverted U-shaped dose-effect curve with peak antinociception at intermediate morphine doses. In these studies, high morphine doses typically depressed the target behavior in the absence of stimulation and produced little or no blockade of pain-induced depression of behavior. Such findings are often interpreted as evidence that sedative effects of high mu agonist doses may obscure expression of antinociception in assays of pain-depressed behavior, in which antinociception is manifested as an increase in rates of the target behavior.

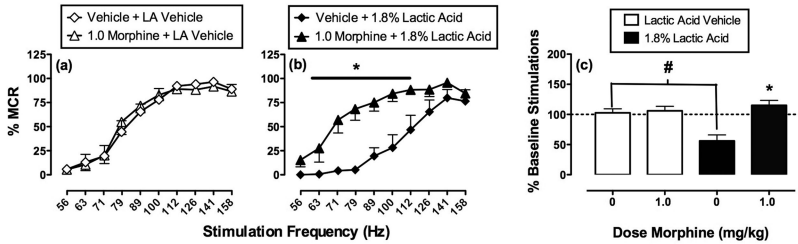

Figure 3. ***Morphine blocks acid-induced depression of ICSS.*** *Panels a and b show morphine effects on ICSS frequency-rate curves in the absence (a) or presence (b) of the noxious acid stimulus. Abscissae: frequency in Hz, log scale. Ordinates: rate of ICSS expressed as percent maximum control rate (%MCR). Asterisk (*) indicates frequencies at which ICSS was higher after morphine+acid than after vehicle+acid as determined by two-way ANOVA followed by a Holm-Sidak post hoc test. Panel c shows the same data in summary form. Abscissa: morphine dose in mg/kg. Ordinate: percentage of baseline number of stimulations per component. Number sign (#) indicates a significant depression of ICSS by acid; asterisk (*) indicates that morphine blocked acid-induced depression of ICSS. All data show mean±SEM from 6 rats. Unpublished data from Altarifi and Negus with procedures as in (7, 16).*

Although data on "sedative" effects can identify drug doses at which antinociception might be obscured, it is also important to consider the degree to which a test drug might produce nonselective *increases* in the target behavior, because nonselective behavioral stimulation can produce false-positive effects in assays of pain-depressed behavior (*6*). Assessment of drug-induced increases in behavior can be difficult with many endpoints, such as feeding, locomotion or food-maintained operant responding under fixed-ratio schedules, because the target behavior occurs at a relatively high and constant rate in the absence of pain, and further increases may be difficult to produce or detect. This issue can be addressed by incorporating conditions that generate both low and high rates of baseline behavior, and ICSS is ideal for this purpose. The magnitude of the brain-stimulation reinforcer can be rapidly manipulated across a range of values to engender a range of baseline behavioral rates such as those shown in Figures 2 and 3. Consequently, drug effects on low rates of pain-depressed ICSS can be compared to drug effects on low rates of behavior maintained by low reinforcer

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magnitudes in the absence of pain. As discussed above with Figure 3, 1.0 mg/kg morphine blocked acid-induced depression of ICSS without increasing low rates of baseline ICSS in the absence of pain, and this finding suggests that morphine effects on acid-depressed ICSS reflect a true blockade of sensory sensitivity to the acid stimulus rather than a nonselective behavioral stimulation. It is important to note that morphine and other mu agonists can facilitate ICSS under other conditions (*21–25*), but this experiment illustrates the potential for mu agonists to selectively block pain-related depression of behavior. Moreover, these results with morphine contrast with effects of cocaine in the same procedure. Cocaine also blocked acid-induced depression of ICSS, but only at doses that also produced robust facilitation of ICSS in the absence of pain (*8*).

Effects of Delta Agonists on Pain-Depressed Behavior

Figure 4 shows that the delta agonist SNC80 also dose-dependently blocked acid-induced depression of ICSS at doses that had no effect on control ICSS in the absence of the noxious stimulus (*9*). Taken together with the efficacy of SNC80 to block acid-stimulated stretching (Figure 1), these results support further consideration of delta agonists as candidate analgesics. However, in contrast with morphine, SNC80 effects on acid-depressed ICSS were determined in part by regimens of repeated dosing. Upon initial administration, or when SNC80 doses were separated by weekly intervals, low doses of SNC80 (e.g. 1.0 mg/kg) only weakly attenuated acid-induced depression of ICSS, and higher doses (e.g. 10 mg/kg) depressed ICSS in the absence of the acid noxious stimulus and failed to block acid-induced depression of ICSS. However, when SNC80 was administered more frequently (e.g. twice per week as shown in Figure 4), higher doses of SNC80 no longer depressed control ICSS, and a full antinociceptive blockade of acid-induced depression of ICSS was observed. These results were interpreted to suggest that initial or intermittent SNC80 produced sedative effects that obscured expression of antinociception in the assay of pain-depressed behavior; however, more frequent injections produced tolerance to sedative effects and unmasked expression of antinociceptive effects. Moreover, dosing regimens that *increased* expression of antinociception in the assay of acid-depressed ICSS also *decreased* expression of antinociception in the assay of acid-stimulated stretching (i.e. SNC80 dosing at short intervals reduced the ability of SNC80 to decrease acid-stimulated stretching). These findings were interpreted to have two implications. First, they suggested that SNC80-induced sedation *contributed to* apparent antinociception in the assay of acid-stimulated stretching but *opposed* antinociception in the assay of acid-depressed ICSS. Second, they provide evidence for differential modulation of drug effects in assays of pain-stimulated and pain-depressed behaviors, even when the putative pain state has been produced with the same noxious stimulus (in this case, intraperitoneal acid injection) (*9*). With SNC80, apparent antinociceptive tolerance in the assay of acid-stimulated stretching was accompanied by enhanced expression of antinociception in the assay of acid-depressed ICSS.

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Table 1. Morphine effects in preclinical assays of pain-depressed behavior.

Species	*Pain Manipulation*	*Behavioral Endpoint*	*Morphine Effective*	*Ref.*
Mouse	Intraperitoneal Acetic Acid	Feeding	Yes	(*36*)
Mouse	Intraperitoneal Acetic Acid	Locomotion	Yes	(*37*)
Mouse	Intraperitoneal Acetic Acid	Wheel Running	Yes[a]	(*38*)
		Feeding	No	
Mouse	Intraplantar Complete Freunds Adjuvant (CFA)	Wheel Running	Yes[a]	(*39*)
Rat	Laparotomy	Locomotion, Food-Maintained Operant Responding	Yes[a]	(*40*)
		Rearing	No	
Rat	Facial Carrageenan +Heat	Food-Maintained Operant Responding	Yes	(*41*)
Rat	Facial Capsaicin +Heat	Food-Maintained Operant Responding	Yes	(*42*)
Rat	Intraperitoneal Acetic Acid	Rearing	Partial	(*26*)
Rat	Intra-articular CFA	Rearing	Yes[a]	(*43*)
Rat	Intraperitoneal Acetic Acid	Intracranial Self-Stimulation	Yes[a]	(*7*)
Dog	Intra-articular Formalin	Locomotion	Yes	(*44*)

[a] Indicates inverted-U shaped morphine dose-effect curve.

In addition to these studies with SNC80, the other putative delta agonist ARM390 was also tested in assays of acid-stimulated stretching and acid-depressed ICSS (*9*). ARM390 is a congener of SNC80 reported to produce delta receptor-mediated antinociception in assays of pain-stimulated behavior in mice but with a lower propensity than SNC80 to internalize delta receptors or produce acute antinociceptive tolerance. However, in contrast to these results in mice, ARM390 failed to produce antinociception in rats in assays of either acid-stimulated stretching or acid-depressed ICSS.

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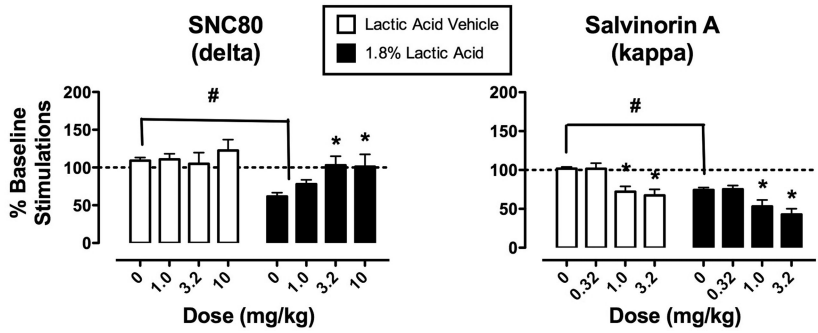

Figure 4. Blockade of acid-induced depression of ICSS by the delta agonist SNC80 but not by the kappa agonist salvinorin A. Abscissae: Dose SNC80 (left panel) or salvinorin A (right panel) in mg/kg. Ordinates. Percentage of baseline number of stimulations per component. Drug effects in the absence or presence of the noxious stimulus are shown by open and filled bars, respectively. Number signs (#) indicates a significant depression of ICSS by acid; asterisks (*) indicate significant effect of test drug on ICSS relative to the "0" dose in the absence or presence of the acid noxious stimulus. Each drug was tested in a group of 6 rats. Data adapted from (8, 9).

Effects of Kappa Agonists on Pain-Depressed Behavior

Kappa opioid receptor agonists do not produce antinociception in the assay of acid-depressed ICSS (*8*, *19*). Figure 4 shows results from one experiment with the kappa agonist salvinorin A, which is an active constituent from the plant salvia divinorum. As has been shown previously with salvinorin A and other kappa agonists, salvinorin A in this study produced a dose-dependent decrease in control ICSS in the absence of noxious stimulation. Low salvinorin A doses that did not affect control ICSS also failed to block acid-induced depression of ICSS, and higher salvinorin A doses that decreased control ICSS also exacerbated acid-induced depression of ICSS. Similar results have been obtained with the other selective and high-efficacy kappa agonist U69,593 in the assay of acid-depressed ICSS. Moreover, the kappa-2 opioid receptor agonist GR89,696 failed to block pain-related depression of rearing and food-maintained operant responding in rats (*26*).

These findings are notable for two general reasons. First, results with these kappa agonists illustrate the potential for a complete dissociation of drug effects in assays of pain-stimulated and pain-depressed behavior. For example, salvinorin A produced antinociception at doses of 1.0 and 3.2 mg/kg in the assay of acid-stimulated stretching in rats (see figure 1), but these same doses of salvinorin A decreased control ICSS and exacerbated pain-related depression of ICSS in the assay of acid-depressed ICSS. Taken together, these results suggest that salvinorin A and other kappa agonists do not produce analgesia, but rather produce nonselective behavioral depression that manifests as "false-positive"

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antinociception in assays of pain-stimulated behavior. Second, the failure of kappa agonists to produce antinociception in assays of pain-depressed behavior agrees with the failure of kappa agonists to produce safe and/or effective analgesia in humans (*27–29*). This provides one source of evidence to suggest that preclinical assays of pain-depressed behavior may have greater predictive validity than assays of pain-stimulated behavior for forecasting analgesic effects of test drugs in humans.

One strategy for improving the safety of kappa agonists has been to develop compounds that do not readily cross the blood-brain barrier and hence remain peripherally restricted after systemic administration (*30*). This approach is founded on the notion that peripherally restricted kappa agonists might retain an ability to produce antinociception by acting at peripheral kappa receptors while displaying reduced potency to produce undesirable effects mediated by central kappa receptors. Consistent with this hypothesis, the peripherally restricted kappa agonists ffir and ICI204,448 both produced a dose-dependent decrease in acid-stimulated stretching at doses that did not alter control ICSS; however, neither drug blocked acid-induced depression of ICSS (*8*). By contrast, the nonsteroidal antiinflammatory drug (NSAID) ketoprofen blocked both acid-stimulated stretching and acid-induced depression of ICSS without affecting control ICSS (*8*). These results were interpreted to suggest that although peripherally restricted kappa agonists may be somewhat safer than centrally penetrating kappa agonists like salvinorin A, they are less efficacious than mu agonists or NSAIDs for blocking pain-related depression of behavior. This conclusion agrees with the poor and inconsistent analgesic efficacy of peripherally restricted kappa agonists studied to date in humans (*31–35*) and provides another example of concordance between preclinical studies of pain-depressed behavior and human studies of pain and analgesia.

Summary and Conclusions

Historically, analgesic drug development has relied almost exclusively on preclinical assays of pain-stimulated behaviors. However, pain states are often associated with depression of behavior, and restoration of pain-related depression is often a goal of treatment. Novel assays of pain-depressed behavior are providing new research tools for evaluating both the expression and treatment of pain-related behavioral depression, and these assays have provided new insights into opioid agonist effects. Consistent with their clinical analgesic efficacy, mu agonists have typically produced antinociception in assays of both pain-stimulated and pain-depressed behavior. The delta agonist SNC80 also produced antinociception in both types of assays, although regimens of repeated treatment produced opposite effects on SNC80 antinociception in assays of pain-stimulated behavior (tolerance) and pain-depressed behavior (enhancement). Further studies with other delta agonists are warranted. Kappa agonists have produced antinociception in most assays of pain-stimulated behavior, but in agreement with their poor clinical efficacy, they have failed to produce antinociception in assays of pain-depressed behavior and often exacerbate pain-related behavioral depression.

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Table 2. Effects of opioids and other drugs in assays of acid-stimulated stretching and acid-depressed ICSS in rats. Drugs in bold are antinociceptive in both assays. References are shown in the footnotes.

	Effective In Assay of Acid-Stimulated Stretching	*Not Effective In Assay of Acid-Stimulated Stretching*
Effective In Assay of Acid-Depressed ICSS	**Methadone**[1] **Fentanyl**[1] **Morphine**[1] **Hydrocodone**[1] **Buprenorphine**[1] **Nalbuphine** **SNC80**[2] **Ketoprofen**[3]	Cocaine[4]
Not Effective In Assay of Acid-Depressed ICSS	Salvinorin A[5] U69,593[5] ffir[6] ICI204,448[6] Δ9-THC[7] CP55940[7] Flupenthixol[8]	Naltrexone[9] Naltrindole[10] Norbinaltorphimine[11]

[1] Mu opioid receptor agonist (*7*, *19*, *20*) [2] Delta opioid receptor agonist (*9*). [3] Nonsteroidal antiinflammatory drug (*8*, *45*) [4] Monoamine reuptake inhibitor (dopamine, serotonin, norepinephrine nonselective) (*8*). [5] Kappa opioid receptor agonist (centrally penetrating) (*8*, *19*) [6] Kappa opioid receptor agonist (peripherally restricted) (*8*). [7] Cannabinoid-1 receptor agonist (*45*). [8] Dopamine receptor antagonist (*8*). [9] Opioid receptor antagonist (unpublished). [10] Delta opioid receptor antagonist (*9*). [11] Kappa opioid receptor antagonist (*19*).

We have also examined other drugs from other drug classes in our assays of acid-stimulated stretching and acid-induced depression of ICSS. Results are summarized in Table 2. An important conclusion from this table is that clinically effective analgesics block both acid-stimulated stretching and acid-induced depression of ICSS, whereas drugs effective in only one or the other type of assay are generally not effective clinical analgesics against acute pain. These findings support the utility of assays of pain-depressed behavior as a complement to more conventional assays in the preclinical evaluation of candidate analgesics. Lastly, it should be noted that research with assays of pain-depressed behavior is in its infancy. Future studies will compare the neural substrates of pain-stimulated and pain-depressed behaviors, evaluate the potential for heterogeneity in substrates for pain-related depression of different behaviors, and develop assays for more chronic pain-related depression of behavior.

Acknowledgments

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Chapter 10

Functionally Biased Agonism of Mu and Kappa Opioid Receptors

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Nearly all opioids approved for human therapeutic use were developed between the early 1900s and the mid 1960's, mostly on the basis of behavioral experiments performed *in vivo*. Starting approximately in the 1970s with the discovery of the mu opioid receptor (MOPr) and the endogenous endorphin/enkephalin system, the 40 years up to today have witnessed major advances in the molecular-level understanding of opioid receptor signal transduction pathways, receptor localization and expression, and regulatory systems including phosphorylation and trafficking. It is concluded that the currently marketed opioids have only marginally benefitted from modern biological insight or technology and that further mechanistic optimization toward the goal of separating side effect(s) from analgesia might be possible. The purpose of this chapter is to review the concept of functionally-selective agonists specifically in the context of mu (MOPr) and kappa (KOPr) opioid receptors. If analgesic and non-analgesic responses to opioid agonists are ultimately associated with non-overlapping signal transduction sub-pathways, then this approach may lead to improved analgesic agents.

Introduction

There is a steady chronology of the inter-relationship between opium and human history that continues to the modern day. Stone tablets from the Assyrian empire serve as the earliest written record of man's experience with opium from

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about 5000-7000 years ago, and then the timeline extends to ancient Egypt where medical applications of opium are described in the oldest known medical text, the Elbers Papyrus. Egyptian trade with Greece was presumably a prelude to the further spread of opium into Southern Europe and beyond. Early Greek and Roman physicians including Hippocrates and Galen praised the medicinal properties of opium, often mistakenly presuming it cured diseases because it removed painful symptoms. Alexander the Great was a likely conduit for the introduced opium to Asia after his victory over the Persian Empire, laying the foundation for a future opium crisis in China that led to two wars with England. The risk-benefit relationship of opium has been recognized throughout history and has under-pinned a long-standing goal to separate the analgesic benefit from the adverse physiological and behavior side effects.

Although the earliest use of opium was primarily via the crude, whitish-brown, dried sap of the poppy (*Papaver somniferum*) seed capsule, later innovations led to the isolation and structure-elucidation of morphine, the major active alkaloid component in opium. Several years after he isolated morphine from opium as a dry powder, Friedrich Serturner (1816) declared, "I flatter myself that my observations have explained to a considerable extent, the constitution of opium and that I have enriched chemistry with a new alkaline base (morphium) a remarkable substance" (*1–3*).

Although Serturner's discovery was a pivotal point in history, the isolation of morphine alone did not solve the opium-derived public health issues of the time, and ironically even he died a morphine addict. His work however allowed, for the first time in history, the administration of a pre-measured dose of morphine to humans and thus eliminated the batch-to-batch variability in potency of crude opium that often led to unexpected overdose and even death.

Some of the earliest synthetic opioids were simple morphine and codeine oxidation products and included heroin, oxycodone and hydrocodone, discovered in 1874, 1916 and 1920 respectively (*4–7*). The elucidation by Robertson (*8*) of the chemical structure of morphine occurred more than a century after Serturner's discovery of the natural product and, for the first time in history, synthetic chemists were enabled to design and synthesize chemical modifications of morphine, still hoping to discover an improved side effect profile. This led to the synthesis of "simpler" structural analogs, the so-called "ring-opened" opioids including demerol, methadone, and fentanyl (*9–11*). Shortly afterwards, another group led initially by Kenneth Bentley in the 1960's began preparing "more complex" analogs of morphine, the so-called Bentley compounds, also known as the orvinols, and included etorphine, dihydoetorphine, and buprenorphine (*12–14*).

While many of these synthetic alterations of morphine ultimately led to commercial products that remain on the market today, they are all burdened to varying degrees by dose-limiting side effects and in some cases recreational abuse (Figure 1).

Starting in the 1970s with the discovery of the mu opioid receptor (MOPr) and the endogenous endorphin/enkephalin systems (*15–18*), the 40 years up to today have witnessed major advances in the understanding of opioid receptor signal transduction pathways, receptor localization and expression levels, regulatory

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systems including phosphorylation and trafficking, and most recently an atomic-level view of a receptor antagonist covalently bound to the human MOPr (*19*). Unfortunately, the opioid chemistry of the 1900's and the pharmacological advances of the 2000's did not overlap in history. Hence, although man's interest in opium and related research has been ongoing for several thousand years, spanned the globe, outlasted empires, and was a primary driver of the modern-day pharmaceutical industry, the development of the currently marketed opioids has only marginally benefitted from modern biological insight or technology. On that basis, one might argue that further mechanistic optimization of the existing commercial opioid should be possible.

The purpose of this chapter is to review the supporting data for new signal transduction mechanisms and the concept of functionally-selective agonists specifically as applied to mu (MOPr) and kappa (KOPr) opioid receptors. Much of this research is just emerging and there are apparent contradictions as well as many unanswered questions, but data from recent publications leads to an optimistic view that this approach may finally lead to the design of a new generation of opioids where analgesia is (ideally) free of adverse events. Such an accomplishment would be historic, of global consequence, and a highly significant advance in the treatment of human pain.

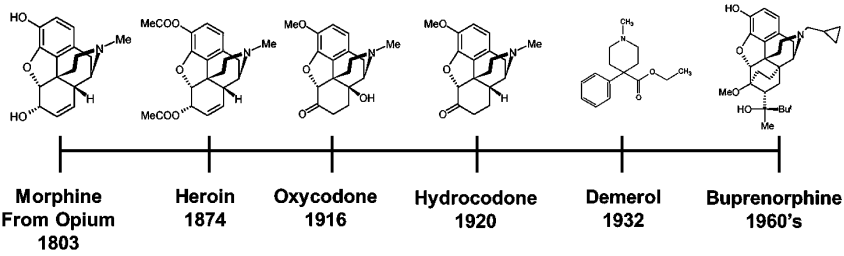

Figure 1. Historical timeline of discovery corresponding to several important opioids including the early synthetic modifications of morphine and codeine (Heroin, oxycodone, and hydrocodone) as well as the simplified "ring-opened" modification (Demerol) and the more complex Bentley compound, buprenorphine.

MOPr Signal Transduction

The opioid receptors are members of a superfamily of receptors known as G protein-coupled receptors (GPCR) and as such, the early receptor theory proposed that they interconvert between two conformational arrangements as a two-state model (*20*, *21*). According to this model (presented in Figure 2), one state is inactive (R), not coupling to G protein and not transmitting intracellular signals. The other state (R*) is active and couples with a heterotrimeric G protein to transduce a signal. The simplest explanation for ligand binding in this model is that antagonists bind and stabilize the former state, while agonists bind and stabilize the latter state. Furthermore in this model, opioid analgesia and side

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effects are further presumed to be on-target consequences of MOPr activation and essentially inseparable.

Observations published in recent reports lead to the conclusion that a two state model is insufficient and that a more complex model involving more than two interconverting, conformational states of the receptor (each with distinct signaling abilities) might be more appropriate. This emerging hypothesis implies that signal transduction pathways may be differentially regulated in a ligand-specific fashion since differing ligands might promote distinct states of a receptor. Hence the intrinsic efficacy of an agonist ligand may differ from one pathway to another. A representation of this multi-state model is shown in Figure 3. If analgesic and non-analgesic responses to opioid agonists are ultimately associated with non-overlapping signal transduction pathways, then this may indeed lead to improved analgesic agents. Several excellent reviews of these concepts were recently published (*22*, *23*).

Like other GPCR, the MOPr is an integral membrane protein localized on the surface of many cell types including neurons. Emerging *in vitro* evidence is supportive of a hypothesis that the MOPr may be further localized on the membrane in microdomains known as lipid rafts (*24*, *25*). A lipid raft is a dynamic plasma membrane domain containing high levels of cholesterol and sphingolipids, and is enriched with a variety of signaling factors including GPCR, G protein, and adenylyl cyclase. In the case of the MOPr receptor, concentration of signaling partner proteins on the raft microdomain may facilitate signaling efficiency, possibly due to inherent stabilization of the MOPr-Gi complex and/or the improved thermodynamics of the close proximity of these signaling partners. A correlation between agonist efficacy and MOPr localization on lipid raft domains has been demonstrated *in vitro* by titrating methyl-β-cyclodextrin (MBCD) into the culture, which in turn extracts the cholesterol from the cellular membrane. Multiple groups have reported that this results in a systematic loss of functional efficacy for otherwise robust MOPr agonists including DAMGO and others (*26*). Control experiments reveal that the cells remain intact and the number of MOPr receptors does not change in response to the MBCD titration. Restoring the cholesterol concentration to the membrane also restores agonist efficacy. A possible implication of this phenomenon will be discussed in a later section of this chapter.

Upon agonist binding to MOPr, the receptor undergoes a conformational change and adopts a state(s) wherein it becomes a guanine-exchange factor (GEF), couples with an inactivated, GDP-containing G_i heterotrimeric protein, and a GDP to GTP conversion occurs to produce an active $G\alpha_i$ protein that further dissociates into the cytosol (*27*).

In subsequent steps, the $G\alpha_i$ subunit acts on multiple targets including adenylyl cyclase (AC), which is acutely inhibited. This results in a reduction of cellular concentration of cyclic AMP (cAMP), an important second messenger, which further attenuates the activity of protein kinase A (PKA). Ultimately, members of the RGS family of proteins binds and prevents further $G\alpha_i$ subunit action, and/or the inherent GTP-ase activity of the $G\alpha_i$ subunit causes self-conversion back to an inactivated state and ultimate re-association with the $\beta\gamma$ subunits.

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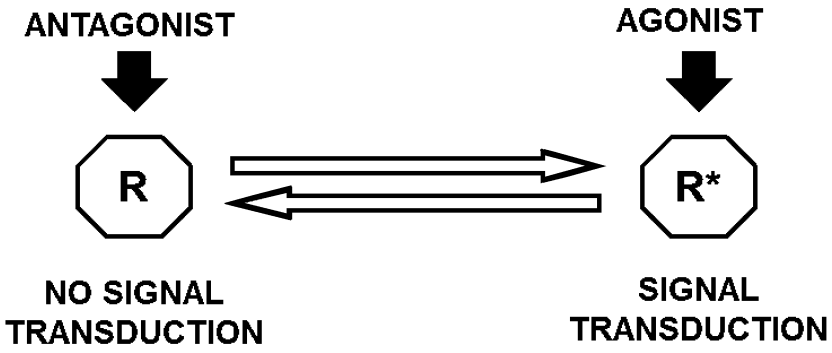

Figure 2. Schematic representing the two-state pharmacological model for G protein-coupled receptors in which state R corresponds to a non-signaling conformation and state R corresponds to a signal transducing conformation. Antagonist ligands bind and promote the former and agonist ligands bind and promote the latter.*

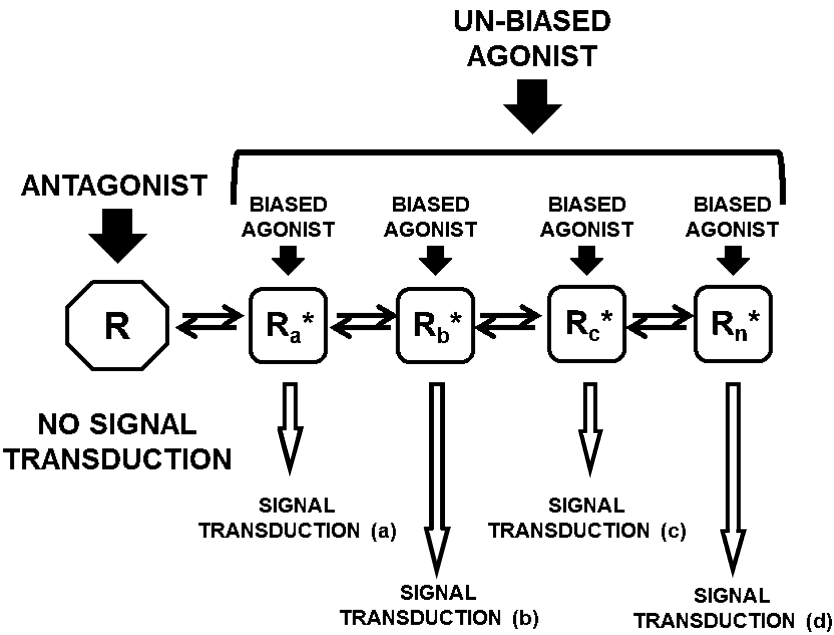

Figure 3. Schematic representing the multi-state pharmacological model for G protein-coupled receptors in which state R corresponds to a non-signaling conformation and states R_a - R_n* correspond to signal transducing conformations. Antagonist ligands bind and promote the former. Unbiased agonist ligands bind and promote all downstream signal pathways and biased agonists selectively bind unique signaling states of the receptor thus selectively activating discrete signal transduction pathway(s).*

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The membrane-bound, activated βγ also couples to various signaling partners as a second arm of the G protein-dependent signal transduction pathway. Specifically, βγ binds the post-synaptic rectifying potassium channel, $K_{IR}3.1$ (GIRK) causing an activation that facilitates K^+ influx, membrane hyperpolarization, and attenuation of action potential (*28*, *29*). βγ also binds and inhibits the voltage gated Ca^{+2} channel ($Ca_v2.2$) localized on pre-synaptic terminals of DRG neurons, attenuating the release of neurotransmitter, particularly glutamate, into the synapse (*30*). These two neuronal mechanisms contribute to the analgesic effect of MOPr agonists.

Ultimately, βγ recruits GRK protein(s) to the membrane where they are activated to phosphorylate one or more serine/threonine sites on the intracellular domain of the MOPr receptor. There are seven GRK isoforms, but overexpression of GRK2 and GRK3 enhances MOPr phosphorylation and internalization, and GRK3 knockout mice are less tolerant to morphine than wild-type litter mates so much attention has focused on them as the primary MOPr -associated GRKs (*31*). Current understanding of GRK phosphorylation of MOPr has recently been reviewed (*32*), but in summary, MOPr phosphorylation can modulate further G protein coupling and can increase the affinity of the receptor to another important group of cellular proteins known as β-arrestins. Although originally considered as a terminator of GPCR signaling via receptor internalization mechanisms dependent upon clathrin3 and AP2, recent publications describe a broad and increasingly complex array of cellular functions for β-arrestins.

Arrestins provide a scaffold for multiple protein signaling partners including mitogen-activated protein kinases (MAPK), PI3Ks, and in some cases switch GPCR signaling to G protein-independent pathways. The affinity of β-arrestin for any GPCR is typically correlated to the pattern and extent of GRK phosphorylation on the cytoplasmic C-terminal domain of the receptor, and has been proposed to be bar code-like, and differentially influenced by full or partial agonism (*33*).

The internalization of GPCRs (including the MOPr receptor) and subsequent events (recycling, degradation, endosomal signaling, etc.), occur through β-arrestin-mediated processes (*34*, *35*). Since the fate of the MOPr receptor after agonist activation influences tolerance, potency, efficacy, and other cellular processes, there is interest in the discovery of ligands that can transduce pathway-specific signals. As new data emerge, various hypotheses have been posed to explain the observations and hopefully guide the design of the ligand bias in the appropriate direction(s). For example, one model proposes that rapid β-arrestin-mediated endocytosis is vital to quench receptor signaling, such that a G protein-pathway-biased agonist may produce abnormal signal patterns due to the receptor's inability to internalize and thus results in prolonged G protein-dependent signaling and abnormal cellular compensatory responses. Another hypothesis proposes that avoidance of the β-arrestin pathway simply avoids signaling events that underpin MOPr side effects and that MOPr analgesia results primarily from the G protein-mediated branch of the signal pathway. A third proposal (*36*) suggests that following MOP activation and $G\alpha_i$ dissociation, the receptor no longer has high affinity for the lipid raft domain, and hence the agonist- MOPr complex translocates to a non-raft domain. If the receptor has been appropriately phosphorylated and has affinity for β-arrestin it binds, thus

blocking further G protein coupling and therefore maintaining its position in the non-raft domain. If the receptor has low affinity for β-arrestin, then it will translocate back to the raft domain by interacting with $G\alpha_i$ again. In this theory, β-arrestin influences the eventual membrane micro-domain location of MOPr but does not initiate the translocation process.

Morphine, as the prototypical MOPr agonist used in many experiments, further complicates these proposals since in most *in vitro* studies it does not promote robust GRK-mediated MOPr phosphorylation and therefore is not a robust activator of β-arrestin when compared to other classical opioid agonists. Surprisingly however, siRNA knockdown (*37*, *38*) or β-arrestin knockout in live animals (*39–41*) usually produces the most profound effects on morphine relative to other opioids. Additional research is required to fully explain this phenomenon and to further clarify the precise cellular mechanisms that underpin the observed results.

MOPr Signal Pathway-Biased Agonism

One of the earliest reports that classical MOPr agonists might have differential G protein versus β-arrestin pathway bias in native CNS neurons was published by Keith and co-workers in 1998 (*42*). In this work, either morphine or etorphine were administered to rats via the intraperitoneal (ip) route and subsequent internalization of MOPr receptors in rat brain neurons was evaluated. In agreement with prior publications that relied on transfected cells or peripheral neurons (*43*), the results demonstrated that etorphine induced a significant MOPr internalization while morphine-induced MOPr internalization was undetectable even when the administered at concentrations exceeding analgesic levels. The published phenotypic description of mice lacking β-arrestin2 (β-arr-/-) add additional intrigue to the possible physiological and behavioral outcomes that might result from a pathway biased MOPr agonist. In the β-arr-/- mice (as compared to wild-type mice) morphine analgesic efficacy, potency, and duration of action were enhanced, tolerance to repeated dose was attenuated as were certain side-effects including respiratory depression and inhibition of gastrointestinal (GI) transit time, both of which are normally hallmarks of on-target MOPr side effects (*41*). Moreover, knock-in mice expressing a mutant MOPr with high morphine-mediated efficiency towards internalization and recycling showed increased analgesia and reward, and reduced tolerance, dependence and addictive behavior (*44*, *45*). An optimistic interpretation of these combined observations is that biological events that are mechanistically triggered down-stream from the point of β-arrestin activation in the signal transduction cascade may be mostly related to non-analgesic responses.

For many GPCRs, the G protein-dependent signal pathway is linked further to mitogen-activated kinase (MAPK) activation, and as such, evidence has been presented (*46*) that supports a direct effect on Ras, leading to the rapid and transient production of soluble, phosphorylated ERK1/2 (p-ERK), which is thought to primarily translocate to the nucleus where transcription factors including CREB, C-myc, and C-fos become targets for activation, thus simulating

varying degrees of important cellular responses. p-ERK production derived from the β-arrestin-dependent branch of the signal cascade is ionically associated with the β-arrestin protein, and therefore is presumably confined to the cytosol.

Activation of MAPK as a result of agonist occupation of MOPr receptors is also well established, and evidence is accumulating that reveals differing mechanistic pathways, differing time-courses, and differing localization may occur as a consequence of alternative G protein signaling vesus β-arrestin signaling. In a recent report, Law and co-workers presented examples of MOPr ligand bias on the dynamics and translocation of ERKs that differs from observations made using other GPCR systems (*47*). In their work, morphine and methadone led to a PKC-dependent activation of ERKs that did not translocate to the nucleus, but instead phosphorylated 90-kDa ribosomal S6 kinase and induced the activity of cAMP response element-binding protein. Other agonists including etorphine and fentanyl activated ERKs in a β-arrestin-dependent fashion and these phosphorylated ERKs (p-ERKs) translocated to the nucleus where a stimulation of Elk-1 occurred. Despite the apparent differences in the cellular localization of the activated ERKs from previous reports with other GPCRs, the authors present a clear example of ligand-induced signal transduction bias between these well-known MOPr agonists. The observed p-ERK localization differences from prior literature and other GPCRs are of interest and more research is needed to characterize possible MOPr -specific effects on cellular ERK patterns.

The time-course and cellular compartmentalization of ERK activation is important in the analysis of ligand-bias at the MOPr receptor since long-lasting, activated ERKs in the cytosol may phosphorylate cytosolic targets thus possibly contributing to pathophysiological conditions. For example, injury to peripheral nerves often causes the production of painful neuromas that can be refractory to current drug therapy. Analysis of the expression of neuronal sodium channels, $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$, and activated MAPKs (p38 and ERK1/2) within neuromas from human patients revealed that $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ are present (*48*). Moreover, activated p38 (p-p38) and p-ERK1/2 were similarly accumulated thus supporting a hypothesis that these channels and kinases are likely to jointly contribute to the pain associated with neuroma formation in man. Of related interest is the more recent observation that cytosolic p-ERK phosphorylates two highly significant voltage-gated ion channels on DRG neurons, $Na_v1.7$ and $Ca_v2.2$ and thus alters their activation thresholds (*49*, *50*). These findings may be a partial mechanistic explanation for opioid -induced hyperalgesia in man and also may indicate that a G protein pathway-biased MOPr agonist may have improved safety and tolerability under certain circumstances. Although just a hypothesis at this time, a similar effect may occur in the enteric neurons of the small bowel where activation of MOPr alters chloride channel activity and alters fluid secretion, an important mechanistic component of opioid-induced constipation. If local MAPK activation plays an analogous role on these ion channels, it may contribute further to the rationale for biased MOPr agonists with improved GI side effect profile, although there is currently no specific data supporting this hypothesis.

Molecular-level insight into the complex protein-protein interactions that are associated with the functional relationship between β-arrestin and ERK activation

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was recently described (*51*). In this work, a series of alanine-scanning mutations of the conserved residues on the non-receptor-binding surface of β-arrestin2 were studied. The authors compared the ability of wild-type and mutant β-arrestin2 to bind rhodopsin, c-Raf1, MEK1, ERK2, and promote ERK1/2 phosphorylation in cells and identified Arg307 in β-arrestin2 as a critically important component of c-Raf1 binding and ERK1/2 activation. The elucidation of atomic-level details such as these will likely be an important component in the further elucidation of these mechanisms.

In addition to the effect on p-ERK, the arrestins are known to interact with other components of the MAP kinase cascade, one of which is the stress-activated protein kinase, cJun-Nterminal kinase (JNK). JNK is of emerging significance since it has recently been shown to play an important role in ligand-directed signaling of the MOPr and KOPr receptors (*52*). It has been reported that β-arr-/- DRG neurons show altered intracellular distribution of JNK and cJun, and that morphine exposure increased the nuclear localization of the phosphorylated (activated) form of cJun (p-cJun), a JNK target in dorsal root ganglia neurons. A very recent publication reported that removing β-arrestin2 from DRG neurons in mice revealed MOPr activation of the JNK cascade in a ligand-specific manner shedding some insight into the previously published morphine-derived behavioral phenotypes of β-arr-/- mice (*53*). Using two different JNK inhibitors the authors reversed the enhanced analgesic effect of morphine, described previously as a known phenotype of β-arr-/- mice, to levels comparable with wild-type mice. Interestingly, the administration of a PKC inhibitor produced a similar result. The behavioral effects of fentanyl under the same study conditions were contrasting and as such revealed that its MOPr agonist-mediated effects were neither genotype-dependent nor affected by JNK inhibition. Analogous to the presumed effect(s) of biased agonist occupation of the MOPr receptor on ERK activation and localization, it appears as though other important MAPK, specifically JNK, may likewise have pathway-specific consequences for activation, translocation, and action. MOPr agonists with appropriate signal bias may therefore have additional benefit as compared with non-signal biased, classical opioids.

Examples of MOPr-Biased Agonists

As mentioned earlier, morphine exhibits some G protein pathway bias in various cellular systems. As an extension of these observations, it was reported that in 293T cells, several metabolites of morphine, specifically normorphine, 6-acetylmorphine, and morphine-6-glucuronide produced higher potencies for β-arrestin recruitment than they did for G protein activation, suggesting an alternate bias in signal transduction (*54*). The authors employed radioligand binding studies and FRET-based methodology to measure these individual potencies in single intact cells. Potency differences ranged from three to ten-fold.

One recent study of interest examined a broad collection of first generation opioids in search of possible G protein pathway bias at MOPr (*55*). Among the agonists studied were “ring-opened” opioids including methadone and fentanyl, “Bentley opioids” including buprenorphine, and “standard opioids”

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including morphine and oxycodone. Direct binding between MOPr/G protein and MOPr/β-arrestin was accomplished via resonance energy transfer (RET) methodology to minimize downstream signal amplifications. Partial agonism in the G protein pathway was invariably associated with lesser (or no) efficacy for β-arrestin, (G pathway bias). Of particular interest was the observation that some of the molecules were described as mixed agonist/antagonists of receptor-transducer interactions, meaning they could activate G protein-mediated signaling on their own, but could antagonize the β-arrestin pathway that would otherwise be stimulated by the un-biased and more potent endogenous agonist enkephalin. Related research from another laboratory demonstrated that buprenorphine decreased calcium currents *in vitro* and eliminated desensitization and internalization induced by the more potent agonists, [Met]^5-enkephalin (ME) and etorphine (*56*). Using the PathHunter β-arrestin assay (DiscoveRx, Fremont, CA USA), additional evidence supporting the pathway bias of buprenorphine, norbuprenorphine, and fentanyl in engineered U2OS cells was recently described (*57*). These cells express human MOPr and β-arrestin, each tagged with a protein fragment of β-galactosidase for enzyme complementation when MOPr and β-arrestin associate. MOPr internalization was also studied in an analogous complementation assay from DiscoveRx. In this assay, U2OS cells express human MOPr and an early endosome marker protein, each tagged with a protein fragment of β-galactosidase for enzyme complementation upon endocytosis of MOPr. Using the same cell background GTPγS binding in membrane preparations was measured, thus enabling a comparison of potency and efficacy of these three opioids in two distinct signaling pathways. The data reveal a profound G protein pathway bias for buprenorphine since it potently and concentration-dependently stimulates GTPγS binding with E_{MAX}=70% (partial agonism) versus the DAMGO control (Figure 4A), but does not stimulate β-arrestin recruitment to the membrane or MOPr internalization (Figure 4B,C). In contrast, fentanyl and norbuprenorphine each show concentration-dependent stimulation of GTPγS as well as concentration-dependent recruitment of β-arrestin. Fentanyl further stimulated MOPr internalization (norbuprenorphine was not tested in this latter assay). Although the concentration-response curves for fentanyl and norbuprenorphine are rightward shifted and produce slightly lower E_{MAX} in the β-arrestin assays relative to the GTPγS assay, suggesting a possible slight G pathway bias based on potency and efficacy in these assays, they did not produce the profound pathway bias as was observed for buprenorphine. The physiological and behavioral impact of these varying degrees of bias remain to be determined but this data illustrates that in spite of acting upon a common receptor target, opioid molecules do not always produce the same cellular effects.

These combined results demonstrate an interesting pharmacology for buprenorphine that is somewhat unique in that alone it is a partial MOPr agonist with bias toward stimulation of the G protein pathway, but if applied in combination with a more potent/efficacious unbiased MOPr agonist, buprenorphine behaves as a modulator of the G protein-dependent signal, and an antagonist of the β-arrestin signal. These data may be a partial explanation for buprenorphine's ability to inhibit or reverse norbuprenorphine-induced respiratory depression in a rodent model (*58*). The extent to which this unique pharmacology

is a basis for buprenorphine's somewhat unique clinical responses as compared to other opioids remains to be determined.

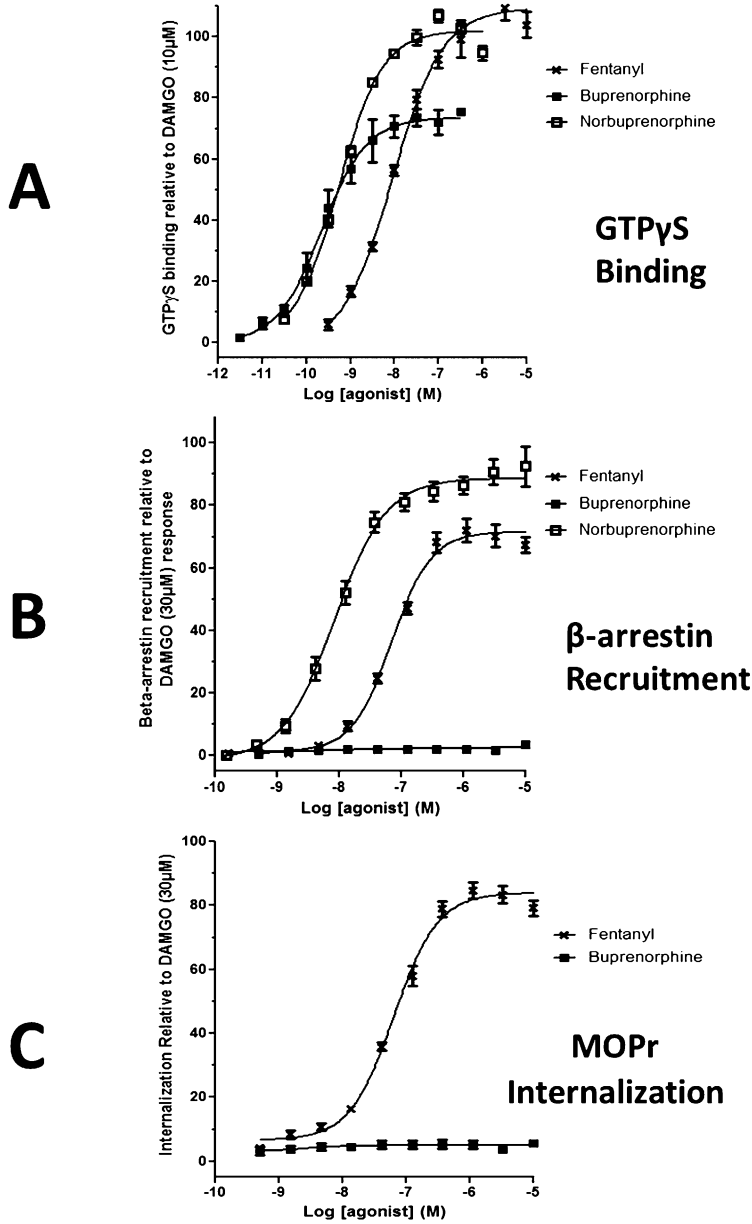

Figure 4. (A) Functional stimulation of GTPγS as a percent of the positive control DAMGO. (B) Concentration-response curves for MOPr -mediated recruitment of β-arrestin, and (C) Concentration-response curves for MOPr internalization.

Buprenorphine may then be considered a prototype for a new type of MOPr agonist and may therefore inspire new possibilities for drug design in the future. The observation that the ring opened opioid fentanyl is, at best, minimally biased and stimulates β-arrestin recruitment and MOPr internalization while the Bentley opioid buprenorphine is G protein pathway-biased and does not stimulate β-arrestin-mediated actions also provides some preliminary insight into the structure-activity-relationship associated with pathway bias at the MOPr receptor, and may therefore be of interest to medicinal chemists.

One other noteworthy study was designed to evaluate endomorphin-2 (EM-2) as an agonist at endogenous MOPr receptors from the locus cerulius of the rat brain, as well as MOPr stably expressed in HEK293 cells (*59*). Against the former, [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin (DAMGO) and EM-2 activated inwardly rectifying K^+ current in a concentration-dependent manner, thus demonstrating their G protein pathway agonism as expected. However, EM-2 induced faster desensitization of the K^+ current than did DAMGO. In the HEK293 cells stably expressing MOPr, the ability of EM-2 to induce phosphorylation of Ser375 in the COOH terminus of the receptor, to induce association of β-arrestin with the receptor, and to induce cell surface loss of receptors was much more efficient than would be predicted from its efficacy for G protein-mediated signaling. From these results, the authors propose that EM-2 is a β-arrestin-biased agonist at MOPr. These emerging examples of ligand bias at the MOPr receptor are likely to be just the beginning and many more examples are expected to appear in the literature in future years.

MOPr Biased Signal Pathways: Considerations

The quantitative assessment of pathway bias remains an ongoing area of discussion and debate. A given MOPr agonist may have potency bias and/or efficacy bias, but may also exhibit on-target and simultaneous agonism/antagonism, the physiological/pathophysiological consequences of each require significantly more research to fully understand. Given the important role(s) of the MOPr receptor, the GRK proteins, and the β-arrestins in the signal transduction cascade, one can imagine that the relative bias of a given agonist may indeed be cell-type or tissue specific, since basal expression levels of these important signaling proteins may vary in differing tissues or neuronal populations. One example is the reported differences in G protein expression levels in the enteric neurons that innervate the colon of guinea pig (*60*). Similarly, striatal neurons are known to have relatively high GRK expression levels as compared to other brain and DRG neuronal populations which may off-set some of the apparent G protein signal bias one might otherwise measure in various *in vitro* systems where GRK expression might be lower (*61*). Related to this point is the reported observation that over-expression of GRK2 causes morphine to induce MOPr phosphorylation and internalization (*62*) Along these lines, it may be possible for a given MOPr agonist to behave as an unbiased agonist in one population of neurons, yet be a biased agonist in another. Moreover, it may be possible that the degree of pathway bias may be a function of drug exposure

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following dosing, for example showing bias a low concentration then becoming unbiased at higher concentration. Examples of these scenarios remain to be discovered but one recent report demonstrates that tolerance to repeated doses of morphine develops in the *ex vivo* isolated ilium of wild-type mice, but not in the colon (*63*). In a subsequent extension of their work, now including other opioids such as DAMGO, fentanyl, and etorphine, a tolerance effect in the isolated ileum of both wild-type and β-arr-/- mice was observed following repeat dose and washout. However in the isolated colon of the wild-type mice comparison of the contractions between the 4th exposure were not different for morphine or DAMGO (100 ± 10%; N = 5) but were reduced for fentanyl (62 ± 13%; N = 8) and etorphine (38 ± 4%; N = 7) indicative of tolerance to fentanyl and etorphine but not DAMGO. In contrast, all agonists produced tolerance in the colon of the β-arr-/- mice. Specifically, DAMGO response at the 4th exposure decreased to 52 ± 10% (N = 5), fentanyl to 20 ± 5% (N = 6) and etorphine 33 ± 7% (N = 6). These interesting data provide evidence for MOPr agonist bias that is tissue specific and related in this case to expression of β-arrestin. This type of tissue-specific nuance in the MOPr signaling pathways may extend into other tissues and cell types, including differing neuronal populations, and if so might have important consequences toward the ultimate design of pathway biased therapeutic opioid analgesics.

Importantly, there are several publications that demonstrate further changes in these protein expression levels as a function of disease state. One recent article of relevance demonstrated that, using a collagen-induced arthritis (CIA) and a human TNF transgenic (TNFtg) mouse model, β-arrestin1/2 expression are significantly increased in joint tissues (*64*). Similar results were reported in cells isolated from rat (*65*). The implication is that a given MOPr agonist may lose or gain signal bias in the setting of pathological states, including inflammation and rheumatoid arthritis. One other important consideration is that intracellular GRK-mediated phosphorylation sites on the MOPr receptor (or other opioid receptors) are not conserved across species, notably rat, mice, and human (Figure 5).

Mu

Species	359
Rat	**T**
Human	N
Mouse	**T**

Kappa

Species	358	369
Rat	N	**S**
Human	**S**	Y
Mouse	N	**S**

Delta

Species	335	343	346	353
Rat	A	G	R	A
Human	K	**S**	**S**	A
Mouse	**T**	G	R	**T**

Figure 5. Comparison of putative intracellular phosphorylation sites on MOPr, DOPr, and KOPr receptors with consideration for species-specific variations in the amino acid sequences. With the exception of position 335 in the DOPr receptor, Rat and Mouse sequences are identical, yet differ significantly from Human, possibly leading to poor translation between data collected in rodent versus Human.

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This may lead to animal-derived conclusions that may not translate well into human clinical experience. In spite of these caveats, the field of MOPr signal transduction bias continues to advance with deeper insights into the biological consequences of ligand bias. Although in its infancy, this approach continues to hold promise for the millennia-long goal of separating MOPr agonist-mediated side effects from analgesia.

KOPr Opioid Receptor: Signal Transduction

The arrangement in the membrane and the key elements of signal transduction for the kappa opioid receptor (KOPr) are similar in many ways to what was previously described for MOPr. Briefly, agonist occupation of KOPr leads to recruitment and activation of $G\alpha_i$ and $\beta\gamma$ subunits that signal further in the cell and act on key effector proteins including the ion channels $K_{IR}3.1$ and $Ca_v2.2$, similar to MOPr, thus contributing to an analgesic effect upon stimulation. Likewise, the ultimate recruitment of GRK to the membrane, phosphorylation of intracellular serine and threonine residues, and subsequent association with β-arrestin occurs, thus initiating internalization, desensitization, and recycling of the receptor. In spite of the somewhat similar signal pathways for MOPr and KOPr receptors, KOPr agonists are not widely used in man as analgesics due to presumed on-target side effects that include dysphoria and diuretic effects, the latter being caused mechanistically by negative regulation of anti-diuretic hormone (ADH). A schematic that relates important elements of the KOPr signal transduction pathways is presented in Figure 6.

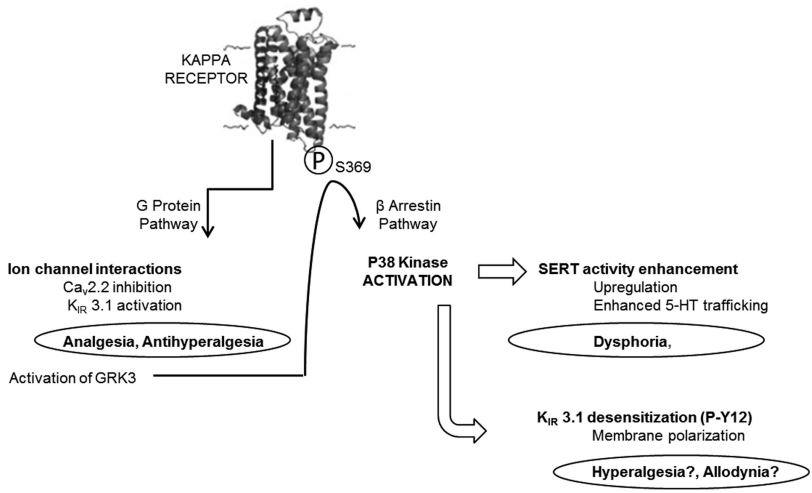

Figure 6. Key elements of the G protein-dependent, and β-arrestin-dependent pathways for KOPr signaling in Rat. The figure illustrates that analgesia and antihyperalgesia might be primarily derived from the G protein-dependent arm, while adverse events such as dysphoria and possibly hyperalgesia are associated with the β-arrestin arm. The latter are further dependent on p38 kinase activation.

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In many physiological systems, activation of KOPr has an opposing effect on the activation of MOPr. For example in the striatum, pre-synaptic stimulation of KOPr attenuates while stimulation of MOPr potentiates dopamine levels. In the nucleus accumbens, the primary reward center of the brain, chronic exposure to MOPr agonists results in a decrease in D2 dopamine receptors on neurons, an effect that is thought to play an important role in opioid addiction. In contrast however, stimulation of KOPr in the same region results in the opposite effect, an increase of D2. These supraspinal actions of KOPr have been concisely summarized in a recent review (*66*).

KOPr stimulation has been shown to activate MAPK, including JNK, p38, and ERK, although the mechanistic pathway(s) leading to this activation likely differ from the MOPr receptor system in certain cell types. Published data obtained from astrocytes implicates calmodulin (CaM) and PKCε in DAMGO (MOPr) stimulation of ERK (*67*). The authors have proposed a mechanism wherein, upon DAMGO binding to MOPr, CaM is released from the MOPr receptor which activates protein kinase C (PKC). Subsequently, PKC generates diacylglycerides that activate PKCε. In contrast, U69593 (a known KOPr agonist) appears to act via phosphoinositide 3-kinase, PKCζ, and Ca2+ mobilization. These signaling components were implicated based on studies with specific inhibitors and a dominant negative mutant of PKCζ. Collectively, the authors suggest that differences in the MOPr and KOPr mechanisms of signaling may contribute to the distinct outcomes on ERK modulation induced by chronic MOPr and KOPr opioids.

Unlike MOPr, the KOPr is further activated under the conditions of stress which is important because stress and anxiety are presumed to contribute further to reinforcement of drug-seeking behavior and depression. Both dynorphin A (the endogenous KOPr agonist (DYNA), and KOPr receptors are expressed in the bed nucleus of the stria terminalis (BNST), a brain region associated with anxiety and stress supporting a hypothesis that KOPr activation in response to stress may play a role in the regulation of certain emotional behaviors (*68*, *69*). Moreover, DYNA is upregulated in this region in response to stress and anxiety.

Recent data from rodent has been presented that mechanistically links the activation of p38 MAPK to stress-mediated KOPr stimulation via a signal transduction pathway that is exclusively β-arrestin-mediated (*70*). The authors measured the production of activated p38 (p-p38) in the dorsal and ventral striatum and observed elevation in p-p38 in both areas (wild-type mice) in response to the forced swim model of stress. If mice were pre-treated with nor-BNI (a KOPr antagonist) or if KOPr knockouts were used in the study, elevation of p-p38 in response to the forced swim stress was not observed, thus linking KOPr uniquely to p-p38 production in mouse. Ser369 has been identified as a critical GRK3 phosphorylation site on the intracellular domain of the rat and mouse KOPr receptors and is therefore of significance when considering signal transduction pathways. This specific phosphorylation is a prerequisite for subsequent KOPr binding to β-arrestin. It has been demonstrated that a single-point, alanine mutation at this position disables the KOPr-β-arrestin-mediated production of p-p38 since it is downstream from the β-arrestin- KOPr coupling event, further demonstrating that the KOPr-G protein-dependent pathway does not

lead to p-p38 production (*71*). Once phosphorylated, p-p38 kinase regulates the central serotonergic system by enhancing the 5-HT reuptake capacity of SERT (the principle target for SSRI inhibitors used in man for depression) *via* a phosphorylation mechanism and by stimulating increased cell surface SERT expression (*72*). This implies that the dysphoria that is dogmatically ascribed to centrally-acting KOPr agonists, may primarily be a result of post-β-arrestin signaling that depletes 5-HT from neuronal synapses. In support of this hypothesis, it has been shown that a non-pathway biased KOPr agonist (U50,488) produced a significant aversive behavior in a standard model of conditioned place preference/aversion but if the animals were pre-treated with SB203580, a selective p38 kinase inhibitor prior to exposure to U50,488 no place aversion was observed (*70*).

Another substrate for p-p38 kinase is the $K_{IR}3.1$ rectifying potassium channel (GIRK). Recall that in the G protein-dependent pathway, this channel is activated in response to agonist occupation of KOPr, causing hyperpolarization of neuronal membranes and dampening of action potential that contributes to KOPr -mediated analgesia. However, as a consequence of the β-arrestin-dependent production of p-p38 kinase, phosphorylation of $K_{IR}3.1$ at position 12 (tyrosine), occurs resulting in desensitization (*73*). The phenotype associated with this modification to the $K_{IR}3.1$ channel is unclear, but it may at least blunt KOPr -mediated analgesia and might contribute to hyperalgesia, allodynia, or related conditions of non-specific pain. This may combine with altered mood described earlier to produce a generalized "unwell" feeling ascribed to KOPr agonists. These data support a hypothesis that a G protein biased KOPr agonist may have enhanced analgesic properties and may further be devoid of dysphoria. If supportive experimental evidence continues to emerge in the literature, it may stimulate a renewed interest in kappa agonists for treating human pain.

It is important to consider the possibility of species-specific effects in these experiments, especially if one considers that the critical GRK substrate (Ser369) on KOPr is not conserved in the human sequence. Evidence for species differences have indeed been reported already. For example U50,488 promoted internalization of human but not rat KOPr *in vitro* (*74*). The internalization was shown to depend on β-arrestin, GRK, and dynamin. The same authors reported that etorphine was a full agonist in the G protein-dependent pathway, it did not cause internalization, suggesting a bias away from β-arrestin-dependent events (*75*). Part of their mechanistic explanation implicates the non-conserved serine residue at position 358 in the human KOPr sequence which is asparagine in the rat. This may result in differential GRK phosphorylation and hence differing trafficking properties. Species-specific caveats notwithstanding, if these systems are preserved and translatable to humans then this body of research might contribute to a new mechanistic rationale for G protein-biased KOPr agonists as analgesics with reduction of dysphoric effect and enhanced analgesia versus first generation KOPr agonists such as pentazocine.

Parkinson's disease (PD) patients suffering from dyskinesia caused by chronic L-DOPA therapy may also benefit from KOPr agonists with G protein pathway bias if the approach successfully eliminates dysphoria from the mechanism. The pathophysiology of L-DOPA-induced dyskinesia is not completely understood

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but the endogenous opioid system is thought to play an important role given the widespread distribution of opioid receptors and their respective endogenous agonists throughout the basal ganglia. Studies have shown that nigrostriatal damage and the subsequent therapeutic treatments (L-DOPA) aimed at alleviating the functional consequences of dopaminergic denervation modulate the activities of the opioid receptors including KOPr. Diminishing reserve of KOPr in various basal ganglia regions is thought to result in sub-optimal basal function that may be improved *via* the treatment with a suitable exogenous KOPr agonist. Support of this hypothesis may be taken from recent observation of the unbiased KOPr agonist, U50,488, modulated L-DOPA induced motor deficits in Parkinsonian rats (*76*). Using the same unbiased KOPr agonist ((U50,488) other authors have demonstrated a similar effect in the MPTP-treated primates, although they also reported a worsening of the Parkinsonism symptoms (*77*).

Similar research published by a separate group revealed that a single subcutaneous administration of another unbiased KOPr agonist, (TRK-820) significantly increased spontaneous ipsilateral rotational behavior of hemi-Parkinsonian rats and also significantly inhibited L-DOPA-induced dyskinesia (*78*). These effects were reversed in the presence of nor-binaltorphimine, a KOPr antagonist. In an *in vivo* microdialysis study, TRK-820 significantly inhibited L-DOPA-derived extracellular dopamine content in the 6-OHDA-treated striatum in dyskinesia rats, but not in hemi-Parkinsonian rats. Moreover, the development of L-DOPA-induced dyskinesia was suppressed by the 3-week co-administration of TRK-820 with L-DOPA. Taken together, the authors concluded that TRK-820 ameliorates L-DOPA-induced dyskinesia with a moderate anti-Parkinsonian effect by inhibiting L-DOPA-induced excessive dopamine release through KOPr only in dyskinesia rats.

In both of these examples, the authors suggest that although the KOPr agonists produced a beneficial effect, side effects including dysphoria would limit the ultimate therapeutic use in man. It is unknown at this time if a G protein pathway-biased KOPr might produce an improved response in these animal models or in human therapy but success in that direction would be considered a major breakthrough for patients currently managing this condition. One important consideration is the recently published evidence that β-arrestin and GRK expression levels are uniquely altered in the striatum of human Parkinson's disease patients (*79*), again raising the possibility that the extent of pathway bias and the resulting pharmacological effect(s) observed *in vitro* or in animal models may not translate well to the human condition. Additional research and human clinical trials will be necessary to evaluate this scenario.

Presently, only a single publication has reported a G protein-biased KOPr agonist that does not recruit β-arrestin (*80*). Similar to the previous description of buprenorphine at the MOPr receptor, 6′-guanidinonaltrindole (6′-GNTI) reportedly acts as a β-arrestin antagonist in the presence of unbiased KOPr agonists, thereby joining buprenorphine as another example of this new pharmacological dual agonist/antagonist concept.

Future Prospects

At the current time in history, the existing first generation opioids on the market as front-line therapy for severe pain are under increased regulatory and public-sector scrutiny due to concerns over abuse and safety. At the same time, exciting new experimental results and mechanistic hypotheses are appearing in the literature with increasing frequency, supporting the basic proposal that pathway biased MOPr and KOPr agonists may become the basis for a new generation of opioid analgesic with an improved safety profile. Given the extensive distribution of the MOPr and KOPr receptor systems throughout the human body, this approach may lead to improvements in the treatment human pain as well as other conditions, for example L-DOPA induced dyskinesia in PD patients. Perhaps this emerging research will indeed produce a new generation of opioid drugs with inherent improvements in safety and side-effect profiles such that a new chapter in this millennia-old field can be written.

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Chapter 11

Peripherally Restricted Opioid Analgesics

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This chapter will review some of the key findings with a few peripheral opioid agonists that have contributed to, and shaped our understanding of peripheral opioid analgesia. Rather then being a comprehensive list of compounds that have been synthesized and tested as peripherally-restricted opioids, this chapter will try to put some of the preclinical and clinical data into context. By highlighting some of the key successes and shortcomings of the peripheral analgesics discussed in this chapter, it may be possible to apply some of our learnings to develop a new generation of peripheral opioid analgesics.

Introduction

There have many reviews written about the activity of peripheral opioid agonists in preclinical models of hyperalgesia and neuropathic pain. There was a concerted effort in the early 1990's by both large and small pharmaceutical companies to develop new peripheral opioid agonists. Although some newer compounds will be mentioned within the context of this review, the focus of this review will not be strictly a laundry list of the pharmacological profiles of compounds that have been discussed in a number of other reviews (*1*, *2*). Rather the scope of this chapter will be an attempt to identify strategies and approaches that may be useful in successfully making the transition between preclinical efficacy and clinical utility of peripheral opioid agonists. To illustrate these strategies, this review will highlight some of the preclinical and clinical data for select peripheral MOR and KOR agonists. The findings will be discussed from the viewpoint of shedding light on factors that can be applied to successfully advance a new generation of peripheral opioid agonists.

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Based in part on early work of Christoph Stein and others (*3*–*5*) demonstrating peripheral effects of locally administered selective MOR and KOR agonists, it has generally been accepted that these opioid receptors represent the most attractive targets for the development of peripheral opioid analgesics. Recent data (*6*, *7*) have raised the possibility that it may be worth putting the time and effort into developing selective, peripheral DOR agonists.

Practical Experimental Issues To Consider

Two of the major issues that always need to be addressed when discussing peripheral opioid analgesics are: whether sufficient efficacy can be achieved if only peripheral opioid sites are activated, and the degree to which compounds retain selectivity for these peripheral sites (ie penetration into CNS and spinal compartments). When CNS penetration is demonstrated in preclinical studies it is important to understand whether efficacy can be observed at doses below that which are required to produce measurable levels of compound in the CNS. If not, one cannot convincingly argue that peripheral mechanisms are solely responsible for the observed level of efficacy in the animal.

A corollary to the degree of peripheral restriction of novel compounds is to have a thorough understanding of the degree to which the antihyperalgesic or antinociceptive effects of the opioids are mediated in the periphery. For example, the acetic acid-induced writhing assay is sometimes used as a "peripheral" analgesic assay for the opioids, as it is believed that opioid agonists can work locally at receptors within the peritoneum cavity or the gastrointestinal (GI) tract to inhibit the writhing response (*8*, *9*). While this may be the case in part, there is also evidence to suggest some level of contribution by central opioid systems as well (*10*). Thus, one should not necessarily rely solely on the use of efficacy of novel opioid agonists in the acetic acid as evidence for peripheral activity of the compounds. Similarly, when physiological effects of opioids such as emesis for MOR agonists, or diuresis for KOR agonists are used as markers for CNS activity of opioids, it must be understood that the sites of action in the brain lie outside or are poorly protected by the blood-brain barrier, or have a peripheral component (*11*, *12*).

Although it may seem straightforward to compare CNS and peripheral (eg blood or tissue) exposures of novel compounds, there are many considerations for choosing an appropriate method. The choice of methods of determining CNS levels of compounds may in part depend on the physicochemical and pharmacokinetic properties of the compounds. Many compounds that are designed specifically for peripheral restriction have inherent physicochemical and/or pharmacokinetic shortcomings, relative to a compound intended for CNS use, or those with good systemic exposures. For compounds that are highly bound to plasma proteins, using CSF measurements may overestimate the degree of peripheral restriction, since only the free fraction of drug will be distributed to the CNS. So if one chooses to use CSF levels as a measurement of central penetration, it is imperative that one knows what the degree of plasma protein binding.

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Compounds that may act as substrates for efflux carriers such as P-glycoprotein (P-gp), or inhibit transporters that serve to move compounds across the blood-brain barrier (eg amino acid transporters) may have a high degree of peripheral restriction. It is important to characterize fully the interactions of the compounds with their cognate transporters because there are a number of known therapeutic agents that also interact with these transporters. For example, if a compound is peripheral due to it being substrate for P-gp, then one should determine plasma and brain concentrations of the compound in the presence of known inhibitors of P-gp or in mdr knockout and wild-type mice. This information is important because one should have an understanding of the range of the degree of peripheral restriction before clinical testing.

In addition to determining CNS exposure, it is important to understand the pharmacokinetic profiles of novel peripheral-acting opioids after systemic and/or local administration. Many compounds that are designed and synthesized to have limited CNS exposure have other pharmacokinetic liabilities which either limit absorption or distribution of the compounds. For example, one chemistry strategy to reduce CNS penetration is to increase the polar surface area (PSA) of compounds (*13*, *14*). However, increasing PSA will also cause compounds to have poor absorption from the gastrointestinal tract (*13*). Thus, brain levels may be low or undetectable due to poor absorption rather than a high degree of peripheral selectivity. Although this may seem like a trivial distinction, it is necessary that sufficient compound needs to reach the site of action. If a compound has poor absorption after oral administration, one might consider that a topical or local administration may be the preferred route of administration. As discussed below, the route of administration is one factor that should also be considered when determining which preclinical pain model(s) should be used to determine the efficacy of novel peripherally selective opioid agonists.

The choice of the preclinical pain model(s) used to demonstrate peripheral efficacy of the opioids should be chosen with careful deliberations. Factors that may influence the choice of the efficacy model include; the intended route of administration (ie local, topical or systemic), the species selected, the target of interest, and the degree to which inflammation may influence the expression and accessibility of the targeted opioid receptors. If possible, it may be prudent to use a battery of efficacy models to demonstrate consistent and robust activity in a number of preclinical pain models with strong peripheral components to the underlying response. Some preclinical pain models including; Complete Freund's Adjuvant (CFA)-induced hyperalgesia, formalin-induced nociception, UVB-induced inflammatory pain, and incisional models of post-surgical pain lend themselves to the use of topical or local administration due in part to the degree of local inflammation, and the ease of administering test compound to the hind paw of a rat or mouse. It should be remembered that inflammation will likely change the degree of dermal penetration of topically-applied test compounds since inflammation will make the skin more permeable (*15*, *16*). The use of mechanical or thermal endpoints in a wide variety of inflammatory pain and hyperalgesia models also allows one to use measurements in the contralateral or untreated paw as an indication of frank antinociceptive responses (*17*). It is

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generally accepted that a frank antinociceptive response has CNS origins and can be used as a surrogate marker for CNS penetration of peripheral opioids.

Another important issue that is often overlooked when studying peripherally-restricted opioid agonists is how to most accurately compare the ratios of ED_{50} values in preclinical pain models and ED_{50} values in side effect models, such as rotarod or spontaneous locomotor activity that are often used to demonstrate a central action of the opioids (*18–20*). This ratio is often referred to as the peripheral restriction index and is used much like plasma to brain concentration ratios to state how much "peripheral selectivity" a compound has. These values may be useful when comparing a number of compounds in a specific chemical series, or across different chemical series to find the one with the most suitable peripheral selectivity. However, when calculating ED_{50} ratios it is important to realize that there are confidence intervals around given ED_{50} values and these confidence intervals should be accounted for when comparing the differences in ED_{50} ratios. While calculating peripheral restriction indices for a series of compounds may help to classify compounds as either having a low or high degree of peripheral restriction, these indices are not particulary suitable for discriminating between compounds with similar, but numerically different peripheral indices (eg peripheral index of 100 *vs* 200 for two different compounds). A full characterization of the dose-response relationships for efficacy and for side effects should be performed. If the slope and maximal effect of the two dose-response relationships are different this may lead to an over or underestimation of the true separation of the two pharmacological effects. When possible the comparisons between therapeutic and side effect potencies of putative peripheral analgesics should be performed in the same species to eliminate any confounding pharmacokinetic differences between species such as plasma protein binding, metabolism, distribution, etc.

Peripheral Opioid Receptor Target Challenges

MOR

Of all the opioid receptors that are expressed in the periphery, MOR systems appear to regulate and impact more physiological functions in the periphery than do KOR or DOR opioid systems. GI function such as transit, secretion, and gastric emptying are controlled in part by MOR within the GI tract (*21*), and as such systemically administered peripheral MOR agonists may be prone to the production of opioid-induced constipation or opioid-induced bowel dysfunction. Additionally, CNS sites such as the area postrema and chemotactic trigger zone which are areas responsible for the emetic and nausea produced by MOR opioids lie outside the protection of the blood-brain barrier (*11*). Thus, systemically administered peripheral MOR agonists may still have some of the same GI liabilities, such as nausea and vomiting that are produced by central MOR agonists such as morphine. Activation of peripheral MOR could also lead to immunosuppressant effects (*22*, *23*), and may have a direct testicular effect on the production of testicular interstitial fluid and testosterone (*24*).

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KOR

The primary CNS effects of KOR agonists that has prevented their therapeutic use as analgesics are dysphoria and psychotmimetic effects, such as hallucinations. Unfortunately, these CNS effects are not easily measurable using preclinical models. Models of sedation such as the rotarod or locomotor activity to detect sedation as a sign of CNS penetration in rodents may not adequately predict the propensity of a compound to produce dysphoria in humans. As is the case for MOR agonists immunosuppressive effects can be produced by KOR agonists (*25*). Additionally, KOR agonists can produce diuresis/aquaresis via a direct effect on the kidneys (*12*). Depending on the clinical indication, the diuresis could be a benefit or a detrimental side effect.

DOR

Perhaps due to the fact that selective, central acting DOR agonists, such as SNC-80 produce convulsions in animals (*26*), the discovery and development of peripherally restricted DOR agonists has lagged behind that of peripheral MOR and KOR agonists. There is very little available data on selective, peripherally-restricted DOR agonists. Much of the pharmacology of DOR in the periphery and in the dorsal root ganglion revolve around the increased trafficking and cell surface expression of receptors following inflammation or administration of MOR agonists such as morphine (*27*, *28*). There is some recent data implicating DOR as playing an important role in peripheral pain transmission (*6*, *7*). This data will be discussed later in this chapter and may serve to spark renewed interest in the development of peripheral DOR opioid agonists.

MOR Agonists

To examine and highlight the potential of MOR agonists for the treatment of pain mediated in the periphery, the antihyperalgesic effects of a locally administered MOR agonist, ADL 2-1294 (loperamide) (*17*) will be compared and contrasted to the that of a systemically administered (intraperitoneal) MOR, DiPOA ([8-(3,3-diphenyl-propyl)-4-oxo-1-phenyl-1,3,8,-triazaspiro [4,5] dec 3-yl] acetic acid) (*29*, *30*).

ADL 2-1294 (Loperamide)

ADL 2-1294, or loperamide is the active ingredient in the over the counter anti-diarrheal, Imodium AD. ADL 2-1294 is a potent, poorly selective (relative to DOR) MOR agonist. ADL 2-1294 inhibited [^{3}H]-diprenorphine binding in cells expressing cloned human MOR with a K_i value of 3.3 nM. ADL 2-1294 was approximately 15-fold more selective for MOR than DOR (K_i = 48 nM) and more than 350-fold selective for MOR, relative to its affinity for KOR. ADL 2-1294 was a full agonist, relative to DAMGO in a GTPγ[^{35}S] binding assay in cells expressing human MOR with an EC_{50} value of 19 nM (*17*).

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ADL 2-1294 was tested for antihyperalgesic efficacy in a variety of inflammatory pain models in rats following local administration (*17*). In rats treated with kaolin-carrageenan in the knee joint, ADL 2-1294 (0.3 mg) injected intra-articular (ia) almost completely reversed joint compression-induced elevation of blood pressure. The efficacy of locally administered ADL 2-1294 was comparable to that of 3 mg of ia morphine. Furthermore, the reversal of blood pressure elevation produced by ADL 2-1294 was antagonized the nonselective opioid antagonist, naloxone (1 mg/kg ip). Additionally, ADL 2-1294 (0.1 mg) injected sc in the dorsal surface of the paw significantly reduced late phase formalin-induced flinching when injected 10 - 360 min prior to the fifteen minute observation period. ADL 2-1294 potently and almost completely inhibited late phase formalin-induced flinching after intrapaw administration with an A_{50} value of 6 µg. ADL 2-1294 was 12 times more potent at inhibiting late phase formalin-induced flinching than morphine following local administration. Unlike morphine which completely inhibited early phase flinching, ADL 2-1294 did not suppress early phase flinching. The ability of locally-administered morphine, but not ADL 2-1294 to inhibit early phase flinching is indicative of a central analgesic action of morphine, even when it is administered locally. ADL 2-1294 was also an effective antihyperalgesic agent when tested in rats whose hind paws were injected and inflamed with CFA for 24 h, or by inflamed by removing the stratum corneum of the hind paw with Scotch Tape. ADL 2-1294 (0.1 mg, intraplantar (ipl)) was effective in completely blocking mechanical hyperalgesia in hind paws that were inflamed with either CFA or by tape stripping. The antihyperalgesic effect of ADL 2-1294 lasted for 6 h in CFA-treated rats, and reversed the hyperalgesia induced by tape stripping for 1 h. Unlike morphine (0.3 mg, ipl) which significantly elevated paw pressure thresholds in both inflamed and untreated hind paws as well, ADL 2-1294 failed to alter paw pressure thresholds in untreated hind paws. In fact, treatment with morphine significantly increased paw pressure thresholds above baseline values indicative of a central antinociceptive effect of morphine.

Catalepsy is known to be produced by the central activity of morphine and other MOR agonists. ADL 2-1294 (0.3 mg) did not produce catalepsy in rats after im administration, whereas significant catalepsy was observed in rats treated with morphine (3 mg im). Loperamide (up to 160 mg/kg po) was not antinociceptive in a tail withdrawal assay or in the tail pinch assay at doses up to 80 mg/kg po (*31*) supporting the fact that ADL 2-1294 is devoid of central antinociceptive activity, and that peripheral mechanisms are responsible for the antihyperalgesic effects of ADL 2-1294. Further support for the peripheral selectivity of loperamide is the fact that it has been shown to have little to no brain penetration and has been shown to be a substrate for P-glycoproteins (P-gp) and is actively pumped from the brain (*32*).

DiPOA

DiPOA is a zwitterionic (See Figure 1), highly potent and selective MOR agonist that inhibited [^{3}H]-diprenorphine binding in cells expressing cloned human MOR with a K_i value of 0.76 nM. DiPOA bound with high selectivity for the

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MOR and had at least 300 times lower affinity for KOR and ORL-1 receptors (*29*). DiPOA did not bind appreciably to DOR receptors. DiPOA was a full agonist (85% E_{max}, relative to DAMGO) in a GTPγ[^{35}S] binding assay in cells expressing human MOR with an EC_{50} value of 33 nM.

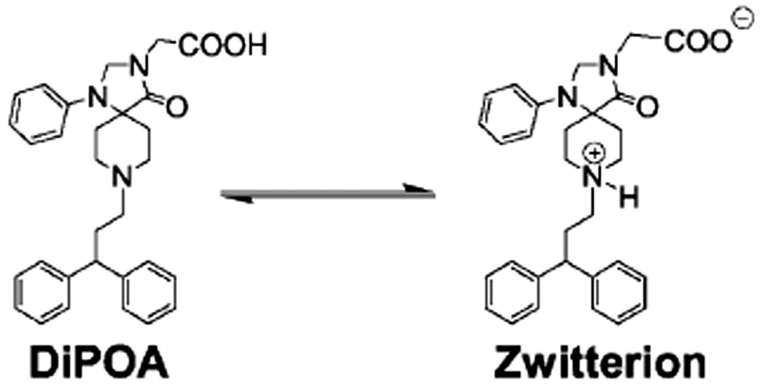

Figure 1. Structure of DiPOA and its zwitterionic form

DiPOA was tested for antinociceptive and antihyperalgesia efficacy in a number of preclinical pain models. Using mechanical hyperalgesia (paw pressure withdrawal thresholds) as an endpoint, DiPOA (ip) displayed significant antihyperalgesic activity in rats treated with CFA for 24 h, and in rats that had received a hind paw incision 24 h prior to testing (*30*). In CFA-treated rats, DiPOA significantly increased paw withdrawal thresholds in rats treated with 1, 3, or 10 mg/kg ip, with a maximal efficacy of 67% reversal relative to baseline thresholds observed 1 h after administration. Twenty-four hours after hind paw incision, rats treated with DiPOA (3 – 30 mg/kg ip) displayed a significant reversal of mechanical hyperalgesia. An almost complete reversal (85% reversal) of mechanical hyperalgesia was observed 1 h after treatment with 30 mg/kg ip of DiPOA and was maintained for at least 5 h. The magnitude of the reversal of mechanical hyperalgesia in rats treated with 3 or 10 mg/kg ip of DiPOA was similar to the magnitude of the reversal observed with the NSAID, indomethacin (30 mg/kg po).

DiPOA at doses up to 10 mg/kg ip was inactive in the rat tail flick test indicating the the lack of an acute antinociceptive effect with these doses of DiPOA. This suggests that the mechanical antihyperalgesic effects of DiPOA in the CFA and Brennan hind paw incision models are due to actions at peripheral MOR. Furthermore, DiPOA at doses up to 30 mg/kg ip did not decrease rotarod performance indicating the lack of a central opioid effect with DiPOA. Pharmacokinetic studies are supportive of low CNS penetration for DiPOA. A time course of DiPOA plasma and brain levels using showed poor CNS penetration by DiPOA, as would be predicted for a zwitterion. On average, the plasma to brain ratios of DiPOA ranged from 0.01 – 0.07% over a 5 h time period after drug administration. Unfortunately, no value for plasma protein binding was

reported for DiPOA so it is unknown as to what fraction of plasma DiPOA was available to cross the blood-brain barrier.

The similarities between the relative antihyperalgesic effects of ADL 2-1294 and DiPOA (see Table I) provide strong evidence for the fact that MOR agonists can produce significant mechanical antihyperalgesic effects via peripheral opioid receptors/mechanisms. It is interesting that whether local or systemic administration, a similar degree of antihyperalgesic efficacy is achieved with these two compounds. This may point to the robust nature of the peripheral MOR system, and suggests that if sufficient concentrations of MOR agonists can be maintained within the inflamed tissue a peripheral antihyperalgesic signal can be observed. Unfortunately, the tissue concentrations of either ADL 2-1294 or DiPOA in the inflamed hind paw are unknown.

Table I. Analgesic Activity of ADL 2-1294 and DiPOA

MODEL	*ADL 2-1294*[a]	*DiPOA*[b]
Late Phase Formalin	ED_{50} = 6 μg ipaw	Not Tested
CFA Mechanical	~ 175% of baseline	67% Max. Reversal @ 10 mg/kg ip
Tape Stripping	~85% of Max. Effect @ 1 mg ipaw	Not Tested
Paw Incision	Not Tested	85% MPE @ 30 mg/kg ip
Tail Flick	Inactive up to 160 mg/kg PO[c]	Inactive up to 10 mg/kg ip

[a] Data from (*17*). [b] Data from (*30*). [c] Data from (*31*).

KOR Agonists

Asimadoline (EMD 61753)

EMD 61753 was designed and synthesized to differentiate itself from other reported peripheral KOR agonists such as ICI 204448, GR94839, and BRL 52974 (*18*). EMD 61753 is an amphiphilic molecule that contains a hydrophobic diphenyl methyl group and a hydrophilic hydroxyl group (See Figure 2). EMD 61753 contains the hydrophobic structural element contained in the peripherally restricted antihistamines, terfenadine and ebastine.

EMD 61753 is a potent KOR agonist with good selectivity relative to MOR and DOR. EMD 61753 bound with high affinity to KOR guinea pig cerebellum membranes displacing [^{3}H]-U69,593 binding with an IC_{50} value of 5.6 nM. Using membranes prepared from rat cerebrum, EMD 61753 bound weakly to MOR and DOR and was 536 and 125 times weaker at displacing specific ligands for MOR and DOR, respectively. EMD 61753 completely inhibited electrically-induced contractions of the rabbit vas deferens with an IC_{50} value of 54 nM demonstrating that EMD 61753 was a full agonist of KOR in this tissue preparation.

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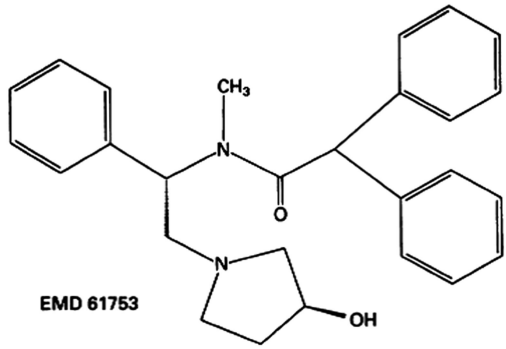

Figure 2. Structure of EMD 61753

EMD 61753 displayed robust antinociceptive and antihyperalgesic effects following both sc and po administration (*18*). In the mouse formalin model, EMD 61753 dose dependently reduced formalin-induced licking in the both early and late phase of the formalin response. The fact that EMD 61753 inhibited early phase licking is indicative of central antinociceptive activity in the mouse. EMD 61753 completely inhibited PBQ-induced abdominal constriction in mice and rats after sc administration. In the mouse there was an approximately 5-fold difference between sc and po potency for inhibiting PBQ-induced abdominal constriction. In contrast, there was an approximately 80-fold difference between the potency of EMD 61753 following sc and po administration in the rat PBQ-induced abdominal constriction. Additionally, EMD 61753 had lower efficacy (~ 50% inhibition vs. ~ 80% inhibition) following po administration in the rat, relative to sc administration. The reason for the discrepancies in potency and efficacy between sc and po administered EMD 61753 in the rat model of abdominal constriction is unknown. It is possible that there are species differences in the degree to which peripheral mechanisms are responsible for the antinociceptive activity of KOR agonists in the abdominal constriction model. A stronger central contribution to elicit an antinociceptive response of KOR agonists may be needed in the rat. Thus, high oral doses are needed to elicit efficacy in the rat PBQ-induced abdominal constriction assay.

To evaluate the effects of EMD 61753 on mechanical nociception and hyperalgesia, pressure was applied to the base of the tail using a cuff, and the effects of EMD 61753 were determined in untreated or carrageenan-treated tails (1% in the tail) (*18*). EMD 61753 was either administered 3 h (prophylactic) or 30 min (remedial) prior to testing. In all cases carrageenan was administered 3 h prior to testing. EMD 61753 (sc) was inactive against pressure nociception when tested in rats whose tails were not treated with carrageenan. In contrast, potent and complete reversal of mechanical pressure hyperalgesia was observed in rats whose tails were inflamed with carrageenan. There were no significant differences between the potency of EMD 61753 against mechanical pressure hyperalgesia with respect to whether the drug was administered prophylactically (at the same time of the carrageenan) or remedially (2.5 h after carrageenan).

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EMD 61753 completely inhibited neurogenic plasma extravasation produced by antidromic stimulation of the saphenous nerve in anesthetized rats. Doses 19 – 46 times higher than those needed to reverse mechanical hyperalgesia were needed to inhibit plasma extravasation. Furthermore, EMD 61753 was ineffective in reducing plasma extravasation produced by substance P injected into a hind paw suggestive of the fact that EMD 61753 was acting presynaptically in the periphery to inhibit neurogenic plasma extravasation as substance P is the putative neurotransmitter mediating neurogenic inflammation (*33*). Chronic asimadoline (5 mg/kg ip; bid) given early (days 1 – 3), or during the course of disease progression (days 1 – 21) effectively reversed or prevented the development of adjuvant-induced polyarthritis in Lewis rats (*34*). Asimadoline reduced edema, mechanical hyperalgesia and reversed joint damage as assessed by radiographic and histological analysis. Asimadoline was much less effective when it was administered late in the disease progression (days 13 – 21) with only a significant reduction in histology scores.

In contrast to the dose dependent and complete reversal of mechanical hyperalgesia observed with asimadoline (*18*, *34*), a time-dependent relationship was observed with locally administered asimadoline (*35*). In both untreated hind paws and hind paws inflamed with CFA (0.15 mL for 4 – 6 days) intraplantar administration of asimadoline (1.6 and 3.2 mg; bilateral) significantly increased paw withdrawal thresholds from 5 to 30 min. At time points ranging from 1 h to 4 days, an enhancement of mechanical hyperalgesia was observed, with the lowest doses (0.1 and 0.4 mg) producing the highest degree of hyperalgesia. Interestingly, while the early mechanical antihyperalgesic effect of asimadoline was significantly antagonized by the peripheral opioid antagonist, naloxone methiodide (sc), the late hyperalgesic response was not. Finally, asimadoline also increased paw volume and temperature in both untreated and inflamed paw after intraplantar, but not sc administration. Due to the underlying inflammation produced by CFA, the increase in paw volume and temperature following asimadoline treatment was more pronounced in untreated hind paws. As was the case with the asimadoline-induced hyperalgesia, the increases in paw volume and temperature were not antagonized by naloxone methiodide (sc). The mechanism(s) responsible for the enhancement of mechanical hyperalgesia, the increase in paw volume, and paw temperature by asimadoline have not been elucidated.

Although there appears to be a good deal of evidence to support the fact that there is a peripheral component to the analgesic and hyperalgesic activity of asimadoline, it is clear that the compound possesses central pharmacological activity. Depending on the pharmacological endpoints used, there is a moderate to large degree of separation between the doses of asimadoline that are likely to produce only peripheral effects and the doses that have CNS activity. EMD 61753 potentiated hexabarbitone-induced sleep time with a minimally effective dose (MED) of 10 mg/kg sc and reversed haloperidol-induced accumulation of DOPA in the nucleus accumbens with a MED of 30 mg/kg sc (*18*). EMD 61753 impaired rotarod function at high doses with an ED_{50} value of 453 mg/kg sc. Unfortunately no mention of the MED for impairment of motor function was made. Additionally, at cumulative intramuscular doses of 0.3 and 1 mg/kg of EMD

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61753 a complete generalization to enadoline was observed in squirrel monkeys trained to discriminate enadoline, a potent centrally acting KOR agonist, from saline in a two-lever drug discrimination task (*36*). EMD 61753 and another peripherally selective KOR agonist, ICI 204448 were the least potent agonists tested for generalization to enadoline suggesting that limited CNS penetration was observed with EMD 61753. Only at high doses of EMD 61753 are brain levels sufficient to produce a discriminative cue similar to enadoline. Response rates were unaffected in monkeys treated with EMD 61753 indicating the lack of sedation at all of the doses tested.

Use of [^{14}C]-EMD 61753 tissue distribution and autoradiographic measurements demonstrated low levels of radioactivity in the brain relative to other tissues such as the lung, liver, and adrenals in rats that were treated with either 1 mg/kg iv or 10 mg/kg po (*18*). Peak brain levels were observed 5 min after iv administration and a plateau was reached 1 h after po administration and maintained for at least 6 h. Relatively high levels of [^{14}C]-EMD 61753 were measured in the pituitary gland and autoradiographic analysis demonstrated that the radioactivity found in the brains was primarily concentrated in the regions of the 3rd, 4th, and lateral ventricles. Histological analyses of these regions of high radioactivity indicated that the regions were the choroid plexus. As is the case with ADL 2-1294, there is evidence that asimadoline is a substrate for P-gp (*37*). Thus, the peripheral selectivity of asimadoline may be due to both its amphiphilic nature and due to it being a target for active transport out of the brain by P-gp.

Asimadoline was tested for efficacy as a postoperative analgesic in a small placebo-controlled, double-blind clinical trial in patients undergoing diagnostic arthroscopic knee surgery (*35*). Patients were treated with placebo or 5 mg of asimadoline 30 min prior to surgery and then again approximately 90 min after surgery. Patients rated their pain on a 100 point visual analog scale (VAS) every hour for up to 8 h. No relief of postoperative knee pain was observed in patients (n = 17) treated with 10 mg of asimadoline. Relative to placebo, VAS scores actually increased, and the time to the initial administration of the rescue medication piritramide, an opioid was reduced. Furthermore, the amount of piritramide used in the 8 h post-surgical period was greater in patients that received asimadoline than in placebo-treated patients. The reason for the pronociceptive effect of asimadoline is unknown, and it may be that the single dose chosen was insufficient to produce an analgesic effect. It is possible that the hyperalgesic effect of local asimadoline that was observed in some CFA studies (*18*, *34*), but not others (*35*) also occurs in humans.

Fortunately, the clinical history of asimadoline did not stop with this one small, failed trial in post-surgical pain. Falling back on the preclinical literature demonstrating that KOR agonists, including asimadoline are effective in reducing visceral sensations after distension (*38*), two studies were performed in healthy subjects to examine the effects of asimadoline on satiation, colonic compliance, perception of colonic distension, and whole gut transit (*39*, *40*). In the initial study, ninety-one healthy subjects (males and females) were treated with placebo, 0.15, 0.5, or 1.5 mg of asimadoline twice a day for 9 days in a double-blind fashion (*39*). In this study, satiation was monitored using the intake of Ensure® Asimadoline had a positive effect on satiation showing an enhancement of the

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amount Ensure® ingested relative to placebo. At the low dose of asimadoline (0.5 mg), the perception of gas at low colonic pressures (8 mm Hg) was reduced. No effects were observed at higher colonic pressures. Interestingly and reminiscent of the pronociceptive effect of asimadoline in the arthroscopic knee study (*35*), in this study an increase in the perception of gas (ie enhanced visceral sensation) was observed in patients treated with 1.5 mg of asimadoline (total bid dose = 3 mg). The changes in liquid nutrient intake and the perception of colonic distension occurred in the absence of any significant alterations in gastrointestinal motor reflexes or transit. Asimadoline was well tolerated in this study with no serious adverse events observed. In a second study (*40*), thirteen patients received either placebo, 0.5, or 1.5 mg of asimadoline in a randomized fashion 1 h prior to ingestion of Ensure®. In subjects that received 0.5 mg of asimadoline a decrease in postprandial fullness was observed without affecting the volume needed to reach full satiation. In subjects treated with 1.5 mg of asimadoline a decrease in satiation was observed such that there was an increase in the volume of Ensure® consumed by the subjects. Interestingly, despite the small number of subjects tested, there appeared to be a gender interaction with respect to gastric volume. In females, an increase in both fasting and postprandial gastric volume was observed after they received 0.5 mg of asimadoline. In contrast, a decrease in fasting volume and no change in postprandial gastric volume were observed in males that received 0.5 mg of asimadoline. No changes in gastric volumes were observed in subjects treated with 1.5 mg of asimadoline. In summary, the results of these two Phase I studies with asimadoline suggest that additional studies with asimadoline in patients with functional dyspepsia are warranted.

Perhaps encouraged by the results with asimadoline in the satiety and gastric compliance studies, asimadoline was tested in two Phase II clinical studies in mixed populations of irritable bowel syndrome (IBS patients) (*41*, *42*). Although asimadoline treatment failed to reach statistical significance with the primary endpoints in any of the studies, there were distinct patient populations that statistically benefitted from the treatment with asimadoline in each study. In a randomized, double-blind, placebo-controlled study (*41*), post-hoc analysis showed that pain scores significantly improved in patients that had mixed IBS symptoms (ie alternating bouts of constipation and diarrhea). Although not statistically significant, pain scores tended to be higher in patients with diarrhea-predominant IBS. In the second study (*42*), patients received placebo, 0.15, 0.5, or 1 mg of asimadoline bid for 12 weeks in a randomized, double-blind manner. The primary endpoint was the number of months with adequate relief of IBS and discomfort. As was the case with the earlier study (*41*), there was no main treatment effect with asimadoline. However in patients with diarrhea-predominant IBS and baseline pain scores of moderate ($\geq$ 2 on a 4 point scale) treatment with 0.5 mg of asimadoline produced significant improvement in the total number of months with adequate relief of IBS pain and discomfort. Additionally, in patients with mixed IBS symptoms a significant increase in adequate pain relief was achieved in patients treated with 1 mg (bid) of asimadoline. Thus, it appears that asimadoline may improve certain subpopulations of IBS patients or certain symptoms of functional dyspepsia, and the clinical challenge and future utility of asimadoline for the treatment of IBS

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and dyspepsia may be dependent on successfully identifying patients that would benefit from the treatment with asimadoline or other peripheral KOR agonists.

CR 665 and CR 845

CR 665 and 845 are tetrapeptides consisting of all D-amino acids that bind very potently and selectively to KOR (*43*). CR 665 was initially synthesized by chemists at the Torrey Pines Institute and along with CR 845 are being developed by Cara Therapeutics. The amino acid sequence of CR 665 is D-Phe-D-Phe-D-Leu-D-Arg-NH, and the amino acid sequence of CR 845 is D-Phe-D-Phe-D-Leu-D-D-lycine [γ-(4-N-piperidynyl)amino carboxylic acid. CR 845 was designed to produce a longer duration of action than CR 665 and to allow oral dosing in clinical settings (*43*). The K_i values for CR 665 and 845 in cells containing cloned human KOR were 0.24 and 0.32 nM and both were full agonists for the inhibition of cAMP in cells containing cloned human KOR. Neither peptide has affinity for MOR or DOR as well as lacking activity in a broad panel (> 90) screen of other receptors, channels, and enzymes (*44*). CR 665 (also known as FE 200665) was tested in mice for antinociceptive activity in the acetic acid-induced writhing assay, and in the rotarod assay to evaluate the propensity for ataxia as an indication of brain penetration. CR 665 (iv) potently inhibited acetic acid-induced writhing with an ED_{50} value of 0.007 mg/kg and only affected rotarod performance at a much higher dose, with an ED_{50} value of 3.8 mg/kg. There was more than a 540-fold difference in the potency of CR 665 for inhibiting acetic acid-induced writhing (peripheral analgesic effect), and the potency of CR 665 for impairment of rotarod performance, a centrally-mediated effect (*20*). In rats treated with CFA for 4 days prior to treatment with FE 200665, a significant reduction in mechanical hyperalgesia, paw volume, and a reduction in histology scores associated with joint and paw swelling was observed (*44*). FE 200665 administered intraplantar (3 – 100 μg) or sc (2 – 20 mg) significantly increased paw withdrawal thresholds 5, 10, and 30 min after treatment with FE 200665. The maximal effect following both local and sc administration of FE 200665 was approximately 80% Maximum Possible Effect, using a cut-off of 250 g of pressure.

CCR 845 also displayed potent activity in a number of preclinical inflammatory pain models (*45*). Following pretreatment with either 0.3 or 1 mg/kg iv of CR 845 mechanical hyperalgesia was completely attenuated in rats that were injected with carrageenan in the hind paw. Additionally, CR 845 significantly reduced the increase in paw volume that occurs after carrageenan treatment. In rats injected in a knee joint with monosodium iodoacetate (MIA) , a model of osteoarthritis pain accompanied by progressive joint degeneration, CR 845 (0.1 or 0.3 mg/kg sc) significantly attenuated the change in hind limb weight bearing distribution that resulted from MIA treatment. Furthermore, CR 845 decreased TNF-α, interleukin-1β, interleukin-8, and granulocyte macrophage-colony stimulating factor in human macrophages that had been treated with lipopolysaccharide and interferon γ. Finally, in synoviocytes taken from patients with rheumatoid arthritis CR 845 suppressed the production and proliferation of TNF-α, and the matrix metalloproteinases, MMP-1 and MMP-3.

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Taken together, the preclinical data obtained with CR 665 and 845 provides compelling evidence that these highly selective and highly peripheralized KOR peptides have robust antihyperalgesic efficacies, and also appear to have the potential to produce strong anti-inflammatory effects by inhibiting a number of pro-inflammatory cytokines. The precise mechanisms and systems that the peripheral KOR agonists and their receptors interact with to produce these changes in inflammatory mediators need to be further elucidated, but offers a chance to explore new approaches that may augment the peripheral analgesic signal that arises from stimulation of KOR.

Importantly, both CR 665 and 845 have both been advanced to clinical trials and Phase I and/or Phase II studies have been completed. This allows us to see how well the preclinical data both in terms of efficacy and in terms of the lack of CNS-mediated side effects with these two peptides translates into the clinical setting. CR 665 was tested in a Phase I dose ascending study in healthy males and females following a 1 h iv infusion at doses ranging from 0.015 – 0.48 mg/kg (*46*). No serious adverse events were observed following administration of CR 665 and more notable is the lack of reported CNS effects such as hallucinations, dysphoria, and emesis. Mild, transient CNS effects were such as paresthesia, dizziness, and somnolence were observed. Treatment with doses of CR 665 of 0.02 mg/kg and above increased serum prolactin levels indicating target engagement in the study, as KOR opioid agonists are known to increase prolactin release via inhibition of dopamine at tuberoinfundibular sites in the pituitary (*47*). In a small (n = 18) study using normal, healthy males, CR 665 (0.36 mg/kg iv; 1 h infusion) was tested in a randomized, double blind, placebo- and positive control 3-way crossover study to evaluate its effects on mechanical distension of the esophagus (*48*). CR 665 and oxycodone (15 mg po) significantly reduced the moderate visceral pain associated with distension of the esophagus. Not surprisingly, oxycodone had a broader range of analgesic efficacy than CR 665 also displaying significant analgesic effects to skin pinch, cuff algometry, and thermal stimulation of the esophagus. This study is important because it was one of the first positive clinical studies with a peripheral KOR agonist and supports the preclinical literature that KOR agonists may be effective in the treatment of visceral pain, especially pain that is associated with the distension of the hollow organs of the gastrointestinal tract.

Based in part on the success of CR 665 in the Phase I clinical studies, CR 845 has been advanced to Phase II studies in to evaluate its efficacy in a post-operative pain setting. CR 845 has an advantage over CR 665 in that it was designed to have oral activity and a longer duration of action. CR 845 was tested in 37 normal human (35 male/2 females) subjects in a Phase I study using a 15 min iv infusion of doses ranging from 0.002 – 0.04 mg/kg (*49*). Dose-proportional exposures were observed after the 15 min exposure, with a t_{max} (time of peak concentration) of approximately 15 min. CR 845 had a plasma half-life of approximately 2 h and there were no detectable metabolites supporting preclinical ADME data showing that CR 845 is excreted as the parent peptide via the urine and feces. CR 845 was well tolerated with no reports of dysphoria, hallucinations, or sedation as measured with the Ramsay Sedation Scale. The fact that dysphoria and hallucinations were not reported by humans that received either CR 665 or CR 845 is evidence that the very high degree of peripheral restriction observed in preclinical studies with

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these two tetrapeptides is maintained in humans. The most common side effect was brief facial tingling or paresthesia that occurred in 65% of the patients (24/37). The facial paresthesia and other mild adverse effects were usually observed within an hour of drug infusion and were transient, resolving within 2 h. Clearly distinguishing itself from MOR agonists, there was very little nausea or vomiting observed in subjects that received CR 845. As was observed in subjects treated with CR 665, an increase in serum prolactin was observed after infusion with CR 845 in patients that received doses of 0.008 mg/kg or above. Consistent with KOR agonist pharmacology, a transient increase in urine was observed in patients that received CR 845.

CR 845 (0.001, 0.003, 0.006 mg/kg; 15 min iv infusion) was also tested in a Phase I study in normal and hemodialysis subjects as a prelude to possibly testing CR 845 for efficacy against hemodialysis-induced pruritis (*50*). Once again, CR 845 was well tolerated in both normal subjects and showed similar tolerability in hemodialysis subjects. No serious adverse events were observed in this study and once again no signs of dysphoria or hallucinations were reported in either treatment group. Not surprisingly, the total exposure (using area under the curve) of CR 845 was three times greater in hemodialysis subjects than in normal subjects. Importantly, despite the 3-fold increase in total exposure of CR 845 in hemodialysis subjects, no serious CNS side effects were observed. These data are supportive of a wide safety margin with CR 845 and suggest that an efficacy study with CR 845 to reduce hemodialysis-induced pruritis is warranted.

CR 845 has been tested in two Phase II studies in women undergoing laparoscopic hysterectomy. In the initial proof-of concept study, CR 845 (0.04 mg/kg; 15 min iv infusion) was administered to 20 patients in a randomized double-blind, placebo-controlled study. Patients received CR 845 or placebo upon reporting a pain intensity of 5 – 8, on an 11-point scale. Pain intensity was measured in all patients up to 8 h or until rescue medication was requested (morphine PCA) (*51*). Relative to placebo-treated patients, there was a significant increase in the pain intensity difference in patients that received CR 845 indicative of a postoperative analgesic effect of CR 845 by itself. Furthermore, morphine consumption was reduced by 49%, relative to placebo-treated patients when it was measured 4 – 8 or 8 -16 h after iv infusion of CR 845. As was the case in the Phase I studies with CR 845, no dysphoria or sedation was reported, and again CR 845 was well tolerated with no incidences leading to drug discontinuation. All adverse events were rated as mild or moderate. Perhaps most importantly, there was a significant reduction in adverse events associated with pre- and or postoperative opiate administration which is likely due in part to the reduction in morphine consumption. Specifically, there was a 72% reduction in nausea in patients treated with CR 845, relative to placebo and there were no incidences of vomiting (0/20 patients) compared to 3/26 patients that received placebo. As was observed in the Phase I study in normal volunteers, a transient increase in urine output was observed in patients that received CR 845.

The results of a larger, more complex designed Phase II study with CR 845 (0.04 mg/kg iv) again in women undergoing laparoscopic hysterectomy were reported in a 6/11/12 press release by Cara Therapeutics (*52*). In this double-randomized, double-blind, placebo-controlled study, patients (N = 203)

were assigned to one of four treatment groups. The treatment groups were as follows: pre and postoperative CR 845, preoperative only, postoperative only, or placebo pre and postoperative. The primary endpoint of the study was the total amount of rescue opioids in the 24 h postoperative period and the secondary endpoint was pain reduction as measured by the summed pain intensity differences over the 24 h postoperative period. In patients that received CR 845 both pre and postoperatively there was an approximately 33% decrease in morphine use, relative to placebo-treated patients. Patients treated with CR 845 pre and postoperatively and patients that received CR 845 only postoperatively showed significant pain intensity difference at 24 h ($PID_{0\text{-}24}$) with a 100% and 50% increase, respectively. As with the other clinical studies, CR 845 was well tolerated although no breakdown of specific adverse events was delineated in the press release.

In a Phase I study designed to potentially expand the therapeutic utility of CR 845, an oral dosage form of CR 845 was tested in an ascending, single dose study in 50 healthy, male volunteers (*53*). The study consisted of a placebo control and four doses of an enteric-coated formulation of CR 845. CR 845 had a mean bioavailability of 16% under fasted conditions. CR 845 appeared to be well tolerated with no signs of dysphoria or psychotomimetic effects. The use of an oral formulation of CR 845 greatly expands the potential range of clinical settings that CR 845 could be tested in, and provides the opportunity to see the potential analgesic range of activity of a peripheral KOR agonist which to date has been shown to be devoid of serious CNS adverse events.

DOR Agonists

There are two recently published findings that may spark a renewed interest in the synthesis and design of peripherally-restricted DOR agonists. Using DOReGFP reporter mice, it was demonstrated that DOR is expressed on peripheral myelinated and nonpeptidergic unmyelinated afferents that selectively suppress mechanical nociceptive stimuli (*7*). Furthermore, this report calls into question findings that indicate that MOR and DOR are co-expressed the same subpopulation of primary afferents. Examining DOReGFP distribution in sensory neurons of the dorsal root ganglia did not overlap with the subpopulation of unmyelinated peptide containing nocicpeptors. Since DOReGFP distribution and cell surface expression differed greatly from the predominant literature (*27*, *28*, *54*), the specificity of the DOR antisera used to localize DOR and follow DOR trafficking was tested. The distribution of antisera agreed with previous reports. However, the staining pattern using the antisera did not change in two different strains of mice with deletion of DOR. The fact that staining was observed in DOR knockout mice suggests that the most widely-used anti-DOR antibody does not recognize DOR in immunohistochemical preparations, but rather cross reacts with an unidentified molecule. Thus, this report changes the view of how peripheral DOR modulates nociceptive inputs and offers a new perspective on the potential uses of selective, peripheral DOR agonists.

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Conditional knockout mice were created in which DOR were specificaclly deleted in peripheral NaV1.8 positive primary nociceptive neurons (*6*). These mutant mice allowed the characterization of the contribution of peripheral DOR in pain control to various stimuli. No deficits to noxious thermal stimuli or to formalin were observed in the conditional knockout mice However, mechanical allodynia assessed with von Frey filaments was significantly enhanced after CFA administration or after partial sciatic ligation (neuropathic pain model) in the mutant mice. Furthermore, the anti-allodynic effect of the selective DOR agonist, SNC80 whether administered systemically (ip) or locally in the hind paw was abolished in the conditional knockout mice, but not control mice. These data strongly support the notion that peripheral DOR are important regulators of peripheral mechanical nociceptors, and therefore should be a considered a good target for the development of novel peripheral opioid analgesics.

Lessons Learned

The data in perclincal models of inflammatory and postoperative pain with ADL 2-1294 and DiPOA provide solid preclinical evidence of the efficacy with peripheral or low CNS penetrating MOR agonists. However, the clinical success of ADL 2-1294 was limited. In small Phase I and Phase II clinical studies, ADL 2-1294 displayed topical antihyperalgesic activity in subjects with sunburn, and using an ophthalmic formulations efficacy was observed in patients with corneal abrasions, post-keratecomy pain and pain associated with the removal of pterygium. It appeared that formulation and solubility issues resulted in the failure of ADL 2-1294 to advance to larger size clinical trials. It appears that DiPOA was not advanced beyond the discovery/preclinical stage. The primary proof of clinical efficacy of peripherally administered morphine comes from the use of morphine (ia) after knee surgery. However, as pointed out in a systematic review of thirty-six studies, many of the randomized, controlled studies had study design flaws, or flaws with data collection, analysis, or reporting (*55*). Due to these design flaws, it was felt that more adequately controlled clinical studies were needed to provide conclusive proof that ia morphine was analgesic and that the analgesia was clinically meaningful.

Based on the similar magnitude of efficacy for ADL 2-1294 and DiPOA, it does not appear to make a difference as to whether peripheral MOR agonists are administered directly in the site of inflammation or if they are administered systemically. If the pharmacokinetics of a systemically administered agonist is sufficient to allow for a broad peripheral distribution, there appears to be a trade-off that needs to be made between the use of local administration of MOR agonists, and the systemic administration of peripherally-restricted MOR agonists. For locally administered compounds there are formulation hurdles that need to be addressed. There needs to be sufficient penetration (if an injection is not used) and sufficient residence time within the inflamed tissue to produce sustained activation of MOR. The major hurdle for systemically administered peripherally-restricted MOR agonists is to reduce the propensity to produce some of the unwanted side effects of central MOR agonists such as nausea,

vomiting, and constipation. Therefore, activation of peripheral MOR at sites distant from the inflammation should be reduced, if at all possible. Decisions regarding the approach with peripheral MOR agonists need to be made early in the discovery process so that the preclinical studies can most closely mirror the pattern of intended use in the clinical setting, if possible. Additionally, the choice of the route of administration may influence the choice of animal model, and the decision tree used to advance compounds.

It appears that the development of peripherally- or site-restricted MOR agonists may represent the greatest challenge for the advancement of a new generation of peripheral opioid agonists. New drug delivery or formulation technologies or new methods to modulate endogenous opioid peptide levels at the site of inflammation (*56*) are alternative approaches that can be explored to exploit the potential of peripheral MOR analgesia.

As is the case for MOR agonists, there is strong preclinical data that provides evidence for peripheral analgesic/anti-hyperalgesic effects of KOR agonists. The preclinical efficacy observed with asimadoline and the Cara tetrapeptides is consistent with the efficacy for many other peripherally-restricted KOR agonists (*1*, *18*, *19*). However until the recent data with CR 665 and CR 845, there were few successes in the clinic with other peripherally-restricted KOR agonists. A small study with ADL 10-0101 (iv) in patients with chronic pancreatitis (*57*) provides additional support that visceral pain is a good therapeutic fit for peripheral KOR agonists. It will be interesting to see whether the oral formulation of CR 845 will show similar efficacy to iv administration after laproscopic surgeries, and to see what other clinical pain states will be pursued with the oral formulation. Knowing the range of clinical efficacies with a peripheral KOR may spark a renewed interest in the discovery and development of novel, peripherally-restricted KOR agonists. The data in IBS patients treated with asimadoline are intriguing, and offer a path forward to more thoroughly understand the impact of KOR on normal and abnormal visceral sensations. Although the IBS patient population is a very heterogeneous one, the initial IBS studies with asimadoline provide some insights into the patient subpopulation that might be best served clinically by treatment with peripheral KOR agonists.

The fact that significant adverse CNS effects were not observed in patients that received either asimadoline, CR 665, or CR 845 is very encouraging, especially in light of the fact that the level of peripheral restriction, as measured by brain levels of compound differs greatly between asimadoline and the tetrapeptides. It would be interesting to test asimadoline in a visceral postoperative setting to see if doses that are devoid of significant CNS adverse effects in IBS studies show efficacy against postoperative pain. As predicted, measurements of serum prolactin and diuresis proved themselves to be good biomarkers for the engagement of KOR after treatment with CR 665 and CR 845. Increases in serum prolactin and urine output will provide a good indication that oral administration of CR 845 has sufficient absorption, and results in plasma concentrations needed to activate KOR.

The recent studies with peripheral DOR systems (*6*, *7*) may serve as the basis for a new body of literature that will greatly increase the understanding of peripheral DOR receptors and possibly expound on the role of opioid receptors in

the modulation of peripheral nociceptive signaling. The finding that mechanical anti-allodynia after inflammation or nerve ligation in conditional knockout mice is a strong demonstration of the potential importance of DOR in peripheral nociception. These studies should serve as the impetus for new drug discovery efforts aimed at the development of novel peripherally-restricted DOR agonists.

Conclusions

In the approximately twenty-five years since peripheral opioid analgesia and its therapeutic benefits began to be fully realized there has been mixed success. There is certainly a greater understanding of the mechanisms by which opioid receptors interact with the other components involved in inflammatory pain processes. Additionally, there has been a great deal of progress demonstrating preclinical efficacy with peripherally-restricted compounds, especially KOR and MOR agonists. Strategies designed to convincingly demonstrate that efficacy is due to activation of peripheral opioid receptors have also evolved as the tools such as more selective agonists and antagonists, transgenic mice, etc have become available. Despite these advances on the preclinical side, successes in the clinical development has lagged. The recent clinical successes with CR 665, CR 845, and the limited success with asimadoline in IBS patients bode well for the future of peripheral KOR in the clinic. It is very encouraging that significant CNS adverse events such as dysphoria and psychotomimetic effects have not been observed to date with these compounds. Hopefully, the successes in the clinic and the progression of an oral form of CR 845 will elucidate the depth and breadth of peripheral KOR analgesia. In light of the recent data about peripheral DOR localization and function, it will be interesting to see if there is a new wave of design, synthesis, and development of novel peripheral DOR agonists. In conclusion, the hope of novel analgesics based on activity of peripheral opioid agonists remains, and the groundwork for a new generation of compounds has been laid.

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Chapter 12

The Delta Opioid Receptor

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The delta opioid receptor has become increasingly relevant to the development of analgesics since its discovery only a few decades past. The endogenous antinociceptive properties of the δOR in conjunction with the potential for δOR ligands to circumvent classical μ-opioid side effects and tolerance make the δOR a provocative alternative to current μOR-specific strategies. This chapter explores the clinical potential of δOR-mediated analgesia and discusses novel biochemical strategies for targeting the δOR. Finally, we evaluate the use of δOR ligands as co-therapies with new and classical analgesics. A greater understanding of δOR function and pharmacology will ultimately contribute to the development of innovative new strategies for the pharmacological management of pain.

Introduction to the δOR

History of the δOR

The existence of the delta opioid receptor was first implicated by the discovery of two endogenous opioid peptides, [Met^5]enkephalin and [Leu^5]enkephalin by Hughs and colleagues (*1*). Early studies compared the differential activity profiles of these enkephalin compounds with morphine on tissues that differentially expressed opioid receptor sub-types, including the guinea pig ileum and mouse vas deferens (*1*, *2*). The ability of [Leu^5]enkephalin to selectively inhibit electrically-induced contraction of the mouse vas deferens, but not the guinea pig ileum, yielded a novel paradigm in which tissue-specific effects of opiate ligands could be evaluated. Further studies in these models elucidated a number of peptide compounds that appeared to have selective action in the mouse vas

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deferens, including the deltorphins I and II and additional specific enkephalin compounds such as [D-Pen^{2,5}]enkephalin (DPDPE). The putative opioid receptor was termed delta (δ) for its characteristic function in the mouse vas deferens.

By 1992, extensive pharmacological characterization of the δ-opioid receptor (δOR) (*3*, *4*) utilizing synthesized, as well as endogenous enkephalins, resulted in the first physical cloning of an opioid receptor. Clones were reported by two independent groups from the mouse neuroblastoma-rat glioma hybrid NG108-15 cell line (*5*, *6*), and later from mouse brain cDNA library (*7*). Within two years of the initial cloning of δOR from NG108-15 cells, δOR was also cloned from rat cerebellum (*8*) and similarly from the human striatum and temporal cortex (*9*). The rapid discovery of the physical δOR heralded the cloning of μ and κ opioid receptors soon thereafter (*10*), and a new era of opioid therapeutic development and research. As a component of endogenous antinociception, δOR and its agonists have since gained attention in the research community as potential analgesics that may rival or replace μOR therapies.

The goal of this chapter is to summarize the progress that has been made with regard to the therapeutic potential of the δOR since its discovery several decades past. Additionally, this chapter emphasizes therapeutic relevance of the δOR's biochemical characteristics and interactions with other proteins such as the μOR, and receptor biased coupling. Finally, this chapter evaluates the clinical prospects of δOR agonists, antagonists and mixed-action opioid pharmacotherapies in the context of pain management. By presenting multiple avenues to target the δOR, we uncover novel and efficacious strategies for the pharmacological management of pain.

Genetic Insights

The first identification of a physical δOR spurred a series of studies that sought to determine the δOR's distribution, physiological properties and biochemical identity. The δOR is encoded by the *Orpd1* gene; as such, examining the phenotype of *Orpd1* -/- mutant mouse strains has yielded important information about the endogenous role of the δOR in antinociception. The generation of *Orpd1*$^{-/-}$ (knockout) mice was first reported by two groups (*11*, *12*), which confirmed the absence of DPDPE and deltprophins I and II binding in knockout animal tissues. δOR knockout animals exhibit hyperlocomotion, anxiogenic and depressive-like behaviors (*12*), delayed wound healing (*13*) and impaired immune response (*14*). Of therapeutic interest, no respiratory effects have been reported in δOR knockout mice. δOR knockout animals do not demonstrate alterations in thermal hyperalgesia, mechanical allodynia, or chemical nociception responses (*11*, *12*, *15*), but do exhibit enhanced neuropathic and inflammatory nociceptive responses (*16*, *17*). These data suggest that the role of the δOR in nociception may relate more closely with chronic rather than acute pain-states. Furthermore, it has been suggested that the emotional, anti-depressive component of δOR function may associate with the experience of pain and therefore provide a unique approach to pain therapy.

The most provocative genetic studies have examined the interplay between the μ-opioid and δ-opioid receptors through morphine administration in

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δOR knockout models. Whereas δORs appear to have no direct function in morphine-mediated analgesia, δOR-deficient animal models exhibit reduced reward behaviors in response to chronic morphine administration, in addition to reduced or absent analgesic tolerance (*11*, *18*, *19*). The relationship between μ-opioid and δ-opioid receptors in pain is not well understood, and is an important topic in this chapter and in the realm of therapeutic development for pain. The prospect of δOR-specific therapies in patients with opiate tolerance is a tantalizing "real-world" solution for pain management that we will seek to explore.

Pattern of Expression

Early pharmacological studies demonstrating δOR-mediated antinociception (*20–22*) and locomotor activation (*23*, *24*) strongly suggested expression of the δOR in the nervous system. As such, much work has characterized the physiological and specific neuroanatomical distribution of the δOR in rodent (*25–27*) and human (*28*) tissues. In rodents, the highest expression of the δOR occurs in the olfactory bulb, followed by expression in dopaminergic neurons of the striatum, pars reticulata of the substantia nigra and nucleus accumbens (*25–28*). The δOR is also detected in the ventromedial hypothalamus (*26*) and areas of the thalamus (*25*), memory-related areas including the hippocampus (*26–28*) and in cortical areas including the temporal lobe (*28*). Most relevant to this chapter is the characterization of the δOR in ascending and descending central nervous system pain areas. Single-cell PCR, *in situ* hybridization and immunostaining corroborates the controversial finding that δOR is expressed in large- and small-diameter rodent dorsal root ganglion neurons (*28*, *29*); furthermore, there is evidence for δOR expression in the spinal cord including the substantia gelatinosa and superficial layers (*30*). These observations suggest that δORs may have a role in modulating nociceptive input, supporting evidence that intrathecal delta agonists have analgesic efficacy (*31*). Expression of the δOR in descending pain structures includes limbic areas amygdala neurons projecting to the periaqueductal grey (PAG) (*26*, *32*), and in neurons of the PAG which project to the rostroventromedial medulla (RVM) (*33*). Pharmacological studies have inferred that within the RVM, δOR is functionally expressed on OFF-cells (*34*), supporting the hypothesis that supraspinally-administered δOR agonists modulate descending inhibition of nociceptive input.

Like other opioid receptors, δOR expression is influenced by nociceptive events. Inflammation and chronic pain induce δOR expression in DRG neurons and the superficial laminae of the dorsal horn (*35*). Additionally, δOR surface targeting can be increased by chronic morphine administration in a μOR-dependent manner; μOR-dependent induction of δOR occurs in the dorsal horn (*36*), PAG (*37*), and amygdala neurons involved in descending nociceptive modulation (*32*). The enhancement of δOR expression in pain-related areas of the nervous system advocate the clinical prospect of δOR agonists in chronic pain patients who have likely received therapeutic μOR-agonist treatment.

Low-to-moderate levels of expression for the δOR have been documented outside of the nervous system, in the human small intestine, skeletal muscle, lung, and on immune cells (*28*). In the enteric nervous system, δOR is expressed in

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neurons of the myenteric and submucosal plexes, and as such is thought to suppress motility and secretion (*38*, *39*); however, recent pharmacological studies contradict the presumed gastrointestinal effects of δOR ligands, and will be discussed later in this chapter. Similarly, the low expression of δOR in the lung appears to be multifaceted, as agonists have demonstrated depressive, stimulatory and absent effects on respiration.

Characteristics of the δOR

δOR Structure

A fundamental understanding of the δOR as a biochemical entity is vital to identifying therapeutic strategies to target the δOR. Similar to the μ and κ opioid receptors, the δOR is a member of the rhodopsin subfamily of GPCRs and is characterized by a 7-transmembrane domain structure with an intracellular carboxyl terminus and an extracellular amino terminus. The human δOR (hδOR) is 372 amino acids in length and shares a 65% sequence homology with mu and kappa receptors (*40*). Notably, hδOR structure shares >90% sequence identity with the mouse and rat δOR. Within opioid receptor subtypes, the locations of greatest sequence divergence occur in the amino- and carboxyl- termini and the extracellular loops (EC2, EC3, 21-52% homology), whereas transmembrane and intracellular domains (TM2, TM3) are highly conserved (86-100% homology) (*41*). As such, a large body of mutagenesis and chimeric receptor work has focused on elucidating the importance of divergent regions.

Chimeric μOR-δOR structures have provided significant insight into the critical binding regions of the δOR. A landmark body of work by Wang and colleagues performed a series of chimeric studies that implicated the third extracellular loop as a critical high-affinity binding site for δOR agonists (*42*); chimeras lacking the third extracellular loop of the δOR fail to bind the selective peptide [D-Ser^2, D-Leu^5]enkephalin-Thr (DLSET). These data contrasted results for the selective μOR agonist [D-Ala^2, MePHe^4, Gly^5-ol]enkephalin (DAMGO), which required the first extracellular loop of the μOR for high-affinity binding. Importantly, an inherent problem with chimeric receptor work is the inability to distinguish between specific local effects on receptor-ligand interaction and overall changes in receptor conformation. The authors partially ameliorated this concern with the demonstration that double-point mutations of Arg291 and 292 of the δOR third extracellular loop are sufficient to abolish the binding of DLSET (*42*).

Subsequent chimeric receptor work corroborated the results of Wang and colleagues and expanded the importance of TM3 to selective δOR agonist binding; splicing the third extracellular loop of the δOR into μOR structure increased the binding affinities of several peptidic and nonpeptidic δOR agonists (*43*, *44*). Additionally, TM3 residues Trp284, Val296 and Val297 were demonstrated to participate synergistically in the selectivity of δOR ligands in concurrent work by Valiquette and colleagues (*45*). The extracellular N-terminal domain of the δOR does not appear to be critical for specific ligand binding (*46*).

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Studies examining the region(s) of the δOR which couple to second messenger cascades and modulate signal transduction are limited. Similar to the role of the N-terminus, the intracellular C-terminus does not appear to be vital for signal transduction: in studies truncating a 31-residue span from the cytoplasmic tail, there was no effect on the inhibition of cAMP production by DPDPE (*47*). These results were corroborated by C-terminal peptide mimetics which failed to antagonize GTPase activity or [35S]GTPyS binding (*48*). However, the recent discovery of the promiscuity of δOR with intracellular protein binding warrants further studies evaluating the δOR cytoplasmic interface. The third intracellular loop appears to be involved in receptor coupling as well as ligand binding: peptides sharing homology with the third intracellular loop of δOR inhibit GTPase activity and [35S]GTPyS binding (*48*) in addition to binding of [H^3]DLSET. Similar work also implicated intracellular loop 4 in receptor G-coupling.

δOR Subtypes

The evidence surrounding the hypothesized δOR subtypes, δ1 and δ2, is limited and remains controversial. The majority of evidence for δOR subtypes is inferred from early pharmacological studies demonstrating distinctive binding patterns of the δOR agonists [^{3}H]DLSET and [^{3}H]DPDPE. Based on the assumption that the existing cloned receptor is δ2, Hiller and colleagues proposed a 9:1 ratio of the δ2:δ1 expression in the rat brain (*49*). This work was founded on earlier studies proposing that δ1 is activated by DPDPE and inhibited by [Ala,Leu,Cys]enkephalin, whereas δ2 is activated by DLSET and deltorphin II and inhibited by naltrindole-5′-isothiocyanate (*50*, *51*). Pharmacological patterns remain the primary evidence for putative δOR subtypes. It has been proposed that insufficient selectivity of the drugs such as DLSET used to identify δOR subtypes weakens the existing evidence; furthermore, a second δOR has not been cloned, nor has evidence for alternative splicing of the *Orpd1* gene been given. On the contrary, splice variants for δOR have been directly refuted in the human neuroblastoma cell line SK-N-BE (*52*).

It is possible that multiple affinity states for the δOR are responsible for differential agonist binding patterns. Later sections of this chapter will address the ability of the δOR to couple to multiple G-proteins and intracellular mechanisms. Other studies have proposed that δ1 is the mischaracterized δOR-κOR heteromer (*53*); work with a δOR-κOR-specific antibody has demonstrated that the δ-κ heteromer exists in rodent peripheral sensory neurons, and that there is a relationship between δOR-κOR signaling and the antinociceptive action of DPDPE *in vivo* (*54*). The existence of independent δOR subtypes presents the opportunity to develop highly-specific strategies for pain, however at the current time there does not exist solid evidence for putative δOR subtypes.

Signal Transduction

As a feature of GPCR proteins, receptors transduce signaling through a variety of heterotrimeric GTP-binding (G) proteins. The δOR is thought to primarily couple with $G_{i/o}$ pathways, as signaling is abolished by the application

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of pertussis toxin (PTX), an ADP-ribosylating agent for $G\alpha_{i/o}$ subunits (*55*). Intracellular signals by the δOR that are conserved across all opioid receptors include inhibition of adenylyl cyclase, inhibition of Ca^{2+} conductance and stimulation of K^+ channels and Na+/H+ exchange (*56*).

The ability of ligands to selectively modify or bias signaling of a receptor is an increasingly important concept for opioid therapeutic development. Ligand-biased signaling has been clearly identified for the μOR (*57*, *58*). Pradhan and colleagues have also provided evidence for biased signaling through the δOR: alkaloid agonists such as etorphine but not peptidic agonists produce βarrestin1 recruitment and antagonism of $G_{i/o}$ signaling (*59*). For similar GPCRs such as the β2-adrenergic receptor, ligand-biased inhibition of $G_{i/o}$ can be associated with agonism of the MAPK pathway (*60*). Interestingly, studies of δOR agonists in neuroprotection have demonstrated the ability of δOR to activate MAPK (*61*, *62*); future work should evaluate whether the δOR mimics the pluridimensional receptor bias of other classical $G_{i/o}$ receptors.

Modifications in receptor coupling have also been demonstrated to result from the physical interactions of the δOR with other receptors. Law and Reisine demonstrated that δOR can physically interact with PTX-insensitive G_q and G_z α-subunits (*63*), and it is thought that oligomerization events may lead to δOR decoupling from $G_{i/o}$ and recoupling to these alternative pathways. Interactions of the δOR with AMPK (*64*) and the M3 muscarinic G_q-coupled receptor (*65*) may promote coupling of the δOR to G_q, and underlie observations that δ-agonism can lead to PLCβ and PKC activation. Furthermore, the μOR-δOR heteromer has been demonstrated to preferentially couple to the G_z pathway *in vitro* (*66*). Opioid receptor activation of $G_{\alpha z}$ is thought to produce a sensitized inhibition of the adenylyl cyclase/cAMP effector pathway in a PTX-insensitive manner (*67*, *68*); *in vivo*, G_z-deficient animals demonstrate hypertolerance to morphine (*69*), leading to the hypothesis that loss of G_z coupling may underly tolerance mechanisms. Therefore, promoting receptor bias to $G_{z\alpha}$-coupling by pharmacologically stabilizing μOR-δOR interactions may present a provocative strategy for avoiding opiate tolerance. Further *in vivo* validation of δOR pathway switching and/or bias signaling is required in order to advance these hypotheses.

Regulation and Surface Expression

The activities of opioid receptor ligands appear to be dependent upon cell surface expression of the receptor. Therefore, a comprehensive understanding of δOR transcriptional regulation and trafficking facilitates the estimation of therapeutic potential and the development of pharmacological trafficking strategies.

The δOR is transcribed from the *Orpd1* gene and is under the transcriptional control of several factors including the Sp family of factors (Sp1/Sp3), which are partially controlled through NF-κB signaling (*70*, *71*). Additionally, AP-1 is a stress- and inflammatory-activated transcription factor that exerts control over *Orpd1* (*72*, *73*). Furthermore, in NG108-15 cells, transcriptional activation of δOR has been shown to occur in an activity-dependent Ca^{2+}/calmodulin fashion (*74*). These data suggest that δOR transcription may relate to stress and cell activity.

Cell surface targeting of the synthesized δOR appears to be more complicated than surface targeting for classical GPCRs. GPCRs are co-translationally translocated to the endoplasmic reticulum for folding and post-translational modification prior to golgi transport and secretory pathway expression. For the δOR, transport through the golgi and to the plasma membrane only occurs for a small fraction (40%) of receptors; the majority of δORs are retrotranslocated to cytosol, deglycosylated, ubiquitinated, and degraded by proteasomes (*75*). This differs from expression of the μOR, which is wholly subject to the constitutive secretory pathway. The concerted regulation of δOR transport to the cell surface is significant in the evaluation of δOR as a therapeutic target.

In neurons, the δOR gains additional mechanisms of trafficking and direction that may relate to its involvement in antinociception. In small nociceptive neurons of the DRG, δORs are sorted into large dense core vesicles (LDCVs) containing substance P, a nociceptive signaling peptide (Bao et al., 2003). As such, δOR is co-localized with substance P in rodent primary afferents (*76*), and nociceptive stimuli eliciting the release of LDCV contents increases δOR surface expression at the synaptic membrane in rat and monkey DRG and spinal cord (*77*, *78*). Sorting of the δOR into LDCVs is thought to result from the interaction of substance-P precursor, protachykinin, with an extracellular domain of the δOR the δOR; protachykinin acts as a chaperone to direct δOR trafficking into LDCVs. Importantly, animals lacking protachykinin show redistribution of the δOR into the constitutive secretory pathway (*76*), suggesting that peptide-receptor trafficking is a vital regulator of δOR externalization. These observations highlight endogenous and pharmacological chaperones as an increasingly important concept in δ-opioid therapeutics. Bao and colleagues demonstrated that δOR-agonists increase LDCV exocytosis and surface-targeting of the δOR (*79*), however it is unclear how this apparently pronociceptive process relates to the ability of δOR-agonists to mediate antihyperalgesia. The development of agonists with pharmacological chaperone properties represents a unique avenue for increasing the antinociceptive potential of the δOR.

Finally, it is important to consider that in target patient populations, expression of the δOR may already be increased as a result of inflammation, chronic pain and/or the prior use of chronic opiates, as previously discussed. Targeting a receptor which increases binding capacity based on pain-related conditions presents a unique opportunity to pharmacologically manage pain. Future studies examining the analgesic efficacy of δOR ligands in naïve and injured models may benefit current understandings of δOR-mediated analgesia.

Internalization and Recycling

Terminal internalization of GPCRs, especially for opioid receptors, is thought to play a large role in tolerance. There is a complex relationship between agonist treatment and the internalization of opioid receptors; most clinically relevant is how agonists determine the fate of receptors, if they are internalized upon activation, and whether receptors are degraded in a desensitizing process or recycled for resensitization.

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In mammalian systems, GPCRs can be subject to agonist-mediated phosphorylation, which facilitates the binding of β-arrestin proteins and the subsequent recruitment of clatharin and associated adaptor proteins for internalization (*80*). Phosphorylation by G-protein coupled receptor kinase (GRK) appears to be an important mechanism for δOR internalization, and can be induced by a number of δOR agonists (*81*); inhibitors of GRK abolish phosphorylation and desensitization of the δOR (*82*, *83*). The δOR C-terminus appears to be a critical site for GRK-mediated phosphorylation. Studies have highlighted Ser363 as a critical primary phosphorylation site for δOR expressed in HEK293 cells (*84*). Furthermore, deletions of the C-terminus reduce agonist-mediated down-regulation and rapid internalization of δOR (*85*, *86*). Subsets of δOR agonists are known to produce C-terminal phosphorylation, including a range of high-efficacy peptide agonists and the non-peptidic agonist, etorphine (*83*). The synthesis of novel peptides that do not direct C-terminal phosphorylation could be of therapeutic value.

As mentioned, binding of clatharin and adaptor proteins to the δOR facilitates internalization of the receptor to agonist-inaccessible compartments such as the early endosome. The early endosome is a site of receptor sorting, wherein the receptor can be directed to the late endosome and proteasome for degradation, or into recycling endosomes for surface targeting (*87*). Several proteins influence the sorting of the δOR; internalized δORs can be trafficked to the late endosome through G-protein coupled receptor associated sorting proteins (GASPs), but are primarily sorted for degradation by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery (*88*). Alternatively, sorting by Rab proteins Rab4 and Rab11 can specifically target internalized receptors for recycling endosomes. The mechanism by which agonists can direct sorting of the δOR is not well-understood. It has been hypothesized that sorting "choices" can occur as early as the recruitment of β-arrestins, and that the arrestin-bound receptor conformation directs the sorting process, but more work is required to elucidate the exact mechanism.

Our current understanding of agonist-directed trafficking for the δOR largely derives from pharmacological observations, and remains complicated due to evidence for multiple mechanisms of directed trafficking. Recent work with bioluminescence resonance energy transfer (BRET) suggests that efficacy of G-protein activation is not related to the efficacy of internalization for δOR agonists: agonists ARM390 and SNC 80 have comparable binding and analgesic properties *in vivo*, yet ARM390 does not result in internalization or acute analgesic tolerance, whereas SNC80 is associated with rapid internalization, receptor downregulation and generalized tolerance to acute doses (*89*). One hypothesis for the complex relationship between agonists and internalization is that different fundamental structures (peptidic, alkaloid) result in bound receptor conformations that are predisposed to one sorting pathway (*90*). Alternatively, Molinari and colleagues provided evidence that some selective opioid agonists also act as competitive antagonists for β-arrestin proteins to mediate differences in internalization and recycling (*91*). On-going work seeks to elucidate the way in which agonists can be designed to direct the recycling of internalized receptors and address one mechanism of tolerance.

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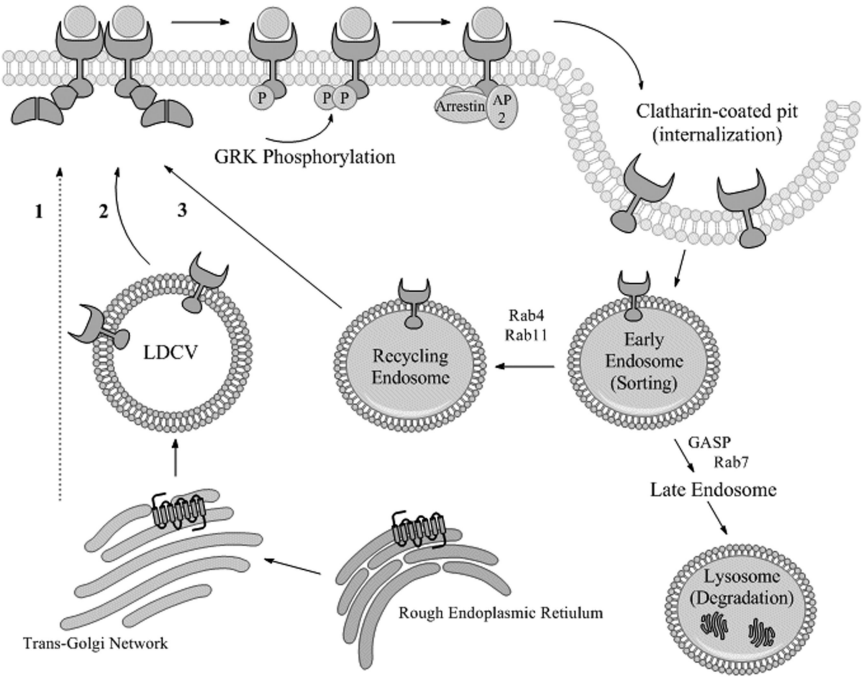

Figure 1. ***Trafficking of the δOR.*** *Surface targeting of the δOR can occur (1) through the constituitive secretory pathway for a small fraction of receptors, and alternatively in neurons through (2) co-localization with peptide transmitters in large dense core vesicles. The δOR is thought to exist at the cell surface as a contact dimer, which is subject to ligand-dependent monomerization and phosphorylation. The recruitment of arrestin and adaptor proteins to the phosphorylated δOR monomer leads to clatharin-mediated internalization and receptor sorting. δORs can be (3) recycled to the surface for resensitization, or alternatively sorted into lysosomes for degradation.*

Dimerization

The manner in which δOR dimerizes with itself and other opioid receptors is relevant to both efficacy of analgesia and tolerance mechanisms. Studies *in vitro* suggest that the δOR exists primarily as a homodimer in cells (*92*), however this finding has yet to be corroborated *in vivo*. Cross-linking experiments have provided two structurally and energetically similar δOR dimers which exist as contact-dimers (as opposed to domain-swapping dimers) via cysteine residues at TM4 and TM5 interfaces (*93*). *In vitro,* a 15-residue deletion from the C-terminal tail of δOR precludes homodimerization (*94*). The dual role of the C-terminus in dimerization and monomeric phosphorylation, as previously discussed, implies that internalization of the δOR may be dependent upon the ability of dimers to dissociate. Accordingly, Cvejic and Devi demonstrated that agonist activity at δ-homodimers *in vitro* results in monomerization prior to receptor internalization (*92*), and similar evidence has been shown for heterodimers (*95*). Therefore,

ligands which selectively stabilize monomeric or dimeric conformations may be able to promote or inhibit internalization of the δOR, respectively. Figure 1 provides a summary of δOR trafficking as discussed.

As referenced earlier in this chapter, chronic administration of μOR agonists is known to increase μOR-δOR heterodimerization in spinal and supraspinal pain areas. The μOR-δOR heterodimer has captured research attention and become increasingly well-represented in pharmacology literature. Similar to the δ-homodimer, the μOR-δOR dimer is a pairwise contact monomer with vital interactions at the C-termini: O'Dowd and colleagues identified a triglycine repeat on the δOR C-terminus and a corresponding triglycine repeat on the μOR C-terminus which equally contribute to heteromer formation (*96*).

The μOR-δOR dimer shows decreased affinity for specific δOR and μOR agonists, but enhanced affinity to endomorphin-1—a ligand with low affinity for either monomeric receptor—suggesting the formation of a novel binding pocket. Furthermore, μOR-δOR dimer mediates PTX-insensitive signaling that is thought to be $G_{z\alpha}$ (*97*), a pathway associated with abolishing tolerance to chronic μ-opiates. Additionally, heterodimer activation *in* vitro causes a slow-onset, sustained phosphorylation of ERK—a feature of δOR agonists that has been related to neuroprotective mechanisms in neurons (*98*). As such, the identification of novel μOR-δOR-specific ligands represents an important possibility for analgesic development.

Finally, δOR antagonists inhibit internalization and trafficking but not signaling of the μOR-δOR heteromer (*99*). As such, co-administration of a δOR antagonist and μOR agonist uncouples dimer signaling from $G_{z\alpha}$ signaling and leads to classical opioid $G\alpha_{i/o}$ signaling. Future work should evaluate the biochemical dynamics that mediate ligand-directed receptor interactions and pathway coupling of opioid receptors.

Therapeutic Applications

δOR-mediated Analgesia

This chapter has evaluated a range of possibilities for producing analgesia through the δOR. Based on the physiological and biochemical characteristics of the receptor, δOR-centric therapeutics should seek to provide analgesia for chronic, neuropathic and inflammatory pain, as well as seek to treat patients with tolerance to μ-opiates. Furthermore, the potential for δOR agonists to circumvent classical μ-opiate side effects including respiratory depression and constipation make δOR an attractive target. Finally, the novel trafficking properties of the δOR may be well-suited for pain management, and provide non-classical target sites (eg. trafficking chaperones). The following sections will briefly summarize what is known regarding the pharmacological use of δOR ligands in the context of nociception.

Whereas clinical opioid analgesics predominantly act at the μOR site, the distribution of the δOR in central pain pathways and on peripheral afferents has provoked pharmacologists to incorporate δOR-targeted strategies into novel pain therapies (*100*). The ability of specific δOR agonists to mediate antinociception

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is well documented (*20–22*); furthermore, δOR agonists can produce analgesia when administered in only the periphery, or supraspinally (*101*). δOR agonists have also been demonstrated as efficacious in models of cancer pain (*102*) and chronic inflammatory pain (*103*). Lastly, the development of novel δOR agonists such as met-kephamide (*104*) have demonstrated the potential of δOR agonists supercede the analgesic efficacy and/or potency of μ-opiates.

The use of antisense oligonucleotides for δOR abolishes specific δOR agonist-mediated nociception, but fails to affect the antinociceptive effects of μOR or κOR selective agonists, suggesting that δOR agonism alone is sufficient to provide antinociception (*105–108*). Importantly, specific δOR agonists do not require functional μORs to mediate antinociception (*109*, *110*), such that δ-agonism may be useful in opiate-tolerant patients. In agreement with this hypothesis, Roerig and colleagues demonstrated the analgesic efficacy of δOR agonists in states of μOR agonist tolerance (*111*).

Like the μOR, δORs act at both spinal and supraspinal sites, suggesting that δOR agonists may have potential to act synergistically within pain pathways. The concomitant spinal and supraspinal administration of DPDPE in rats has demonstrated synergistic antinociceptive action (*112–114*). Similarly, spinal-supraspinal synergy was reported for [Ala^2,Glu^4]deltorphin (*115*).

δOR analgesic compounds have been demonstrated to produce fewer side effects than conventional μOR analgesics (*116*); preclinical evaluations have identified δOR agonists with reduced addictive potential (*117*, *118*), and without classical μ-opioid side effects including respiratory depression (*119*) and constipation (*120*). DPDPE produces antinociception without GI or straub tail phenomenon attributed to mu-selectivity (*20*, *21*). Furthermore, a novel δOR agonist, JNJ-20788560, efficaciously produces analgesia without respiratory depression, pharmacological tolerance or physical dependence (*118*).

In spite of the strong foundation of evidence for the use of novel δOR analgesics in the clinic, the progress of development has not been simple. Early studies evaluating the clinical prospect of δOR agonists were marred by evidence of convulsant side effects for the non-peptidic δOR agonist SNC-80 (*121*, *122*); convulsant action was determined to have a lower efficacy requirement than antinociception, which further hindered the popularity of δOR agonists in pain management. Upon closer examination, it appears that not all δOR agonists have anti-convulsant activity, and it is unclear whether convulsion relates to interactive effects with the μOR (*123*), or agonist-directed signaling by SNC-80 (*124*). More recent development of specific non-convulsant δOR agonists including DPI-3290 has allowed δOR ligands to regain popularity as a potential therapeutic agent.

δOR Co-Therapies

Perhaps the most realistic and clinically useful concept for the use of δOR agonists in the management of pain is as a co-therapeutic with μOR agonists. A significant body of work suggests that concomitant administration of δOR agonists can enhance μOR-mediated analgesia. Vanderah and colleagues provided endogenous evidence for this hypothesis by showing that stress-associated enhancements in morphine potency may be due to increases in enkephalin

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activity at the δOR (*125*). Historically, a wealth of literature suggests that δOR ligands may be used to increase the potency and efficacy of μOR agonists (*126–129*). More recent work has directly demonstrated that analgesia produced by combined μOR and δOR agonists is synergistic (*130*) and reduces tolerance (*131*). Furthermore, the use of opioids in conjunction with non-steroidal anti-inflammatory drugs (*132*) or novel analgesics including cholecystokinin antagonists (*133*) has significant potential for synergism. Targeting the δOR as a cotherapy may aid the transition of δ-ligands into the clinical setting and increase the efficacy of novel δOR strategies.

Finally, it is important to comment on the use of δOR antagonists. As previously discussed, δOR-deficient mice show resistance to the development of μ-opiate tolerance. Development of clinical tolerance mandates dose escalation in order to maintain analgesic effects, which increases the likelihood of adverse side-effects such as nausea, constipation and respiratory depression. Rapid development of opiate tolerance is also thought to underlie the analgesic inefficacy of opiates in neuropathic pain (*134*). The use of δOR antagonists may provide a novel approach to circumventing tolerance. Studies utilizing μOR and δOR agonists have also combined μOR agonists with δOR antagonists, and demonstrated a marked reduction in the development of tolerance and physical dependence (*135*, *136*). Furthermore, the use of δOR antagonists successfully reverses tolerance to μ-opiates in animal models (*136*). It is known that δOR antagonist receptor occupation is sufficient to enhance the binding of the μOR and signaling activity in vitro (*137*), and may be one explanation for observed tolerance reversal by δOR agonists. However, based on the knowledge that chronic μOR agonist administration can increase the heterodimerization of μOR-δOR, and we have discussed the ability of δOR antagonists to promote classical μOR signaling from the μOR-δOR heterodimer, future studies should also investigate the specific role of the μOR-δOR interaction in tolerance reversal.

Targeting μOR-δOR Heterodimers

The formation of the μOR-δOR heterodimer is abundant in both spinal and supraspinal areas involved in nociceptive processing (*138*, *139*). Furthermore, it is hypothesized that the ability of the μOR-δOR heterodimer to selectively couple to $G_{\alpha z}$ signaling may circumvent mechanisms of tolerance. However, an important question is whether it is possible to selectively target and stabilize the μOR-δOR heterodimer. While several conventional μOR and δOR ligands have been shown to bind and elicit signaling from the μOR-δOR heterodimer, it is hypothesized that a pharmacologically distinct binding pocket is generated by the interaction of μOR-δOR and the G_z complex. So far, the benzomorphan-based ligand LP1 has been identified as one such ligand which specifically binds and activates the μOR-δOR dimer; comparisons of LP1 to morphine in rodent models of chronic pain show that LP1 has analgesic efficacy, but is not subject to tolerance (*131*, *140*). μOR-δOR agonists are relatively new on the therapeutic horizon and warrant investigation in order to benefit not only the evaluation of μOR-δOR as a therapeutic target, but also to enhance our current understanding of the complex interactions between opioid receptors.

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Conclusion

The enormous potential of the δOR in analgesic development is largely untapped, and necessitates further research and evaluation as both a biological receptor and a therapeutic target. The distribution of the δOR in both ascending and descending pain pathways, both spinally and supraspinally, suggests that multi-site synergism of δOR agonism may compete with that of μOR agonism if targeted properly. The unique biochemical characteristics of the δOR present opportunity for innovative pharmacotherapies including pharmacological chaperones to increase availability of target opioid receptors, as well as the prospect of designing molecules specific for unique conformations and oligomeric states of the receptor. Finally, targeting the δOR as a co-therapy may provide a tool for amplifying current and upcoming analgesic therapeutics. Additional chapters in this textbook will address the usefulness of multifunctional ligands which act at multiple receptors to increase analgesic efficacy and reduce unfavorable side effects; the δOR is an excellent candidate for such multifunctional strategies. Understanding and further evaluating the multi-faceted potential of the δOR is vital to the progression of opioid analgesics and pain managment.

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Chapter 13

Kappa Opioids: Problems and Opportunities in Analgesia

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KOP-r have been studied as potential targets for novel analgesics for a considerable period of time. Early studies showed that acutely administered high efficacy centrally-penetrating KOP-r agonists were problematic due to considerable central side effects, including dysphoria and psychotomimesis. Current opportunities for KOP-r ligands in analgesia rest primarily on agonists with high peripheral selectivity in humans, to avoid the aforementioned central side effects. More recent preclinical studies show that the KOP-r / dynorphin system is upregulated in response to stress, or to certain pain conditions, in neuroanatomical areas mediating mood, reward and emotion. Such upregulation may result in neuropsychiatric states including dysphoria, anxiety or depression, which can accompany severe or chronic pain states. Blockade KOP-r with novel selective antagonists may therefore offer an opportunity to reduce the burden of morbidity or suffering in such pain states.

Brief Overview and State of the Field

KOP-r receptors are widely distributed in the central and peripheral nervous systems (CNS and PNS), and modulate sensory, perceptual, autonomic and neuroendocrine function (*1*, *2*), through their activation by the endogenous neuropeptide high-efficacy agonists, the dynorphins (*3*–*5*). Since the discovery of heterocyclic selective KOP-r agonists, there has been interest in their potential as analgesics without the main side effects of MOP-r agonist prescription analgesics

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such as morphine (especially constipation, respiratory depression, pruritus, abuse potential). However, early studies found that acute doses of these KOP-r agonists produced dose-dependent and reversible psychotomimetic, dysphoric and sedative effects (*6–8*). These undesirable effects have been an insurmountable obstacle for study and progression of centrally-penetrating KOP-r agonists, for pain-related indications. More recent studies and approaches described below support a continued interest in the role of peripheral KOP-r in analgesia (*9*), in central KOP-r blockade for the treatment of pain-related morbidity (e.g., dysphoria and depression) (*10*), and in the pharmacotherapy of addictions to illicit drugs and prescription analgesics (*11*).

Basic Neuroscience of the KOP-r / Dynorphin System, of Relevance to Pain-Related Indications

KOP-r (encoded by gene *OPRK1* in humans) are 7-transmembrane domain G_i/G_o -coupled receptors, widely distributed in CNS, and dorsal spinal cord. KOP-r can mediate perceptual/sensory mechanisms, and also neuroendocrine function (including activation in the HPA axis, and prolactin release) (*1*, *2*, *12*, *13*). In preclinical models, KOP-r mediated antinociception can be detected in various assays thought to be mediated by spinal and supra-spinal sites (*14–17*), and also by activation of KOP-r located in the PNS (*18–21*), by acting directly or indirectly on primary afferent signals (*22*, *23*).

Of relevance to potential undesirable effects of centrally-penetrating high efficacy KOP-r agonists, KOP-r are present in several cortical, nigrostriatal and meso-limbic areas (*1*), potentially mediating perception, cognition, mood, anxiety and reward. For example, KOP-r are present in the nigrostriatal and meso-limbic dopaminergic pathways (*1*, *2*), where they counter-modulate dopaminergic activation, critical for natural homeostasis of mood and reward (and also drug-induced reward) (*24–26*). It is generally postulated that activity at these or other supraspinal CNS sites by high efficacy KOP-r agonists mediates the perceptual distortions, psychotomimetic effects, anhedonia and dysphoria and sedation observed in human studies, in non-human primates, and in rodent models (e.g., depressant-like, sedative-like and aversive effects) (*7*, *15*, *27–29*). Of interest, one KOP-r agonist, nalfurafine [TRK-820], has been approved for clinical use (anti-pruritus) in Japan (*30–33*). It would be of interest to determine whether the pharmacodynamic (*33*, *34*) or pharmacokinetic qualities of nalfurafine vs. other KOP-r agonists (or the requirements of pruritus pharmacotherapy vs. those of pain pharmacotherapy) (*35*) underlie the clinical effectiveness of this ligand.

Centrally-Penetrating High Efficacy Agonists in Humans

Studies indicated dose-limiting central side effects of KOP-r agonists in human analgesia assays, as mentioned above (*8*). The profile of these effects (e.g., sedation, psychotomimesis, dysphoria) is consistent with acute effects of centrally-penetrating high efficacy KOP-r agonists in non-pain related clinical studies (*6*, *7*, *28*, *36*).

Thus, clinical development of **high efficacy centrally-penetrating KOP-r agonists for pain indications** has not been the focus of recent studies or publications, to our knowledge. It is currently unknown whether the aforementioned undesirable effects would be ameliorated by "tapering up" KOP-r agonist doses slowly. Likewise, it is unknown whether differential tolerance (to undesirable vs. analgesic effects) would occur, to reveal an actual "therapeutic window" for this approach. Studies have not explored to date whether there are subsets of patients who have a differential pre-existing sensitivity to sedative/psychotomimetic effects vs. analgesic effects of KOP-r agonists, based on genetic polymorphisms at *OPRK1* (the gene encoding the KOP-r target). *ORPK1* polymorphisms have been associated with differential clinical characteristics, in other fields, especially the addictions (*37*, *38*).

Peripheral KOP-r Receptors - A Further Target for Analgesia

Activation of KOP-r in the PNS can mediate antinociceptive effects in certain models, particularly involving anti-hyperalgesia or anti-allodynia (*18*, *19*, *21*, *22*, *39*). Therefore, a number of groups have followed the postulation that a peripherally-selective KOP-r agonist would produce analgesic effects, with a reduced burden of centrally-mediated KOP-r related activation (*40*, *41*).

Of note, the shortened natural sequence KOP-r agonist peptide dynorphin A(1-13) (administered i.v.) is devoid of substantial negative subjective effects in humans, likely due to its relative ineffectiveness in penetrating into the CNS. However, dynorphin A(1-13) is able to produce KOP-r mediated neuroendocrine biomarker effects which are mediated outside the blood-brain barrier (i.e., prolactin release) (*42–44*). Prolactin release has in fact been used successfully as a quantitative biomarker for KOP-r mediated effects in human clinical trials of novel pharmacotherapeutic agents (*45*, *46*).

Candidate peripherally selective KOP-r agonists have been studied, based on synthetic peptide structures; positive clinical results have been obtained to date, in particular pain modalities (e.g., visceral pain) (*9*, *47*).

In general, there may be a potential opportunity for further study in this area, in that species differences (e.g., human, non-human primate or rodent) have been reported in blood-brain barrier passage for a given ligand, or in active transport mechanisms, such as the p-glycoprotein ABCB1 efflux transporter (*48–50*). Thus, appropriate modeling of BBB passage across appropriate species (or *in vitro* systems) may be approached early in the development process, to optimize lead compound selection, for compounds with maximal potential peripheral selectivity in humans.

The KOP-r System as an Adjunct Analgesic to MOP-r Agonist Approaches

The KOP-r system, when activated by its endogenous neuropeptide agonists (the dynorphins), or by exogenous ligands, can act in a manner opposite to that of classic MOP-r agonists. For example, MOP-r agonists and other compounds

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with abuse potential (e.g., cocaine) tend to increase dopamine dialysates in dorsal and ventral striatum, whereas KOP-r agonists tend to have an opposite effect (*51–53*). Other undesirable effects of MOP-r agonists, observed in the clinical context of analgesia, such as pruritus, are also blocked by KOP-r agonists (*33*, *54*). Crucially this desirable effect of KOP-r agonists occurs at doses that do not cause sedation (*54*). Of note, translational data in non-human primates have revealed that co-administration of small intrathecal doses of a KOP-r agonist blocked MOP-r agonist-induced pruritus, but not MOP-r induced analgesia (*31*). Thus appropriate administration of KOP-r agonists may be considered as an opportunity to decrease some common undesirable effects of classic MOP-r analgesics. *Another chapter in this book (by Dr. J. Bidlack) focuses on the exciting possibility of bivalent MOP-r/KOP-r analgesics to exploit the divergent actions of these two receptor systems.*

Sex Difference in KOP-r Analgesia

Several clinical and preclinical papers have pointed to sex-differences in KOP-r mediated analgesia (*14*, *55–57*). These illustrate the opportunity of improved prescription of analgesics based on sex-specific pharmacology. As a potential obstacle in the interpretation of the cross-species profile of these sex differences, is a lack of selective KOP-r compounds available for studies in humans. Thus, clinically approved compounds such as pentazocine, nalbuphine and butorphanol have intermediate pharmacodynamic efficacy (partial agonism) at KOP-r, and also differing efficacy at MOP-r, with limited binding selectivity (*4*, *5*, *58*). A potential opportunity would therefore be investigation of sex-specific clinical analgesia with more KOP-r selective ligands, particularly more selective KOP-r partial agonists (were they to become available). KOP-r partial agonists would be expected to have a relatively smaller incidence of the aforementioned undesirable effects of high efficacy centrally mediated KOP-r agonists.

The Endogenous KOP-r/Dynorphin System as a Target in Neuropsychiatric States Secondary to Chronic Pain (i.e., Dysphoria, Depression, Anxiety)

A tenet of modern analgesia is that clinical pain states are composed of nociceptive sensory/perceptual components, and also of emotional/psychiatric components. These latter components can strongly affect the suffering, distress and morbidity that the patient may undergo. Thus, major chronic pain states are associated with sequelae such as dysphoria, depression and anxiety, that may in themselves decrease quality of life (*59–62*).

Preclinical studies show that exposure to stress (*63–66*), or to certain types of pain (*10*, *67*, *68*), or to chronic MOP-r agonists (*69*, *70*), can result in upregulation in "tone" in the KOP-r/dynorphin system (or *Pdyn* mRNA) in supraspinal sites. Several preclinical lines of evidence also show that increased KOP-r activation (including activation by endogenous dynorphins at specific supraspinal sites) can cause aversion/dysphoria, and depression-like or anxiety-like behaviors (*66*,

71–74). Furthermore, KOP-r antagonism can block such effects (*66*, *75–77*). Thus a current opportunity in the field is blockade of central (likely supraspinal) KOP-r for the management of such components of clinical pain states.

At a translational level, novel heterocyclic KOP-r antagonists have been recently developed, and have even reached clinical stages of development (*45*, *78*). Therefore, pharmacological tools may be available in the foreseeable to future, to test the hypothesis that blockade of central KOP-r may ameliorate these comorbid neuropsychiatric sequeleae of pain states.

Summary

The KOP-r / dynorphin system has been, since its discovery and characterization, a target in the development of analgesics. High efficacy centrally penetrating KOP-r agonists have considerable shortcomings as analgesics, due to their central side effects, which include dysphoria and psychotomimesis. Current opportunities for KOP-r ligands in analgesia are focused primarily on compounds with high peripheral selectivity in humans, studied in clinical pain states that may benefit from such activity (e.g., those including inflammatory or visceral components). Actions of selective KOP-r partial agonists (which can be postulated to have lesser undesirable central effects) remain understudied due to the relative lack of clinically available ligands. A further current opportunity exists in the blockade of supraspinal KOP-r sites, as a means to block neuropsychiatric morbidity (e.g., dysphoria, depression, anxiety) that accompanies certain chronic or severe pain states in humans.

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Chapter 14

Mixed Mu/Kappa Opioid Agonists

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Partial μ/κ opioid agonists produce analgesia and may have fewer side effects than full μ or κ agonists. Also, a ceiling effect has been observed with many μ/κ partial agonists, which may account for attenuation in side effects. Many benzomorphans, including cyclazocine and pentazocine, are mixed μ/κ partial agonists. Recent efforts have been focused on the synthesis of long-acting benzomorphans. Aminothiazolomorphinans are an example of novel morphinans having mixed κ and μ activity. The recognition of receptor dimerization and that μ and κ monomeric opioid receptors may be in close proximity lead to the synthesis of bivalent ligands. The two pharmacophores comprising the bivalent ligands determine their μ/κ pharmacological properties. Bivalent opioid ligands represent a relatively new class of opioid ligands.

Mixed Mu/Kappa Partial Agonists Overview

Partial agonists range from having an efficacious agonist component to being primarily an antagonist with a very small agonist component. Some compounds may be primarily an agonist or antagonist at one receptor and be a partial agonist at another opioid receptor. For example, nalmefene is primarily an antagonist at the μ opioid receptor, but is a partial agonist at the κ receptor, exhibiting both agonist and antagonist properties (*1*). In contrast, ketocyclazocine is a partial agonist at both κ and μ opioid receptors (*2*). Its κ agonist properties were first recognized, and the κ opioid receptor was named for its affinity for ketocyclazocine (*3*). Compounds that act as both μ and κ partial agonists range from having equal affinity and the same efficacy at both receptors to having a much higher affinity and/or efficacy for one receptor over the other receptor. Thus, each mixed μ/κ

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agonist has its own unique pharmacological properties depending on its interaction with both receptors. Likewise, opioid receptors may be monomeric, homodimers, or heterodimers, as well as oligomers (*4*).

Kappa opioid receptors are found in pain-relaying neurocircuitry alongside μ opioid receptors. The close anatomical distribution of these two types of opioid receptors suggests possible physiological interactions, particularly in nuclei-relaying nociceptive stimuli (*5–7*). Mu and κ opioid receptors in the spinal cord form heterodimers (*8*). Gintzler and colleagues (*9*) have hypothesized the monomeric κ opioid receptors mediate nociception, whereas κ opioid receptors that heterodimerize with μ opioid receptors mediate antinociception (*9*).

In the 1950s, it was recognized that some antagonists of the effects of morphine could be agonists themselves. Lasagna and Beecher (*10*), and Keats and Telford (*11*) showed in humans that nalorphine (*N*-allyl-normorphine) antagonized the analgesic effects produced by morphine, and produced analgesia when administered by itself. Isbell reported that when nalorphine was given with morphine or 1¾ hours after morphine, nalorphine blocked the euphoric and miosis effects of morphine, but not the morphine-induced depression in body temperature or respiratory depression (*12*). These studies were some of the earliest reports of the use of mixed μ/κ agonists and antagonists as analgesics.

Table 1 summarizes the desirable and undesirable properties of μ and κ opioid agonists. There have been relatively few κ analgesic studies in humans because of dose-limiting side effects associated with the full κ agonists, such as enadoline (*13*). The μ and κ components of mixed μ/κ agonists may be able to balance or complement each other. For example, the euphoric and sense of well-being associated with μ agonists may be able to offset the dysphoria associated with κ agonists. Mu opioids have the potential for abuse, while κ opioids do not have abuse potential. While μ agonists produce pruritus, κ agonists have anti-pruritus effects. The mixed μ/κ opioids have the potential to combine optimal components to minimize the undesirable effects associated with the μ and κ opioid receptors.

Efficacy As Measured in Second Messenger Assays

Second messenger assays, such as the [^{35}S]GTPγS binding assay and adenylyl cyclase activity, are methods that allow for the thorough characterization of the efficacy of compounds. Compounds can range from full agonist activity such as fentanyl and DAMGO (*14*) to partial agonists, such as nalmefene (*1*), to antagonists such as the κ-selective antagonist, nor-binaltorphimine (nor-BNI). Efficacy is a graded continuum. Many compounds are partial agonists in second messenger assays, but some are not recognized as partial agonists *in vivo*. For example, Figure 1 shows the effect of naloxone in the [^{35}S]GTPγS binding assay using membranes from Chinese hamster ovary (CHO) cells expressing the human κ opioid receptor. Figure 1A shows that naloxone stimulated [^{35}S]GTPγS binding with a B_{max} value of 44%. Naloxone also inhibited U50,488-stimulated [^{35}S]GTPγS binding as shown in Figure 1B. In the second messenger assays, agonist properties are observed at lower concentrations than antagonist properties. From *in vivo* studies, relatively few investigators view naloxone as a partial κ

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agonist. Most of the traditional opioids were labeled as agonists or antagonists based on their *in vivo* properties, often obtaining these classifications before the μ, δ, and κ opioid receptors had been identified molecularly. Instead, the efficacy of a compound should be viewed as a continuum from very efficacious to having no efficacy in a given assay. As the late Professor Sydney Archer used to say, "Antagonists are just lousy agonists" (Bidlack, personal communication).

The terms full agonist, partial agonist, and antagonist are categorical descriptions. However, there is a wide continuum with regard to the efficacy of partial agonists, ranging from having just a little efficacy, such as 20% of less stimulation in the [^{35}S]GTPγS binding assay to having at least 80% of the efficacy of a full agonist such as DAMGO for the μ receptor and U50,488 for the κ receptor. Buprenorphine, a medication used to treat opioid dependence and pain, has been shown to be a partial agonist at both μ and κ opioid receptors as measured by the [^{35}S]GTPγS binding assay (*15*). Buprenorphine has been shown to be a low-efficacy opioid in humans (*16*). *Two other chapters in this book (by Dr. G. Hans and by Dr. Husbands) discuss the use of buprenorphine in patients with neuropathic pain, and on the development of novel orvinols related to buprenorphine.* With buprenorphine being an exception, the analgesic efficacy of most opioids has not been determined in humans (*17*). The lack of knowledge about clinical analgesic efficacy is due to the problem that large-scale clinical trials to determine relative analgesic efficacy are difficult to conduct (*17*).

Table 1. The *In Vivo* Desirable and Undesirable Properties of Mu and Kappa Opioid Agonists

	Desirable Properties	***Undesirable Properties***
Mu Agonists	Analgesia- thermal, mechanical, somatic stimuli Euphoria	Abuse Potential Tolerance Dependence Sedation Respiratory Depression Constipation Pruritus
Kappa Agonists	Analgesia- visceral chemical stimuli Anti-Pruritus	Dysphoria Sedation Psychotomimesis Diuresis

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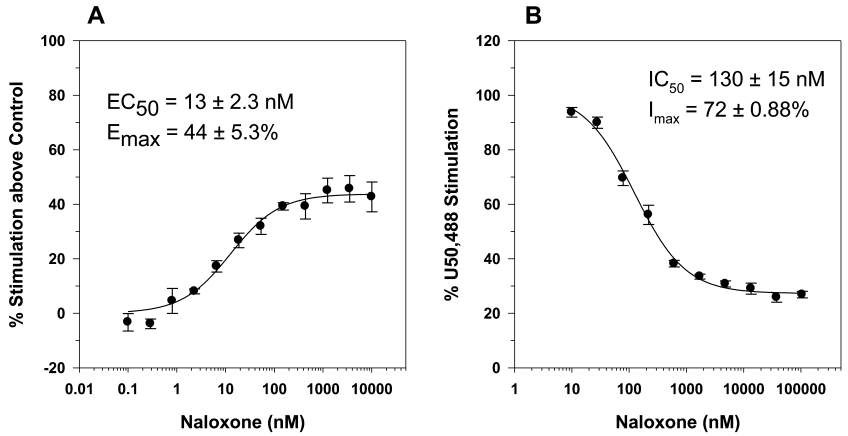

Figure 1. ***Effect of Naloxone alone (A) and in the presence of the κ agonist U50,488 (B) on [^{35}S]GTPγS binding in membranes from CHO cells expressing the human κ opioid receptor alone.*** *[^{35}S]GTPγS binding was measured as previously described (18). Basal [^{35}S]GTPγS binding, measured in the presence of 10 μM GTPγS, was set at 0%. To detect antagonist activity, [^{35}S]GTPγS binding was stimulated with 100 nM U50,488.*

Benzomorphans

The benzomorphan series is comprised of numerous compounds, including pentazocine, cyclazocine, phenazocine, and dezocine. Archer and colleagues reported on the synthesis and pharmacological properties of phenazocine and three derivatives, which antagonized analgesic effects of morphine and meperidine (*19*). Pentazocine, a mixed μ/κ partial opioid agonist was approved as an analgesic by the FDA in 1967 and has been used as an analgesic for almost 50 years (*20*, *21*). The (-)enantiomer of pentazocine binds to μ and κ opioid receptors (*22*), while (+) pentazocine binds to σ receptors, which do not mediate analgesia (*23*). Both μ and κ opioid receptors contribute antinociceptive effects to somatic, as well as, visceral pain induced by pentazocine in animals (*22*, *24*). Recently, pentazocine-induced antinociception was shown to be mediated primarily by μ opioid receptors in mice (*25*). Pentazocine mediated analgesia induced by a thermal, mechanical, or somatic chemical stimulus was abolished in μ-opioid receptor knockout mice, but the visceral chemical analgesic effects of (-) pentazocine were retained (*26*). In humans, pentazocine has been shown to produce μ-like subjective effects that were antagonized by naltrexone (*27*). The analgesic effects of pentazocine have been postulated to be mediated by the κ opioid receptor in humans (*28*). However, it is not clear that pentazocine does not produce some analgesia mediated by the μ opioid receptor.

With the benzomorphans, the group attached to the basic nitrogen contributes substantially to the affinity of the benzomorphan for certain types of opioid receptors. Benzomorphans with an *N*-methyl substituent have highest affinity for

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the μ opioid receptor. Changing from an *N*-methyl to an *N*-cyclopropylmethyl (*N*-CPM) or *N*-cyclobutylmethyl (*N*-CBM) group results in compounds with higher affinity for the κ receptor. This substitution also usually converts a μ full agonist to a μ partial agonist. For example, the benzomorphan cyclazocine is a μ partial agonist and a κ full agonist (*29*, *30*). Table 2 shows the structures and pharmacological properties of some benzomorphans. While both pentazocine and cyclazocine have a similar receptor-preferring profile based on receptor binding assays, cyclazocine had a 60-110- fold greater affinity for the μ, κ, and δ opioid receptors than pentazocine (*22*). Potent analgesia was observed in humans dosed with cyclazocine, and after abrupt cyclazocine cessation, patients did not display drug-seeking behavior (*31*). Cyclazocine produced dysphoric side effects and a short duration of analgesic activity, which lead to a discontinuation of clinical trials, undoubtedly due to its κ agonist properties (*29*, *31*, *32*). The short duration of action produced by cyclazocine in animals and humans may be due to *O*-glucuronidation; this metabolite was observed in humans (*32*) and animals (*33*, *34*).

In an attempt to retard *O*-glucuronidation of cyclazocine, the 8-OH group of cyclazocine was replaced with a NH_2 group (*30*, *35*), and subsequently with a $CONH_2$ group to produce 8-carboxamidocyclazocine (8-CAC) (*26*). 8-CAC retained most of the high affinity properties that cyclazocine possessed (*36*). In the mouse warm-water tail withdrawal assay, both 8-CAC and cyclazocine produced analgesia that was mediated by both μ and κ receptors (*37*). After an i.p. injection, the duration of antinociception produced by cyclazocine lasted for less than 2 hr, while 8-CAC produced antinociception in the mouse writhing assay for up to 14 hr (*37*). Both cyclazocine and 8-CAC partially inhibited morphine-induced antinociception (*37*).

A $CONH_2$ group has been added at the C-8 position of the benzomorphans pentazocine, metazocine, phenazocine, Mr2034, ketocyclazocine, and ethylketocyclazocine (*38*). The affinities of these benzomorphan derivatives for the μ and κ receptors either remained the same or, particularly in the case of phenazocine, the $CONH_2$ derivative had a 4- to 10- fold greater affinity for the μ and κ receptors, respectively (*38*). The parent compound and the $CONH_2$ derivative of the parent benzomorphan retained similar pharmacological properties as measured in the [^{35}S]GTPγS binding assay (*38*). The benzomorphan structure containing either an *N*-CPM or *N*-CBM group represents a core structure that has high affinity for both μ and κ opioid receptors.

Morphinans

Morphinans are often thought of as μ-preferring compounds. However, like the benzomorphans, the preference for one type of opioid receptor over another often depends on the group attached to the basic nitrogen group. Morphinans with an *N*-methyl group comprise the compounds that have higher affinity for μ opioid receptors than any other opioid receptor. The *N*-methyl group also confers agonist properties to the μ-preferring morphinan. As with the benzomorphans, the

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addition of an *N*-CPM or an *N*-CBM group increases the affinity of the morphinan for the κ opioid receptor. Table 3 shows the structures and pharmacological properties of some morphinans. Of the classical opioids, nalbuphine has similar high affinity for the μ and κ opioid receptors, but its affinity for the δ opioid receptor is approximately 200-fold lower than its affinity for either the μ or κ opioid receptor (*39*). Clinically, nalbuphine has been regarded as a μ antagonist and a κ agonist (*40*). In monkeys, nalbuphine produced antinociception in only some subjects and only when the water temperature of the tail withdrawal assay was at or below 50°C, suggesting that nalbuphine was a low efficacy or partial μ agonist (*41*). Nalbuphine was a partial agonist at the μ opioid receptor as measured by [^{35}S]GTPγS binding (*39*). Its antagonist properties at the μ receptor were greater than its agonist effects. Nalbuphine increased [^{35}S]GTPγS binding mediated by the μ receptor by only 26% (*39*). At the κ opioid receptor, nalbuphine did not stimulate [^{35}S]GTPγS binding as much as the full agonist U50,488 (*39*, *42*), but nalbuphine inhibited U50,488-stimulated [^{35}S]GTPγS binding by less than 20%. Nalbuphine was a partial agonist for the β-arrestin-mediated pathway activated by the κ opioid receptor (*42*). Nalbuphine has been an opioid of choice when the properties of mixed μ/κ opioids were studied in behavioral assays (*43*). Like nalbuphine, nalmefene has a high affinity for the μ and κ opioid receptors. Its affinity for μ and κ receptors was approximately 20-fold higher than its affinity for the δ opioid receptor (*39*). Clinically, nalmefene was recognized to have κ agonist properties when it was discovered that nalmefene increased prolactin levels in humans (*1*). Nalmefene was an antagonist at the μ receptor and a partial agonist at the κ receptor as measured by [^{35}S]GTPγS binding (*1*).

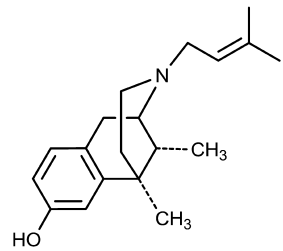
Naltrexone has been regarded primarily as a μ and κ antagonist, with lower affinity for the δ opioid receptor. While naltrexone is primarily an antagonist at the μ receptor, with less than 20% stimulation of [^{35}S]GTPγS binding, naltrexone is a partial agonist at the κ receptor (*38*). Naltrexone's κ agonist properties may account for why some patients experience adverse effects when taking naltrexone.

Cyclorphan Derivatives

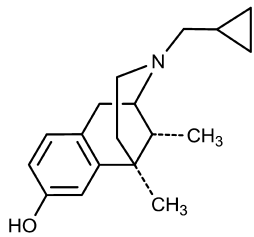
Open-ring morphinans such as cyclorphan (*44*) and butorphanol analogues have been synthesized as mixed μ/κ opioids (*45*). Table 2 shows the structures of cyclorphan and its derivative butorphan. Cyclorphan had high affinity at κ and μ opioid receptors (*18*, *45*). Cyclorphan has a 75-fold lower affinity for the δ receptor than the κ receptor (*18*). Cyclorphan, which contains a *N*-CPM, and butorphan (MCL-101), which contains an *N*-CBM were μ partial agonists, exhibiting both agonist and antagonist properties (*45–47*). Likewise, cyclorphan and butorphan (MCL-101 were κ full agonists (*45*, *46*). Butorphanol and butorphan are quite similar with butorphanol having a hydroxyl group at C-8, while butorphan and cyclorphan have a H atom. Both cyclorphan and butorphan produced antinociception that was mediated by μ, κ, and δ opioid receptors. However, both compounds only antagonized antinociception mediated by the μ opioid receptor (*45*). The open ring structure of cyclorphan is similar to the

morphinan butorphanol, which had the highest affinity for the μ and κ receptors, and 100-fold lower affinity for the δ receptor (*18*). Butorphanol is a partial agonist at the μ receptor and a full agonist at the κ opioid receptor (*18*). The pharmacological properties of butorphanol were not changed when a $CONH_2$ group was added at *C*-3 position (*18*).

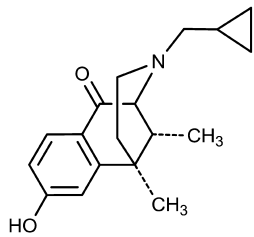
Table 2. Structures and Pharmacological Properties of Some Benzomorphans as Measured by [^{35}S]GTPγS Binding



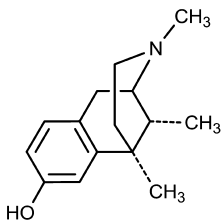
Pentazocine



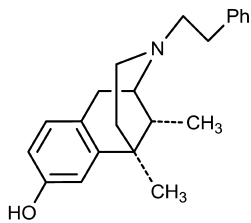
Cyclazocine



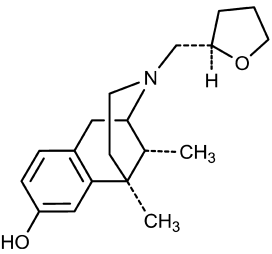
Ketocyclazocine



Metazocine



Phenazocine



Mr2034

	Mu Opioid Receptor	Kappa Opioid Receptor	
Compound	**Partial Agonist**	**Full Agonist**	**Antagonist**
Pentazocine[a]	Yes	Yes	No
Cyclazocine	Yes	Yes	No
Ketocyclazocine	Yes	Yes	No
Metazocine	Yes	Yes	No
Phenazocine	Yes	Yes	No
Mr2034	Yes	Yes	No

Data for the [^{35}S]GTPγS binding results using membranes from CHO cells expressing either the human μ or κ opioid receptor are from Wentland et al. (38). With the benzomorphans, κ partial agonist activity has not been observed. [a]Pentazocine data are unpublished results (Bidlack and Knapp). A partial agonist both stimulated [^{35}S]GTPγS binding and inhibited agonist-stimulated [^{35}S]GTPγS binding.

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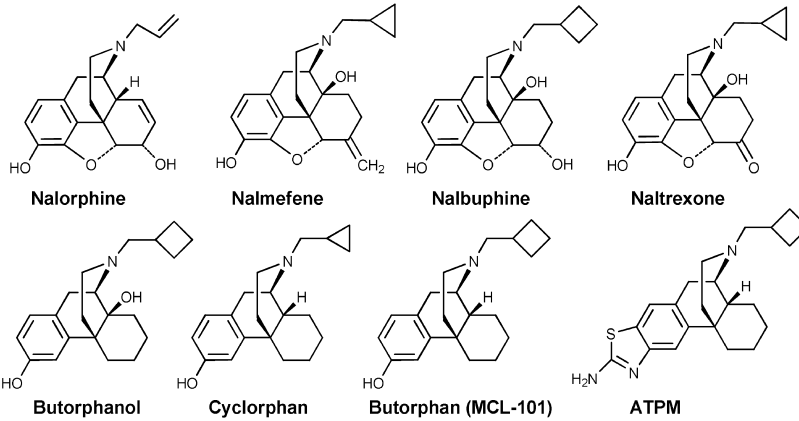
Table 3. Structures and Pharmacological Properties of Some Morphinans Measured by [^{35}S]GTPγS Binding



	Mu Opioid Receptor		Kappa Opioid Receptor		
Compound	**Partial Agonist**	**Antagonist**	**Full Agonist**	**Partial Agonist**	**Antagonist**
Nalorphine[a]	Yes	---	---	Yes	---
Nalmefene[b]	No	Yes	---	Yes	---
Nalbuphine[c]	Yes[e]	---	---	Yes	---
Naltrexone[c]	Yes[e]	---	---	Yes	---
Butorphanol[a]	Yes[e]	---	---	Yes	---
Cyclorphan[d]	Yes	---	Yes	---	---
Butorphan[d]	Yes	---	Yes	---	---
ATPM[d]	Yes	---	Yes	---	---

Data for the [^{35}S]GTPγS binding results using membranes from CHO cells expressing either the human μ or κ opioid receptor are from the following publications. [a]Wentland et al. (38); [b]Bart et al. (1) and Wentland et al. (38);[c]Wentland et al. (39); [d]Neumeyer et al. (18); [e]Stimulation of [^{35}S]GTPγS binding mediated by the μ opioid receptor was less than 30% above basal levels. A partial agonist both stimulated [^{35}S]GTPγS binding and inhibited agonist-stimulated [^{35}S]GTPγS binding.

Two new derivatives of cyclorphan with high affinity have been described recently (*18*). The most selective compound, 3-(3′-hydroxybenzyl)amino-17-methylmorphinan was 24-fold more selective for the μ receptor than the κ receptor, and this compound was 1700-fold more selective for the μ than the δ receptor. It was a full agonist at both the μ and κ receptors (*18*). A structurally related compound, 2-(3′hydroxybenzyl)amino-17-cyclopropyl-methylmorphinan was κ selective, having a K_i value in receptor binding that was 150-fold selective for κ over μ, and greater than 10,000-fold more selective for the κ than the δ receptor (*47*). These two compounds show that it possible to synthesize selective benzomorphinans.

Aminothiazolomorphinans represent a new class of compounds with high affinity for μ and κ opioid receptors (*48*, *49*). These compounds were full agonists at the κ receptor and had activities at the μ receptor ranging from a weak partial agonist to a full agonist (*49*). (-)-3-Amino-thiazolo-[5,4,b]-N-cyclopropylmethylmorphinan (ATPM), shown in Table 3, produced antinociception mediated by both μ and κ opioid receptors with less potential for tolerance development than morphine (*50*). Also, ATPM reduced heroin self-administration in rats (*50*).

Naltrexamine Derivatives

Naltrexamine analogues have repeatedly shown agonist activity at the κ opioid receptor and mixed agonist and antagonist activity at μ receptors (*51*). *N*-Naphthoyl-β-naltrexamine (NNTA), a derivative of β-naltrexamine, has been reported to selectively activate μ−κ heteromers in HEK-293 cells and to produce potent antinociception following intrathecal administration in mice (*52*). Two naltrexamine derivatives have been shown to be partial agonists at the μ receptor and they were very selective for the μ receptor over the κ and δ receptors (*53*).

Bivalent Opioid Ligands

With the demonstration of μ/κ dimers, there has been a desire to design compounds that would target these dimers. A series of dimeric ligands were synthesized from the monomeric cyclorphan and butorphan (MCL-101) (*54*). Figure 2 shows the structures of some bivalent ligands. The monomers were connected with an ester spacer of varying lengths at the 3-hydroxyl positions. MCL-144, containing a 10-carbon ester spacer between two butorphan molecules, exhibited the highest affinity for μ and κ opioid receptors with K_i values of 0.090 nM and 0.049 nM, respectively (*54*). Both shorter and longer spacers attenuated the affinity of the bivalent compound for the receptor. MCL-144 was a full agonist at the κ receptor and a partial agonist at the μ opioid receptor (*47*, *54*). MCL-144 containing two stereoselectively active enantiomers was compared with MCL-193, which contained one stereoselective (-) enantiomer and one inactive (+) enantiomer of butorphan. These two bivalent ligands were compared with the parent compound (-)butorphan (MCL-101). *In vitro* analysis showed all three compounds to be κ full agonists and μ partial agonists. Both (-)(-)MCL-144 and (-)(+)MCL-193 produced full dose-response curves in the mouse 55°C warm-water tail withdrawal test (*47*). In antinociceptive tests, (-)(-)MCL-144 and (+)(-)MCL-193 had the same pharmacological properties, demonstrating that having two active pharmacophores separated by a 10-carbon spacer group did not increase the antinociceptive efficacy of the compound (*47*).

MCL-145 with a conformationally constrained fumaryl spacer had very high affinity for the μ and κ receptors. MCL-145 was a partial agonist at both the μ and κ receptors (*47*, *54*). Linking two butorphan molecules via an ether hydrocarbon chain greatly reduced the binding affinities of the resulting bivalent ligands relative to butorphan. Replacing the either linkage by an ester linkage restored

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binding affinity, but allowed the compound to be metabolized by esterases. When a hydroxyl group on the carbon atom beta to the ether linkage was included, the bivalent compound produced had high affinity for μ and κ receptors and was metabolically stable (*56*). Comparing MCL-144 with MCL-145 was interesting because the only difference between these bivalent ligands was the spacer region connecting the two pharmacophores. However, MCL-145 had an ED_{50} value in the mouse 55°C tail withdrawal assay that was 10-fold lower than MCL-144 (*55*).

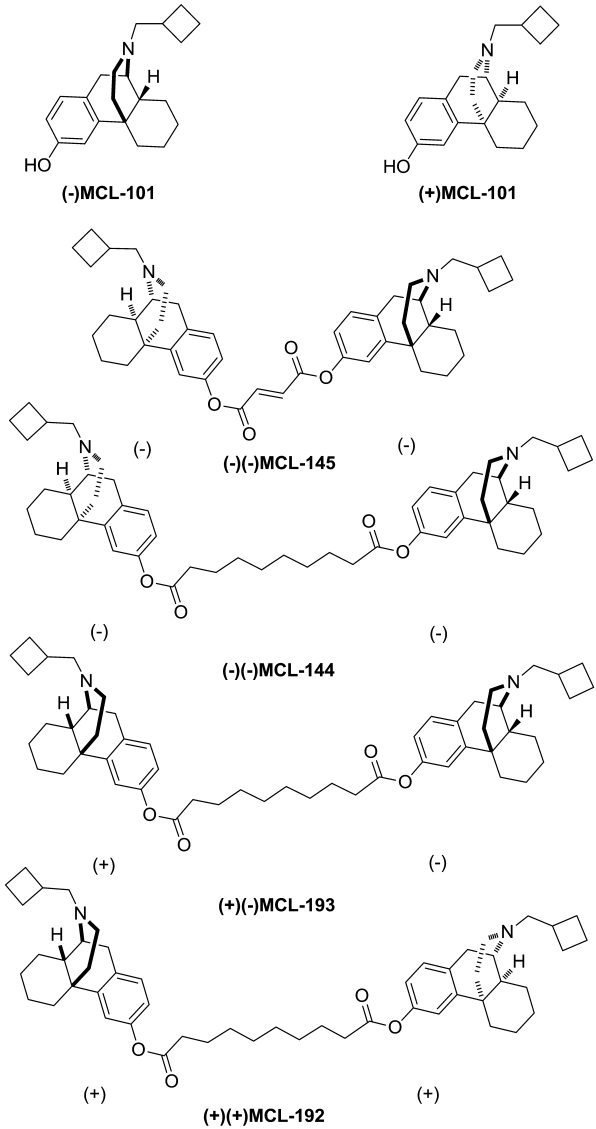

Figure 2. Structure of Bivalent Ligands Derived from Butorphan (MCL-101).

A series of homo- and heterodimeric ligands containing κ/μ agonist pharmacophores joined by a 10-carbon ester linker chain were synthesized and evaluated for their pharmacological properties (*57*). MCL-695, shown in Figure 3, contains butorphan at one end of the linking chain and butorphanol at the other end. MCL-695 was the most potent ligand in this series with binding affinities at μ and κ receptors of less than 0.10 nM. These affinities were better than the parent compounds butorphan and butorphanol (*57*). All of the morphinan-derived ligands were partial κ and μ agonists. ATPM -derived bivalent ligands, such as MCL-714 shown in Figure 3, were full κ agonists and partial μ agonists (*50*, *56*, *57*).

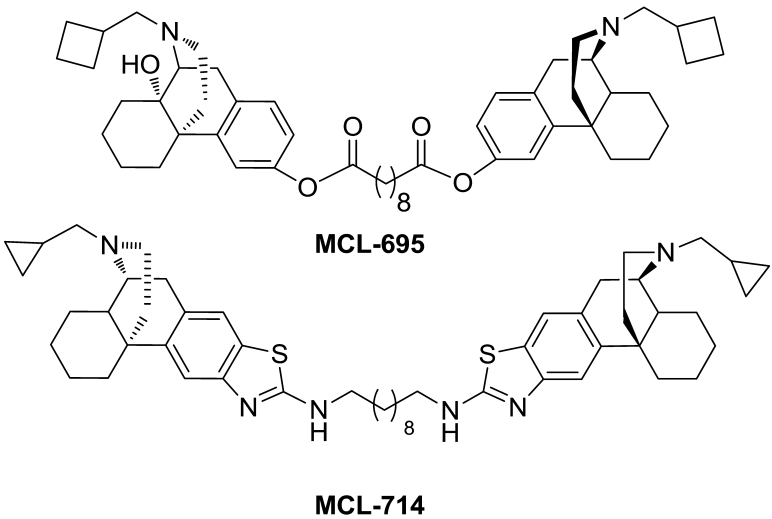

Figure 3. ***Structures of MCL-695 and MCL-714.*** *MCL-695 contains one butorphanol and one butorphan (MCL-101) molecule. MCL-714 is a bivalent ligand derived from the aminothiazolomorphinan ATPM.*

A series of bivalent ligands containing κ- and μ-antagonist pharmacophores, 5′guanidinonaltrindole (5′-GNTI) (*58*) and β-naltrexamine, respectively, were synthesized and evaluated as tools to study μ/κ heterodimeric opioid receptors (*59*, *60*). KMN-21 selectively antagonized the activation of κ−μ heterodimers (*59*).

Summary

The classical benzomorphans are primarily partial μ agonists and full κ agonists. Morphinans have been synthesized that bind with higher affinity to μ and κ receptors than to δ receptors. Morphinans can range from being partial agonists at the μ receptor to being pure antagonists at the μ receptor. At the κ receptor, most morphinans are either full or partial agonists, not pure antagonists or inverse agonists. Derivatives of morphinans and benzo-morphinans show a

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wide range of pharmacological activity at μ and κ receptors. Bivalent ligands have been synthesized recently. These bivalent ligands retain many of the properties of the parent compound, but have been shown to bind with higher affinity than the parent, depending on the spacer length and composition. The wide varieties of mixed μ/κ agonists are valuable tools to assist in the understanding of the function of μ and κ receptors, and their homo- and hetero-dimers. Mixed μ/κ agonists also may be useful analgesics with fewer side effects than full μ agonists.

Acknowledgments

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Chapter 15

Medicinal Chemistry, Pharmacology, and Biological Actions of Peptide Ligands Selective for the Nociceptin/Orphanin FQ Receptor

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Nociceptin/orphanin FQ (N/OFQ; FGGFTGARKSARKL-ANQ) was identified via reverse pharmacology strategies as the endogenous ligand of a previously orphan GPCR now referred to as N/OFQ peptide (NOP) receptor. The N/OFQ – NOP receptor system is widely distributed in the nervous system where it modulates several different biological functions. Structure relationship studies performed on the N/OFQ sequence allowed to generate NOP selective ligands encompassing full and partial agonist as well as pure antagonist activity, to increase their potency, metabolic stability, and in vivo duration of action. These peptide NOP ligands were used to investigate the consequences of NOP receptor activation and block thus suggesting the possible therapeutic indications of drugs interacting with this receptor. Evidence coming from these studies, together with findings obtained with knockout animals and non peptide NOP ligands, suggests that the most promising indications for NOP antagonists are depression and Parkinson disease and for agonists anxiety, drug abuse, cough, and pain (after spinal administration). In addition, clinical studies demonstrated that intravesical instillation of N/OFQ elicits beneficial effects in patients with overactive bladder.

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Keywords: nociceptin/orphanin FQ; NOP receptor; NOP peptide ligands; pain; anxiety; depression; Parkinson disease; urinary incontinence

The N/OFQ – NOP Receptor System

Discovery

In 1994, soon after the cloning of classical opioid receptors namely the delta (*1*, *2*) opioid peptide (DOP), the kappa (*3*) (KOP), and the mu (*4*) (MOP) receptors, several research groups identified a G protein-coupled receptor showing high homology with opioid receptors (*5–9*) but not able to bind opioid ligands. On this basis the protein was named opioid receptor like 1 (*6*). This protein was used to fish for its natural ligand from brain extracts according to the strategy named reverse pharmacology (*10*). This approach was indeed successful since one year later two groups independently reported the identification of the same heptadecapeptide, FGGFTGARKSARKLANQ, which was named nociceptin (*11*) for its ability to elicit hyperalgesia after supraspinal administration in mice and orphanin FQ (*12*) for its ability to recognize a previously orphan receptor and for its first and last aminoacid residues (F and Q). Nociceptin/orphanin FQ (N/OFQ) displays a primary sequence very similar to that of endogenous opioid peptides, however the presence of Phe in position 1 instead of Tyr makes this peptide highly selective for its receptor over classical opioid receptors. After the identification of N/OFQ as the naturally occurring ligand of the opioid receptor like 1, the receptor was renamed, according to IUPHAR recommendations, N/OFQ peptide receptor and abbreviated as NOP (*13*).

The N/OFQ Peptide

N/OFQ derives from the peptide precursor preproN/OFQ (ppN/OFQ) (*14–17*). The ppN/OFQ gene is comprised of at least four exons separated by three introns. Exon I contains exclusively 5′ noncoding sequence, exons II and III share the open reading frame of the gene, and exon IV contains most of the 3′ noncoding region of the message. The sequence of murine and human ppN/OFQ genes displays organizational and structural features that are very similar to those of the genes encoding the precursors of the endogenous opioid peptides namely prepro-enkephalin, -dynorphin and -opiomelanocortin, suggesting that the N/OFQ and opioid peptide genes have evolved from a common ancestor (*18*). The ppN/OFQ amino acid sequence is highly conserved across animal species, especially at the C-terminus where the sequence of the mature peptide is located between canonical Lys-Arg excision motifs. However, in addition to those framing N/OFQ, the ppN/OFQ sequence contains other cleavage sites, suggesting that ppN/OFQ may generate other biologically active peptides. In fact, the peptide nocistatin has been demonstrated to be biologically active (*19*); in most cases nocistatin does not produce any effect per se but is able to counteract

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the actions of N/OFQ (*20*). Importantly nocistatin does not bind to the NOP receptor. Despite considerable efforts by several groups the receptor mediating the pharmacological actions of nocistatin has not been identified. However recent evidence suggests that nocistatin is able to interact with NIPSNAP1, a molecule of physiologically unknown function, predominantly expressed in the brain, spinal cord, liver, and kidney, and more importantly, nocistatin effects are no longer evident in NIPSNAP1 knockout mice (*21*). Another peptide encoded by ppN/OFQ but unable to bind the NOP receptor is known as N/OFQ II: this peptide stimulates locomotor activity in mice (*22*).

ppN/OFQ mRNA and the N/OFQ peptide are broadly distributed in the central nervous system of the rat (*23*). In most brain areas peptide immunostaining and mRNA expression are very similar thus suggesting that the peptide is mainly produced by interneurons. mRNA and peptide are particularly abundant in cortical and limbic structures (hippocampus, dentate gyrus, septal areas, and amygdala), a number of hypothalamic and brain stem nuclei, the dorsal and ventral horns of the spinal cord, and in cell bodies of the dorsal root ganglion. This N/OFQ distribution in the central nervous system suggests that the peptide is potentially involved in the regulation of a variety of brain functions, including emotional processing, learning and memory, locomotion, reward, pain transmission, and autonomic regulation of peripheral organs and systems.

Little is known about the biosynthesis of N/OFQ, apart from the involvement of prohormone convertase 2 as demonstrated by studies performed in mice knockout for this enzyme (*24*). As far as N/OFQ metabolism is concerned, Montiel et al. (*25*) demonstrated the involvement of aminopeptidase that generates [desPhe^1]N/OFQ a peptide lacking affinity for the NOP receptor (*26*, *27*). This has been later confirmed in non human primates measuring [desPhe^1]N/OFQ levels after spinal injection of N/OFQ (*28*). However different endopeptidases are also involved in N/OFQ metabolism (*29*, *30*). Peptidase inhibitors are able to increase N/OFQ potency and, in some cases, maximal effects both in vitro (*31*) and in vivo (*29*, *32*) thus demonstrating an important role of peptidases in N/OFQ signaling. Indeed the inhibitory effect of the peptide in the human vas deferens can be detected in the presence of a cocktail of peptidase inhibitors but not in their absence (*33*).

The NOP Receptor

The NOP receptor belongs to the superfamily of G protein-coupled receptors, characterized by seven transmembrane helices interconnected by alternate intra- and extra-cellular loops. It displays high homology with opioid receptors, but has no affinity for opioid ligands (*34*). The primary structure of the NOP receptor is highly conserved across mammalian species with mouse and human sequences being >95% identical. The NOP receptor gene is located in the distal region of mouse chromosome 2, and in the q13.2-13.3 region of human chromosome 20 (*35*, *36*). NOP coding sequence is organized into introns and exons in a similar manner to the MOP, DOP and KOP opioid receptor genes, suggesting that the four genes have all evolved from a common ancestor, *i.e.* they belong to the same family (*37*). Like other members of the opioid receptor family the NOP receptor gene

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undergoes alternative splicing (*38*, *39*). The biological significance of these splice variants is unknown since pharmacological studies have not clearly established the existence of functionally distinct NOP receptor subtypes.

The NOP receptor mRNA is particularly abundant in the central nervous system, notably in the cortical and limbic areas, hypothalamus, brain stem, the dorsal and ventral horns of the spinal cord, and in cell bodies of the dorsal root ganglion (*40*). Extensive autoradiographic mapping of the NOP receptor protein shows similar distribution patterns to those of the mRNA, indicating that the receptor is expressed predominantly in local-circuit neurons. However, detailed studies on the expression of NOP protein by the different cell types have been limited to date by the lack of a specific and selective antibody.

The NOP receptor is coupled to Gi proteins that inhibit adenylate cyclase and calcium currents, and activate potassium channels (*41*). These cellular actions reduce synaptic efficacy, either by reducing transmitter release when the receptor is located presynaptically, or by reducing neuronal firing or excitability when the receptor is located postsynaptically. Indeed, several in vitro and in vivo studies have shown that NOP activation inhibits basal and/or stimulated release of various neurotransmitters, including acetylcholine, dopamine, serotonin, noradrenaline, GABA, glutamate and substance P in central as well as peripheral nerve tissues (*42*). Similarly, when tested electrophysiologically on individual neurons, NOP activation produces an inhibition of basal and/or stimulated electrical activity (*43*). These cellular effects produce inhibitory effects but can also be associated with circuit dependent disinhibitory actions, allowing an explanation of most, if not all, of the biological actions elicited by N/OFQ.

There is evidence in the literature that NOP receptors can form heterodimers with classical opioid receptors particularly with MOP (*44*). This may have profound implication in terms of receptor signaling and trafficking. For instance NOP/MOP heterodimers were shown to associate with N-type calcium channels, with activation of MOP receptors triggering N-type channel internalization, but only in the presence of NOP. Furthermore, the formation of OP/NOP receptor heterodimers attenuated the NOP inhibition of N-type channels (*45*). This kind of mechanisms might be operative in primary sensory neurons as well as in brain areas relevant for nociceptive transmission, thus they may have potentially profound effects on nociceptive processing.

Milestones in N/OFQ – NOP Receptor Research

Figure 1 chronologically summarizes the main milestones in the field of N/OFQ both in terms of research tools (top side) and biological actions of NOP ligands (bottom side).

Research Tools

The electrically stimulated mouse vas deferens was identified as N/OFQ sensitive preparation (*46*, *47*) and used to evaluate the biological activity of

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N/OFQ related peptides. This first generation structure activity relationship studies (*48–51*) allowed to indentify several peptide NOP ligands useful for pharmacological as well as pathophysiological investigations, including: i) N/OFQ(1-13)-NH_2, the smallest fragment maintaining the same potency and efficacy of the natural peptide (*46*), ii) [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1-13)-$NH_2$ ([F/G]N/OFQ(1-13)-NH_2) a partial agonist (*52*), iii) [$NPhe^1$]N/OFQ(1-13)-NH_2 a low potency pure antagonist (*53*), iv) [(pF)Phe^4]N/OFQ(1-13)-NH_2, a potent full agonist (*54*, *55*).

Other chemical modifications leading to the generation of interesting peptide ligands were described in the early 00's. For instance the Aib substitution in position 7 and/or 11 of N/OFQ generated potent NOP agonists (*56*). Similarly, the replacement of Leu-Ala with an extra couple of Arg-Lys residues in position 14-15 was found to be useful for generating the highly potent agonist [$Arg^{14}Lys^{15}$]N/OFQ (*57*). Further studies demonstrated that this NOP ligand displayed high potency and NOP selectivity in vitro and these features are associated in vivo with long lasting effects (*58*).

In subsequent structure activity relationship studies (*59*, *60*) the above mentioned chemical modifications were combined into single molecules to identify the second generation of NOP selective peptide ligands encompassing partial ([$Phe^1\psi(CH_2\text{-}NH)Gly^2(pF)Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$, UFP-113 (*60*)) and full ([(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$, UFP-112 (*61*, *62*)) agonist as well as pure antagonist ([$NPhe^1Arg^{14}Lys^{15}$]N/OFQ-NH_2, UFP-101 (*63*, *64*)) activities. These peptide ligands associated high potency with very high NOP selectivity and were instrumental in advancing our knowledge related to the N/OFQ – NOP receptor system (*36*).

Other NOP peptide ligands have been identified by screening of synthetic peptide combinatorial libraries. With this approach the potent and NOP selective partial agonists Ac-RYYRWK-NH_2 and Ac-RYYRIK-NH_2 were identified (*65*). These peptide sequences were used in subsequent studies for generating other interesting NOP ligands including the peptide ZP120 (*66*) that has been investigated and developed for its aquaretic activity (*67*). Another example of NOP ligand identified with combinatorial peptide chemistry approach is the non selective NOP antagonist peptide III BTD (*68*, *69*).

The different biological functions controlled by the N/OFQ – NOP receptor system and the therapeutic potential of agents able to selectively activate or block the NOP receptor stimulated industrial interest in this field of research and several companies activated medicinal chemistry programs aimed at the identification of drug-like non peptide NOP ligands (*70*, *71*). This lead to the identification and characterization of interesting and useful molecules. In 2000, J-113397 has been identified as the first non peptide NOP antagonist by Banyu researchers (*72*) and Ro 64-6198 as a potent and selective NOP agonist by Roche (*73*). These molecules were widely used for investigating the role played by the N/OFQ – NOP receptor system in physiological as well as pathological conditions and still represent standard ligands for the NOP receptor. Later, other non peptide NOP ligands were published including the highly potent and pure antagonists SB-612111 (*74*) and C-24 (*75*) and different series of full agonists identified by Pfizer (*76–78*) and Schering-Plough (*79–82*) researchers.

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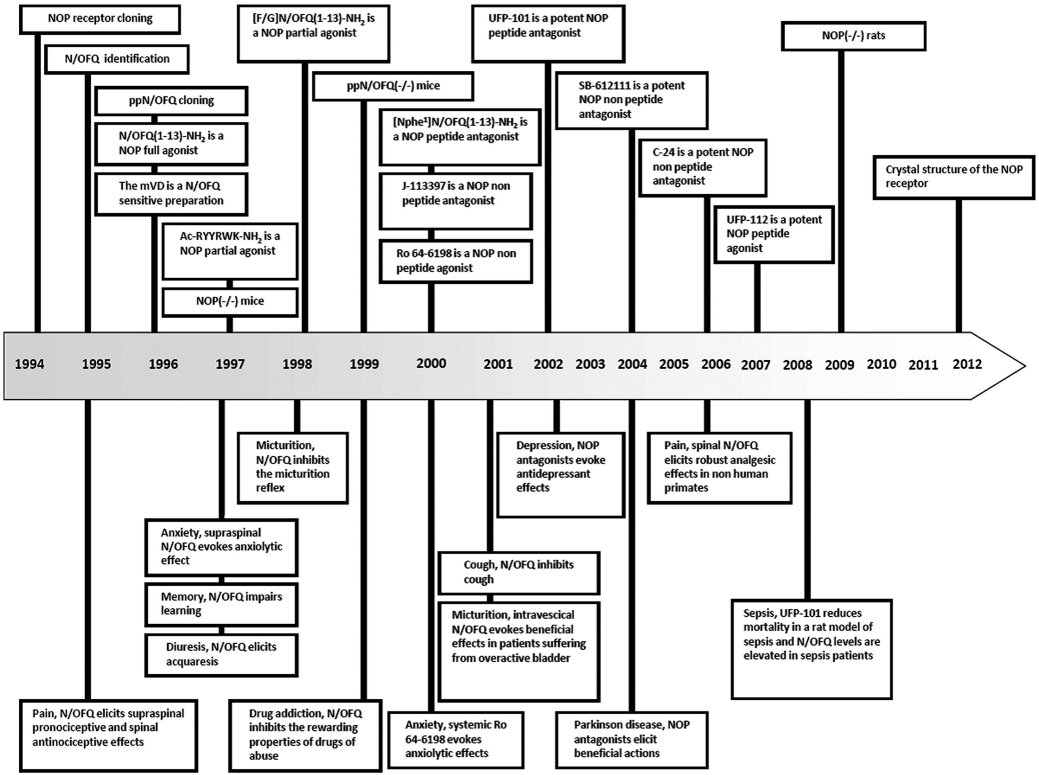

Figure 1. This scheme chronologically summarizes the main milestones in the field of N/OFQ in terms of research tools (top side) and biological actions and possible indications of drugs interacting with the NOP receptor (bottom side). For relative references see text.

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Together with receptor selective ligands, transgenic animals, particularly knockouts, represent useful research tools in modern pharmacology. Receptor knockout animals allow to perform simple and meaningful experiments to investigate the in vivo selectivity of action of standard and novel ligands and the involvement of the receptor of interest in the control of a given biological function. In most cases the phenotype of knockout animals is similar to the effect elicited by a selective receptor antagonist in normal animals.

NOP(-/-) mice were first generated in 1997 (*83*). Autoradiography studies demonstrated complete loss of N/OFQ binding in the brain of these animals (*84*). Subsequent in vitro functional studies demonstrated that in tissues taken from NOP(-/-) mice N/OFQ no longer elicits any effect. This includes data from bioassay studies (contractile action in the colon (*85*), inhibitory effect in the electrically stimulated vas deferens (*86*), inhibition of capsaicin induced bronchoconstriction (*87*)), neurochemical investigations (inhibition of serotonin release from cerebral cortex synaptosomes (*88*)), as well as electrophysiological studies (inhibition of excitatory transmission in the spinal cord (*89*)). In addition, in vivo studies on NOP(-/-) mice demonstrated that the N/OFQ actions examined to date are solely mediated by the NOP receptor. Receptor knockout studies are available in the literature regarding the following biological actions of N/OFQ: supraspinal pronociceptive (*83*, *86*) and spinal antinociceptive effects (*90*), induction of bradycardia, hypotension and diuresis (*91*), stimulation of food intake (*92*) and inhibition of locomotor activity (*83*, *86*). NOP(-/-) mice were also used for investigating their phenotype. It has been demonstrated that these animals show i) normal response to acute pain (*83*, *93*) while they display a pronociceptive phenotype in response to prolonged nociceptive stimulation (i.e. the formalin test) (*93*, *94*), ii) an antidepressant phenotype in the forced swimming (*95*) and tail suspension test (*96*), iii) increased anxiety-related behavior in the elevated plus-maze and light-dark box while in other anxiety related assays no differences were found compared to normal animals (*97*), iv) a better locomotor performance in the rotarod test (*98*), v) facilitated long term potentiation and greater learning and memory abilities than control mice (*99*). Recently rats knockout for the NOP receptor gene were also generated (*100*). In the brain of these animals N/OFQ binding is not detectable (*100*) and the inhibitory effect elicited by the peptide in the electrically stimulated vas deferens is no longer evident (*101*). In vivo studies performed in NOP(-/-) rats demonstrated that these animals display phenotype differences superimposable to those described in mice in the formalin, rotarod, forced swimming, and elevated plus maze assays (*101*). In addition in conditioned place preference experiments NOP(-/-) rats are more sensitive to the rewarding effect of morphine than normal animals (*102*). This is in line with the finding that NOP(-/-) mice displayed slightly enhanced methamphetamine and ethanol conditioned place preferences compared to wild type mice (*103*). Collectively these findings demonstrated that the control exerted by the N/OFQ – NOP receptor system on these biological functions is very robust across animal species.

Mice knockout for the ppN/OFQ gene have been also generated (*104*). However these ppN/OFQ(-/-) animals were only used in a small number of studies. The limited information available suggests that these animals behave in

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a similar manner as NOP(-/-) mice in terms of pain transmission (*93*) while they display differences from receptor knockout animals in terms of response to stress. However parallel experiments with NOP(-/-) and ppN/OFQ(-/-) mice should be performed before drawing firm conclusions on behavioral differences between the two mutant genotypes. Moreover the following consideration is worthy of mention. As mentioned before the ppN/OFQ gene contains in addition to N/OFQ itself, other biologically active peptides. Since ppN/OFQ null mice do not express N/OFQ as well as the other biologically active peptides encoded by the same gene caution should be exerted in interpreting behavioral differences between ppN/OFQ(-/-) and ppN/OFQ(+/+) mice as solely due to the lack of N/OFQ.

In the last years, methodological advances in X-ray crystallography, protein expression and purification, as well as techniques required to stabilize and crystallize receptor proteins have made possible the determination of the crystal structure of several G protein-coupled receptors, including receptors for adrenaline, dopamine, histamine, acetylcholine, adenosine, sphingolipid, and peptides (*105*). In May 2012 in the same issue of Nature the crystal structure of the NOP receptor (*106*) and those of classical opioid receptors (the MOP (*107*), DOP (*108*) and KOP receptor (*109*)) have been published. All these protein structures were obtained in complex with antagonists (C-24 for NOP) and thus represent the inactive state of the receptor. The availability of the NOP receptor crystal structure now enables rational drug design efforts directed to the identification of innovative potent and selective ligands.

Biological Actions

As far as biological functions controlled by the N/OFQ-NOP receptor system and possible indications of drugs interacting with the NOP receptor (Figure 1, bottom side) are concerned, studies performed during the period 1995-99 were mainly carried measuring the responses to the administration of the natural peptide N/OFQ. Based on the structural similarities of the N/OFQ-NOP receptor with classical opioid systems several studies investigated the effects of N/OFQ on pain transmission. These studies generated contentious results, in fact N/OFQ has been variously reported to cause hyperalgesia, allodynia, analgesia, and even nocifensive behaviors (*35*, *110*). One reason, among others, is that N/OFQ effects on nociception are strongly dependent by the range of doses and the route of administration. The supraspinal injection (i.c.v.) of N/OFQ was reported to cause thermal hyperalgesia in mice (*11*, *12*). However, this effect was soon shown to reflect reversal of stress-induced analgesia, rather than a decrease of nociceptive threshold (*111*). Indeed, i.c.v. N/OFQ attenuates the analgesic action of opioid as well as non opioid drugs (*35*). One mechanism whereby N/OFQ attenuates opioid induced analgesia is by directly inhibiting a descending antinociceptive pathway which is itself indirectly activated (disinhibited) by opioids (*112*). This mechanism might be activated during chronic opioid administration since NOP receptor knockout (*113*) as well as antagonist studies (*74*, *114*) demonstrated that blocking N/OFQ – NOP receptor signaling counteracts morphine tolerance.

When administered intrathecally (i.t.) in rodents, very low doses (femto to picomole range) of N/OFQ are reported to cause pronociceptive effects, whilst

higher doses (nanomole range) produce antinociceptive effects (*35*, *110*). Several studies have shown that intrathecal administration of nanomoles of N/OFQ produces robust antinociceptive effects and this holds true in a wide variety of animal models of phasic (mechanical or thermal) as well as of tonic (inflammatory or neuropathic) pain. The spinal antinociceptive action of N/OFQ is consistent with the well documented ability of the peptide to block excitatory (glutamate) transmission in the dorsal horn of the spinal cord (*110*). Robust evidence first obtained with NOP(-/-) and ppN/OFQ(-/-) mice (*93*) and then confirmed with receptor antagonists (*94*, *115*) and rat NOP(-/-) (*101*) studies, demonstrates that endogenous N/OFQ – NOP receptor signaling in the spinal cord is activated by prolonged (e.g. formalin, zymosan A, writhing tests, SBL response to i.t. substance P), but not acute (tail flick/immersion test) nociceptive stimulation and the consequence of its activation is the appearance of antinociceptive effects. Finally in a elegant series of studies the group of MC Ko demonstrated that in non human primates spinal administration of N/OFQ or synthetic NOP ligands i) does not elicit any effect at low doses, ii) in the nanomole range of doses induces a robust antinociceptive action that is sensitive to NOP antagonists but not naloxone, iii) in contrast to morphine, does not induce pruritus and iv) elicits a synergistic antinociceptive effect when given in association with morphine (*28*, *116*). Thus evidence coming from non human primates strongly suggest NOP receptor agonists as innovative spinal analgesics. Interestingly, this same research group demonstrated that the NOP selective non peptide agonist Ro 64-6198 given systemically is able to induce dose dependent antinociceptive effects in non human primates while being inactive in rodents. In monkeys antinociceptive doses of alfentanil (and in generally of opioids) are associated with respiratory depression, itch/scratching responses and reinforcing effects under self-administration procedures while in parallel experiments antinociceptive doses of Ro 64-6198 did not produced these side effects (*117*).

Similar to what seen at the spinal level, in the periphery both pro and antinociceptive effects were reported for N/OFQ. For instance intradermal administration of very low doses of N/OFQ stimulate the flexor reflex in mice. This effect involves stimulation of the release of substance P from peripheral nerve endings. However at higher doses N/OFQ prevented the facilitatory effect of substance P (*118*, *119*). In addition several groups reported the ability of N/OFQ to inhibit neuropeptide release from peripheral sensory neuron terminals in different organs including the airways (*120*, *121*), heart (*122*), and renal pelvis (*123*, *124*). Finally in non human primates the coadministration of N/OFQ with capsaicin into the tail dose dependently inhibited thermal nociception suggesting that activation of peripheral NOP receptors produces antinociceptive effects (*125*).

One of the most highly investigated actions of N/OFQ is its ability to counteract stress related behaviors and promote anxiolytic like effects. The pivotal study in this field demonstrated that N/OFQ acts as anxiolytic in several benzodiazepine-sensitive behavioral tests (*126*). Similar results were later reported in response to the systemic administration of Ro 64-6198 (*73*) and these findings were confirmed in different assays, species and laboratories (*127*). Interestingly the anxiolytic like action of Ro 64-6198 did not show tolerance

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liability after daily administration for two weeks (*128*). In addition, anxiolytic like effects were reported for several non peptide NOP agonists developed by Roche, Shering-Plough, and Pfizer laboratories (*70*, *71*). Knockout studies corroborated these findings. Indeed, in comparison with wild type animals, ppN/OFQ(-/-) mice have a greater tendency towards anxiety-like behavior when exposed to a novel and threatening environment, and have increased basal and stress-induced levels of plasma corticosterone (*104*). In addition NOP(-/-) mice display a mild anxiogenic phenotype in same anxiety related assays (*97*). Superimposable results were recently obtained investigating the phenotype of receptor knockout rats (*101*). The mechanisms by which N/OFQ exerts its anxiolytic effects are not fully understood but there is evidence for the involvement of $GABA_A$ receptor signaling (*129*, *130*), however CRFergic and serotonergic pathways might be also implicated (*131*).

It was initially shown that microinjection of N/OFQ into the CA3 region of the dorsal hippocampus caused profound impairment of spatial learning in rats trained in the Morris water task (*132*), and effect sensitive to NOP antagonists (*133*). In addition N/OFQ was reported to inhibit long-term potentiation in rat hippocampal slices (*134*). These observations received strong support from the observation that NOP(-/-) mice not only displayed greater learning ability and have better memory than NOP(+/+) animals, but also showed increased long-term potentiation in the hippocampal CA1 region (*99*). However systematic studies on the possible cognitive enhancing properties of selective NOP antagonists have not yet been performed.

By acting both on the central and peripheral nervous systems, N/OFQ modulates the functioning of several organs and systems including the heart and vessels, airways, kidney, urinary bladder, the gastrointestinal and immune system (*36*). Thus, N/OFQ has been shown to induce bradycardia and hypotension (*135*), to possess vasorelaxant properties (*136*), to stimulate diuresis and in particular to promote aquaresis (*137*), to control several gastrointestinal functions under physiological as well as pathological conditions (*138*), to modulate immune functions (*139*), and to inhibit some reflexes mediated by sensory fibers such as cough and micturition. These latter actions of N/OFQ deserve further comments since NOP agonists are under clinical development as antitussive agents (*140*) and as innovative drugs to treat urinary incontinence due to overactive bladder (*141*).

The antitussive action of N/OFQ has been first described in the guinea pig (*142*) and then confirmed in the cat (*143*). The non peptide NOP agonist Ro 64-6198 mimicked the antitussive action of N/OFQ (*144*). Similar results were obtained by testing in various preclinical models of cough a large series of non peptide NOP agonists developed by Schering-Plough researchers (*80*, *81*, *145*). Among these molecules, the compound SCH 486757 has been selected and characterized in details (*146*). SCH 486757 selectively binds human NOP receptor over classical opioid receptors. In a guinea pig capsaicin cough model, SCH 486757 displayed similar antitussive efficacy as codeine, hydrocodone, dextromethorphan and baclofen. The antitussive effects of SCH 486757 were sensitive to the NOP receptor antagonist J-113397 but not to naltrexone and did not undergo tolerance after 5 days of treatment. Importantly, SCH 486757 did not display abuse liability. Finally, SCH 486757 displayed a good oral

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pharmacokinetic profile in the guinea pig, rat and dog. In summary, SCH 486757 showed a favorable antitussive profile in preclinical animal models. This molecule was evaluated clinically in a multicentre, double-blind study in patients with subacute cough (*147*). Both SCH 486757 and codeine reduced symptoms to a similar degree but not statistically different than placebo. However the maximum clinical dose of SCH 486757 was limited by its tendency to produce somnolence. Further studies are therefore needed before drawing firm conclusions on the therapeutic value of NOP agonists as innovative antitussive drugs.

In an elegant series of rodent studies (reviewed in (*148*)) Menarini researchers demonstrated that intravenous administration of N/OFQ inhibits the micturition reflex and this effect is no longer evident in capsaicin-pretreated rats indicating the involvement of capsaicin-sensitive nerve fibers innervating the urinary bladder. In support of this interpretation, N/OFQ also inhibited the reflex, but not the local bladder contraction, induced by topical capsaicin. Intrathecal N/OFQ produces urodynamic modifications similar to those induced by systemic administration. Intracerebroventricular administration of the peptide also inhibited the micturition reflex in a naloxone-resistant manner suggesting a direct effect on supraspinal sites controlling micturition. Beyond the inhibitory effects exerted by N/OFQ on the micturition reflex, a peripheral excitatory effect mediated by capsaicin-sensitive fibers was also detected. Indeed, application of the peptide onto the bladder serosa when the intravesical volume was subthreshold for triggering of the micturition reflex, activated the reflex and this was associated with a local tonic-type contraction that was abolished by the coadministration of tachykinin NK_1 and NK_2 receptor antagonists. Collectively, these results indicate that in the rat, NOP receptors are present at several sites for the integration of the micturition reflex and that their activation produces mainly inhibitory effects. Based on these findings, Lazzeri and coworkers performed the first clinical investigation with N/OFQ by testing the urodynamic effects of intravesical application of the peptide in normal subjects and in patients suffering from an overactive bladder (*149*). In normal subjects N/OFQ did not modify urodynamic parameters while in patients the peptide produced a statistically significant increase in mean bladder capacity and volume threshold for the appearance of detrusor hyperreflexia. Maximum bladder pressure was not significantly affected. These results were later confirmed in a randomized, placebo controlled, double-blind study in which [desPhe^1]N/OFQ (a N/OFQ metabolite that lacks affinity for the NOP receptor (*26*)) was used as placebo, demonstrating that N/OFQ, but not the placebo, elicits a robust acute inhibitory effect on the micturition reflex in patients with neurogenic bladder (*150*). These promising results obtained after acute administration of N/OFQ allowed the investigation of the feasibility, safety and efficacy of daily intravesical instillation of 1 mg of N/OFQ for 10 days in patients who perform clean intermittent self-catheterization for neurogenic detrusor overactivity incontinence (*151*). Urodynamic parameters recorded during the study confirmed previous findings. Moreover, during N/OFQ treatment, daily urine leakage episodes were approximately halved and bladder capacity increased by 172%. No significant problems related to feasibility of the procedure as well as significant side effects were reported by patients. This study demonstrated the clinical efficacy of N/OFQ during 10 days of treatment supporting the use of NOP

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receptor agonists in the control of detrusor overactivity incontinence. Collectively these studies suggest that NOP selective agonists are worthy of development as innovative drugs to treat urinary incontinence due to overactive bladder.

The rewarding properties of N/OFQ have been examined using the conditioned place preference test that pairs administration of the drug with a particular set of environmental cues. In rats, supraspinal injection of N/OFQ does not induce place preference or aversion, an indication that the peptide lacks intrinsic motivational properties (*152*). Most significantly however, N/OFQ was shown to block morphine-induced place preference (*153*, *154*), an effect that was later extended to other drugs of abuse, all known to acutely stimulate the dopaminergic mesocorticolimbic pathway, such as alcohol, amphetamine and cocaine (*155*). One mechanism whereby N/OFQ attenuates reward elicited by drugs of abuse is by directly inhibiting dopaminergic mesocorticolimbic neurons, which express the NOP receptor (*156*, *157*). Of particular relevance to addiction are the observations that N/OFQ blocks reinstatement of alcohol-seeking behavior in alcohol-prefering rats (*158*), and that the non peptide NOP receptor agonist Ro 64-6198 blocks reinstatement of morphine place preference in mice (*159*). The endogenous N/OFQ – NOP receptor system seems to contribute to the control of rewarding functions since pharmacological blockade or genetic knockout of the NOP receptor potentiates the rewarding effect of morphine in rats (*102*) and similar finding were obtained with methamphetamine and ethanol in NOP(-/-) mice (*103*).

In the early 00's the availability of NOP selective antagonists and NOP(-/-) mice made possible to the scientific community to investigate the consequences of blocking endogenous N/OFQergic signaling and to foresee the possible therapeutic indications of drugs acting as NOP blockers.

The seminal study by Redrobe et al. (*160*) demonstrated that NOP antagonists elicit antidepressant like effects in the forced swimming test in mice. This initial finding was later confirmed and extended in subsequent studies demonstrating that antidepressant like effects are measured in response to chemically different NOP antagonists including the peptides [NPhe^1]N/OFQ(1-13)-NH_2 and UFP-101 and the non peptides J-113397 and SB-612111 (*92*, *95*, *96*, *160*) and that NOP(-/-) mice and rats display an antidepressant like phenotype (*95*, *96*, *101*). Moreover UFP-101 injected into the dorsal hippocampus produced antidepressant-like effects in the mouse forced swim and tail suspension tests (*161*). This evidence obtained using despair tests has been corroborated by findings obtained in rats subjected to chronic mild stress: three week treatment with UFP-101, similar to imipramine, dose and time dependently reinstated sucrose consumption (*162*). Interestingly clinical studies suggest that N/OFQ levels are increased in depressed patients (*163*, *164*). Little is known about the possible mechanism(s) involved in the antidepressant effects of NOP antagonists. However it has been reported that N/OFQ is able to inhibit noradrenaline and serotonin release from cerebral cortex as well as neuronal firing in the dorsal raphe and locus coeruleus (*131*). Therefore assuming that chronic stress/despair conditions stimulate the release of N/OFQ, the peptide may reduce monoaminergic signaling acting both at presynaptic and postsynaptic sites. By preventing such effects of N/OFQ NOP antagonists may restore normal levels of noradrenaline and serotonin at their

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respective synaptic clefts. Thus, NOP receptor antagonists, by acting at different levels and with different mechanisms, may achieve a similar endpoint to that of classical antidepressants, i.e., an increase in cortical synaptic concentrations of monoamines (*131*).

In the paper reporting its identification as the endogenous ligand of the NOP receptor, N/OFQ has been shown to inhibit spontaneous locomotor activity (*12*). This effect has been later confirmed in mice and rats by different laboratories (*29*, *165*) and the involvement of the NOP receptor has been demonstrated by receptor antagonist (*63*, *166*) and knockout studies (*83*, *86*). However the endogenous N/OFQ – NOP receptor system does not tonically control spontaneous locomotion since NOP antagonists do not modify per se this behavior and knockout animals do not show any phenotype. In the seminal paper by Marti et al. (*98*) it has been demonstrated that systemic administration of J-113397 or intranigral injection of UFP-101 facilitated, in a dose-dependent manner, rat performance in the drag and rotarod tests. Moreover, NOP(-/-) outperformed NOP(+/+) mice in the same assays (a finding later confirmed in NOP receptor knockout rats (*101*)). These results suggest that endogenous N/OFQ may indeed exert an inhibitory influence over motor activity that becomes relevant during exercise rather than at rest. Consistent with these observations, systemic administration of J-113397 and its analogues Trap-101 (*167*) and GF-4 (*168*) increased motor performance in normal rats and in NOP(+/+) mice but was ineffective in NOP(-/-) mice. Collectively, these findings reinforce the view that NOP receptor blockade may represent a new strategy for the control of hypokinetic disorders. Indeed, a series of elegant studies demonstrated that NOP receptor antagonists attenuated motor deficits in rodent and non human primate models (6-hydroxydopamine (*169*), haloperidol (*170*, *171*), reserpine (*172*), 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (*173–175*)) of parkinsonism. The finding that N/OFQ levels are 3.5 fold elevated in the cerebrospinal fluid of parkinsonian patients compared to controls (*176*) further strengthens the rational for developing NOP receptor antagonists as drugs to treat Parkinson's disease.

Another field in which NOP receptor antagonists may produce beneficial effects is that of inflammation and sepsis. The N/OFQ-NOP system is present in immune cells and N/OFQ modifies immunocyte functions. On the basis of various in vitro and in vivo studies, N/OFQ increases the inflammatory response in healthy animals and in those with a septic or inflammatory process. N/OFQ affects tissue perfusion, increases capillary leakage and inflammatory markers, and leads to immune cell chemotaxis. Moreover, NOP activation produces bradycardia and hypotension (for reviews on this topic see (*139*, *177*)). Thus the block of the NOP receptor may elicit beneficial effects in some inflammatory diseases. For instance, in the dextran sodium sulfate murine model of colitis it has been reported that NOP(-/-) mice are less vulnerable than wild type animals. Moreover expression level of mucosal addressin cell adhesion molecule-1 and infiltrating cells were significantly decreased in NOP(-/-) compared to NOP(+/+) mice (*178*). These results suggest that N/OFQ-NOP receptor signaling deteriorates colonic inflammation. This proposal has been recently confirmed in receptor antagonist studies. In fact SB-612111 significantly ameliorated the clinical disease course of mice with dextran sodium sulfate-induced colitis as indicated by reduced fecal

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bleeding, improved recovery from diarrhea and weight loss, and a reduction in histopathological alterations. In addition, the inflammatory response in the colon was diminished, as demonstrated by reduced protein and messenger RNA expression for different proinflammatory agents including interleukin 1β and 6, and tumor necrosis factor alpha (*179*). Collectively this limited evidence suggests that the actions of NOP antagonists in models of inflammatory bowel diseases should be further and carefully investigated.

Another condition in which the block of the NOP receptor may provide beneficial effects is sepsis. In fact, systemic N/OFQ administration increased mortality in the cecal ligation and puncture model of sepsis in rats. On the contrary, under the same experimental conditions, systemic treatment with the selective NOP receptor antagonist UFP-101 reduced animal mortality. This was associated with reduced cells migration, bacterial dissemination, and plasma levels of interleukin 1β and tumor necrosis factor alpha (*180*). There is also clinical evidence for increased plasma N/OFQ concentrations in septic patients. In fact it has been reported that plasma N/OFQ levels were higher in patients with sepsis who died compared with those who survived (*181*). In addition a recent study demonstrated that mRNA expression of NOP and ppN/OFQ in peripheral blood cells are higher in septic patients compared with healthy controls (*182*). There is clearly a need for further preclinical and clinical studies on the role of the N/OFQ system in inflammatory processes or sepsis. However the available evidence suggests that NOP antagonists may provide beneficial effects in inflammatory bowel diseases and during sepsis.

Structure Activity Relationship (SAR) Studies on NOP Peptide Ligands

N/OFQ Related Peptides

The primary sequence of N/OFQ shows strictly similarities with endogenous opioid peptides in particular with dynorphin A. The N-terminus of N/OFQ, FGGF, is highly reminiscent of the canonical YGGF of the opioid peptides, and N/OFQ and dynorphin A have the same length and are characterised by the presence of basic residues in their C-terminal.

Despite the close similarity between N/OFQ and opioid sequences, it has been demonstrated that N/OFQ does not bind opioid receptors and opioid peptides do not elicit biological activities via the NOP receptor. The structural bases of this high selectivity over classical opioid receptors can be ascribed to the absence of the phenol moiety in position 1 of N/OFQ and, more specifically over KOP, to the presence in the middle of the N/OFQ and dynorphin A of specific residues that prevent NOP/KOP cross activation (*183*).

The molecular mechanism that makes Tyr^1 essential for the binding to classical opioid receptors but not NOP has been recently discovered. In fact, in the ligand binding pocked of all the three classical opioid receptors there is a hydrogen bond network formed by an His residue of the TM6 of the receptor, two water molecules, and a phenol moiety of the ligand employed for generating

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the crystal (*107–109*). Most probably the phenol moiety of Tyr[1] of endogenous peptide ligands establishes similar interactions with the hydrogen bond network. On the contrary, in the same region of the ligand binding pocked of the NOP receptor there are no water molecules and only hydrophobic interactions are present between receptor residues and the phenyl ring of the benzofurane moiety of the C-24 ligand which likely mimics the Phe[1] phenyl ring of N/OFQ related peptides (*106*).

As far as the importance of the C-terminal basic residues of N/OFQ are concerned, molecular modeling studies performed with the peptide antagonist UFP-101 showed that the Arg-Lys couples are instrumental for inducing an alpha helix conformation and forming ionic interactions with acidic side chains of NOP receptor residues positioned in ECL2, TM2 and TM7 (*106*). Interestingly, these same mechanisms of interactions were previously suggested based on photocrosslinking experiments (*184*).

Classical peptide SAR studies performed soon after the identification of N/OFQ demonstrated that the natural sequence can be shortened at its C- but not N-terminal and that N/OFQ Phe[1], Phe[4], and Arg[8] are the most important residues for NOP binding (*26*, *185*). Further SAR studies were then performed by us and other groups investigating several positions of the N/OFQ sequence; these are briefly summarized in the following paragraphs also in light of the recent availability of the NOP receptor crystal structure (*106*).

The substitution of Phe[1] with Ala produces an inactive peptide (*26*, *185*, *186*) however the use of Leu or Cha generates peptides showing the same potency as N/OFQ (*48*, *50*). This demonstrates that, contrary to opioids, aromaticity in position 1 is not essential for NOP binding and activation. Interestingly Phe[1] can be also substituted with Tyr (*50*, *187*), 2′,6′-dimethylphenylalanine (Dmp) (*188*), or 2′,6′-dimethyltyrosine (Dmt) (*189*, *190*) with no changes in NOP potency but moderate (Tyr and Dmp) or extreme (Dmt) loss of selectivity over classical opioid receptors. The spatial disposition of the benzyl side chain is important for both NOP receptor binding and activation. In fact D-Phe[1] displayed very low potency at the NOP receptor (*48*). The increase of conformational freedom obtained by reducing the Phe[1]-Gly[2] peptide bond, i.e. [Phe[1]ψ(CH_2-NH)Gly[2]], produces a slight loss of potency and of efficacy generating a NOP partial agonist (*49*, *52*). Similar results were obtained using methyleneoxy ([Phe[1]ψ(CH_2-O)Gly[2]]) or methylenethio ([Phe[1]ψ(CH_2-S)Gly[2]]) bonds (*191*). More importantly, the shift of the benzyl side chain from the C alpha to the N atom generates a peptide that behaves as a low potency NOP antagonist (*50*). Interestingly, the substitution of Nphe with a rather large series of Nxaa analogues always produced inactive peptides (*50*, *192*). Collectively this evidence indicate that the spatial disposition of the benzyl side chain of Phe[1] is crucial not only for receptor binding but also for receptor activation. In the inactive state of the NOP receptor, the phenyl ring is buried in a hydrophobic pocket created by residues from TM3, 5 and 6, including Tyr131, Met134 and Ile219 (Figure 2 and (*106*)). It is possible that the interaction of these residues with Phe or Nphe may induce different rearrangements of the hydrophobic pocket with consequent changes in receptor active or inactive state. This suggestion will be experimentally validated when the NOP receptor crystal in complex with an agonist will be available.

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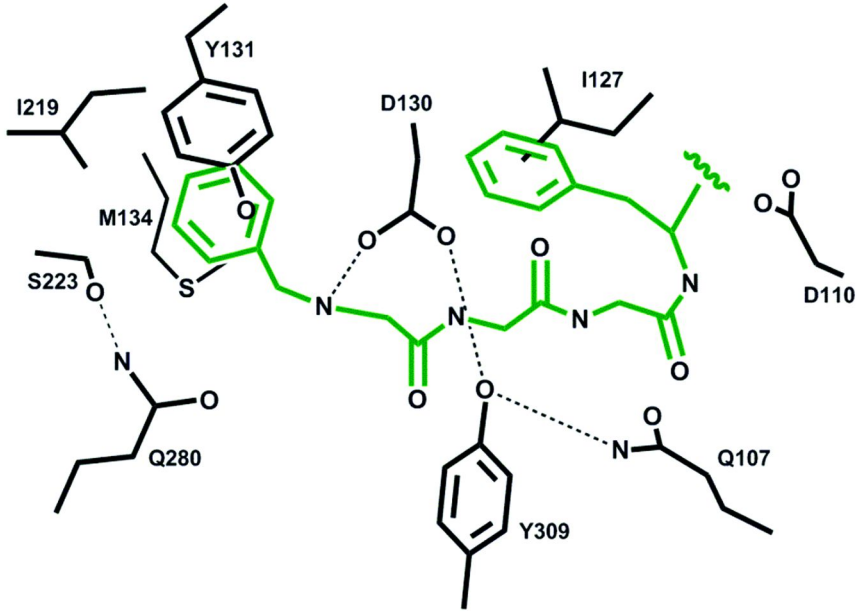

Figure 2. Schematic representation of the interactions between the N-terminal Nphe-Gly-Gly-Phe sequence of UFP-101 (colored in green) and crucial residues (colored in black) of the NOP receptor binding pocket.

The Gly^2-Gly^3 dipeptide may probably act as a conformation inducing spacer between the pharmacophores Phe^1 and Phe^4. In opioid peptides Gly^2-Gly^3 can be substituted with D-Ala (e.g. deltorphin or dermorphin) or Pro (e.g. casomorphin) without loss of activity. However these substitutions are not tolerated when applied to the N/OFQ sequence (*48*, *49*). Similar results were obtained using sarcosine for substituting Gly in positions 2 and 3 (*193*). In addition, the distance between Phe^1 and Phe^4 of N/OFQ appears to be critical, since any alteration of it leads to a marked decrease or a total elimination of biological activity (*48*, *49*). Indication coming from crystallographic analysis and docking investigations suggests that Gly^2-Gly^3 might be instrumental for placing in the right position the Phe^1 and Phe^4 pharmacophores and the N-terminal nitrogen atom of the peptide which forms a ionic interaction with the Asp130 of the NOP receptor (Figure 2 and (*106*)).

The crucial role of the Phe^4 residue of N/OFQ has been demonstrated in pivotal Ala- and D-scan studies (*26*, *185*, *186*). A systematic SAR analysis of the Phe^4 pharmacophore of N/OFQ has been performed (*51*). All attempts to reduce conformational freedom or to modify the aromaticity of the Phe^4 side chain were found to be detrimental for biological activity. Introduction of halogens into the phenyl ring of Phe^4 led to significant changes in activity with the most potent compound being the pF analogue. NO_2 and CN groups in the same position also increased the ability of the analogues to bind to and activate the NOP receptor. In addition a quantitative SAR investigation of the para position of the Phe^4 phenyl ring indicated that the lipophilic character of the substituents is not relevant for

biological activity, the electron withdrawal effect from the Phe[4] aromatic ring is crucial, and the steric features of the substituents are not critical per se (*51*). Molecular docking studies performed with the NOP crystal and the antagonist UFP-101 demonstrated that the phenyl ring of Phe[4] interacts with Ile127 of the receptor (Figure 2 and (*106*)). Modulation of the electronic asset of the Phe[4] phenyl ring induced by para substituents may likely affect the force of Van der Waals interactions between Phe and Ile possibly by changing the aromatic ring polarizabilities and inducing preferred interaction geometries.

The N-terminal tetrapeptide (Nphe-Gly-Gly-Phe) of UFP-101 (or [NPhe[1]]N/OFQ(1-13)-NH_2) can be perfectly superimposed to C-24 into the NOP receptor binding pocked as demonstrated by docking studies (*106*). Comparison of Figure 2 with Figure 2e of reference (*106*) indicates that the two aromatic rings and the basic nitrogen of Nphe-Gly-Gly-Phe and C-24 interact with the same NOP receptor residues.

The C-terminal truncation down to the 1-13 sequence of N/OFQ does not affect its biological activity; further shortening of the peptide produces a progressive reduction of peptide affinity/potency (*26*, *48*). NMR investigations indicated that C-terminal region of N/OFQ prefers alpha helix conformations (*186*). This result has been later confirmed by an independent NMR study (*194*). In addition the following findings corroborate this proposal: i) the substitution of Ala[7], Ala[11], and Ala[15] with the alpha helix inducer residue Aib (alpha-aminoisobutyric acid) generated peptides that are slightly more potent than N/OFQ (*56*); ii) the use of the alpha helix breaker residue Pro in different positions (5, 6, 7, and 11) always produced low potency or inactive peptides (*189*, *195*); iii) adequate C-terminal cyclizations, which are known to favour alpha helix conformation, lead to biologically active N/OFQ analogues (*196–198*); iv) molecular modelling studies suggested an alpha helix organization as preferred conformation of the C-terminal of N/OFQ related peptides (*106*, *199*).

One of the structural characteristics of the N/OFQ C-terminal region is the presence of two couples of Arg-Lys residues at positions 8–9 and 12–13; these have been suggested to bind to the acidic amino acid cluster in the ECL2 of the NOP receptor (*199*). With a design strategy of attempting to obtain highly potent peptide ligands, a series of N/OFQ analogs in which the Arg-Lys dipeptide unit was placed at positions 6–7, 10–11, or 14–15 was synthesized. Among these peptides, [Arg[14]Lys[15]]N/OFQ was found to behave as a highly potent NOP agonist (*57*). A SAR study focused on position 14 and 15 demonstrated that similar results can be obtained using the dipeptides Lys-Arg, Lys-Lys, Arg-Arg while smaller effects were measured with single modifications at positions 14 or 15 (*200*). It is worthy of note that contiguous charged residues are known to favour alpha helix structures and this mechanism may contribute to the high potency of this series of N/OFQ related peptides. Further studies also demonstrated that Arg/Lys can be substituted with Trp without loss of potency in position 14 but not 15. This suggests that the chemical nature of the peptide / receptor interactions are different for position 14 (ionic or π/π) and 15 (only ionic) (*201*).

N- and C-terminal chemical modifications affecting peptide potency and/or efficacy have been variously combined into N/OFQ sequence to generate highly potent NOP ligands encompassing full and partial agonist as well as antagonist

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activity. The pharmacological properties of the most interesting ligands will be described and analysed in details in the next chapter section.

The most important chemical modifications useful for generating N/OFQ related NOP ligands have been summarized in a schematic form in Figure 3.

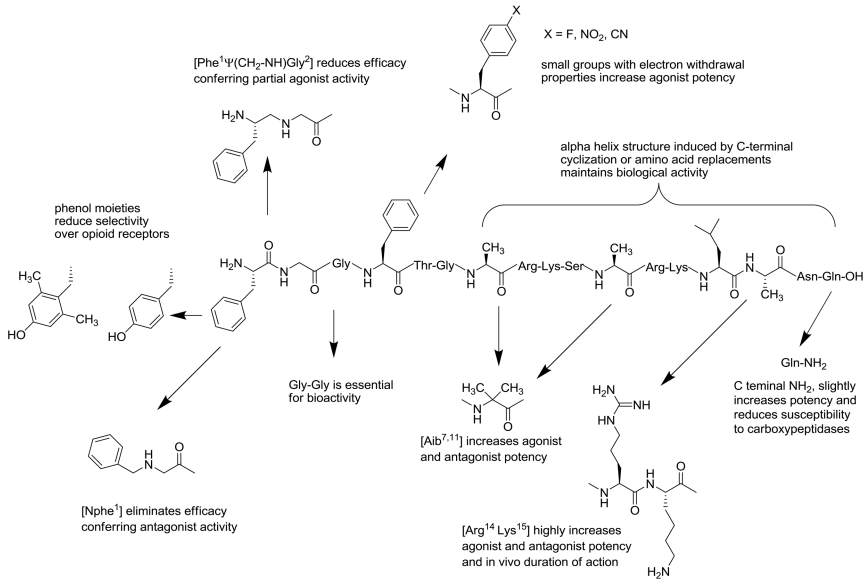

Figure 3. Schematic summary of structure activity relationship findings on N/OFQ peptide sequence.

N/OFQ Unrelated Peptide NOP Ligands

These molecules were identified by screening of synthetic peptide combinatorial libraries. From a library of more than 52 million hexapeptides, few peptides having high affinity for the NOP receptor were selected and demonstrated to behave as selective NOP partial agonists (*65*). Among this series of compounds Ac-RYYRWK-NH_2 (Figure 4) and Ac-RYYRIK-NH_2 were the most investigated. Interestingly photoaffinity labelling studies demonstrated that these hexapeptides bind the NOP receptor in a distinct, although partially overlapped, site compared to the natural ligand N/OFQ (*202*). The substitution of the N-terminal acetyl group of RYYRWK-NH_2 with different moieties produced modifications of both peptide potency and efficacy. In particular, pentanoyl-RYYRWK-NH_2 behaved as an highly potent NOP antagonist although this peptide maintained some residual agonist activity (*203*). Moreover, a similar study performed using Ac-RYYRIK-NH_2 as template confirmed these observations and identified Isovaleryl-RYYRIK-NH_2 as a pure NOP antagonist (*204*). SAR studies performed on Ac-RYYRWK-NH_2 with non natural amino acids demonstrated that: i) each Arg of the hexapeptide is required to maintain high binding affinity (*205*), ii)

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Tyr[2] and Tyr[3] are not required, but at least one of these residues must maintain its hydroxyl group to keep the intrinsic activity of the peptide (*205*), iii) the indole moiety of the Trp[5] side chain is not essential, being a phenyl-ethyl side chain already sufficient for maintaining high potency (*206*), iv) shortening of the Lys[6] side such as in Orn, Dab, or Dap is tolerated (*207*). It has been also demonstrated that the substitution of the C-terminal amide of Ac-RYYRIK-NH_2 with a primary alcoholic function produced a reduction of efficacy thus generating a NOP antagonist (*208*, *209*). Finally Ac-RYYRWK-NH_2 was used by Zealand researchers as parent molecule to generate the NOP ligand ZP120 (*66*) with the use of the proprietary structure-inducing probe (SIP) technology (*210*). This technology consists in the addition to a given peptide of the $(Lys)_6$ sequence. The SIP effect is to increase enzymatic stability without affecting biological activity. Most probably this effect derives from the ability of the $(Lys)_6$ sequence to promote alpha helix structure with subsequent reduction of susceptibility to peptidase action.

The pseudohexapeptide III-BTD (Ac-Arg-DCha-BTD-DArg-D(pCl)Phe-NH_2, Figure 4) was identified as non selective NOP ligand via the screening of a combinatorial beta-turn-constrained peptide library generated using a limited number of beta-inducer probes placed in the middle of the peptide sequence (*68*). Peptide III-BTD behaved as a NOP antagonist but displayed poor selectivity over classical opioid receptors. Further studies in which the 7-thia-1-aza-bicyclo[4.3.0]nonane nucleus of BTD was substituted with a quinolizidinone derivative produced a more selective NOP ligand (*211*). The structure-activity requirements of this latter peptide were then investigated by varying the position, structure, and charge of the Arg residues. Attempts to shorten the peptide abolished affinity for the NOP receptor, whereas deletion of the acetamido N-terminus maintained receptor affinity and selectivity (*212*).

NOP Peptide Ligands

This section describes and discusses the pharmacological features of a selected panel of NOP selective peptide ligands encompassing full (N/OFQ(1-13)-NH_2, [(pF)Phe[4]]N/OFQ(1-13)-NH_2, [Arg[14]Lys[15]]N/OFQ, UFP-112) and partial ([F/G]N/OFQ(1-13)-NH_2, UFP-113, Ac-RYYRWK-NH_2, ZP120) agonist as well as pure antagonist ([NPhe[1]]N/OFQ(1-13)-NH_2, UFP-101) activities, their usefulness in pharmacological and target validation studies, and in some limited cases, their possible development as drugs. The basic pharmacological profile of these molecules at human and rodent NOP receptors is summarized in Table I.

Full Agonists

The reference full agonist N/OFQ produced a concentration dependent stimulation of [^{35}S]GTPγS binding in membranes from CHO cells expressing the human NOP receptor as well as of calcium mobilization in CHO cells coexpressing the NOP receptor and the $G\alpha_{qi5}$ protein displaying very high

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potency. N/OFQ was able to inhibit the electrically induced twitch response both in the mouse and rat vas deferens (Table I). The exclusive involvement of the NOP receptor in the action of N/OFQ in these tissues has been demonstrated not only with a large panel of receptor antagonists but also with knockout studies (*86*, *101*).

N/OFQ(1-13)-NH_2 mimicked N/OFQ actions showing similar potency and efficacy as the natural peptide both at human and rodent NOP receptors (Table I). Moreover N/OFQ(1-13)-NH_2 displayed high affinity for NOP (pKi 9.00) associated with high selectivity over classical opioid receptors in guinea pig brain membranes (*187*). The NOP full agonist properties of N/OFQ(1-13)-NH_2 have been confirmed in vivo where the peptide mimicked the following actions of N/OFQ: supraspinal pronociceptive effect and inhibition of morphine analgesia, stimulation of food intake, inhibition of spontaneous locomotor activity, diuretic action, inhibition of heart rate and blood pressure (reviewed in (*31*)). Interestingly amidation of the C terminal of the peptide full sequence, i.e. N/OFQ-NH_2, slightly increased its potency both in vitro and in vivo (reviewed in (*31*)); this might be due to lower susceptibility to carboxypeptidases.

[(pF)Phe^4]N/OFQ(1-13)-NH_2 also behaved as NOP full agonist in the assays reported in Table I, however its potency was 3 to 10 fold higher than that of N/OFQ. This holds true in other in vitro assays/preparations such as cAMP levels in CHO_{NOP} cells, bioassay in the guinea pig ileum and mouse colon, and [^{35}S]GTPγS binding in membranes prepared from rat cerebral cortex membranes (*54*, *213*). In these preparations the effects of [(pF)Phe^4]N/OFQ(1-13)-NH_2 were sensitive to the NOP antagonist [NPhe^1]N/OFQ(1-13)-NH_2 that showed similar pA_2 values vs [(pF)Phe^4]N/OFQ(1-13)-NH_2 and N/OFQ. In vivo in mice (locomotor activity, pain threshold, cardiovascular parameters) and in rats (food intake) [(pF)Phe^4]N/OFQ(1-13)-NH_2 not only displayed higher potency than N/OFQ (or N/OFQ(1-13)-NH_2) but also produced longer lasting effects (*55*). The involvement of NOP receptor activation into the in vivo action of [(pF)Phe^4]N/OFQ(1-13)-NH_2 has been demonstrated in mouse locomotor activity studies using [NPhe^1]N/OFQ(1-13)-NH_2 as NOP antagonist.

After its identification by Okada et al. (*57*), [Arg^{14}Lys^{15}]N/OFQ has been pharmacologically characterized in detail. In vitro in the assays described in Table I [Arg^{14}Lys^{15}]N/OFQ displayed full agonist activity and values of potency approximately 10 fold higher than those of N/OFQ. Similar results were obtained in different laboratories in bioassay studies (mouse colon, guinea pig ileum, human bronchus) (*58*, *214*), potassium evoked amylase secretion from pancreatic lobules (*215*), stimulation of human monocyte chemotaxis (*216*). In isolated tissues the effects of [Arg^{14}Lys^{15}]N/OFQ were resistant to naloxone while antagonized by J-113397 and [NPhe^1]N/OFQ(1-13)-NH_2. Interestingly, in the rat vas deferens, a cocktail of peptidase inhibitors increased the potency of N/OFQ by 4-fold but not that of [Arg^{14}Lys^{15}]N/OFQ, thus suggesting that the chemical modification confers to the peptide some resistance to enzymatic degradation (*58*). This was recently confirmed by demonstrating that the half-life of N/OFQ and [Arg^{14}Lys^{15}]N/OFQ in the presence of trypsin are 13 and 30 min, respectively (*200*). In in vivo experiments in mice, [Arg^{14}Lys^{15}]N/OFQ mimicked the effects of N/OFQ administered i.c.v. producing pronociceptive

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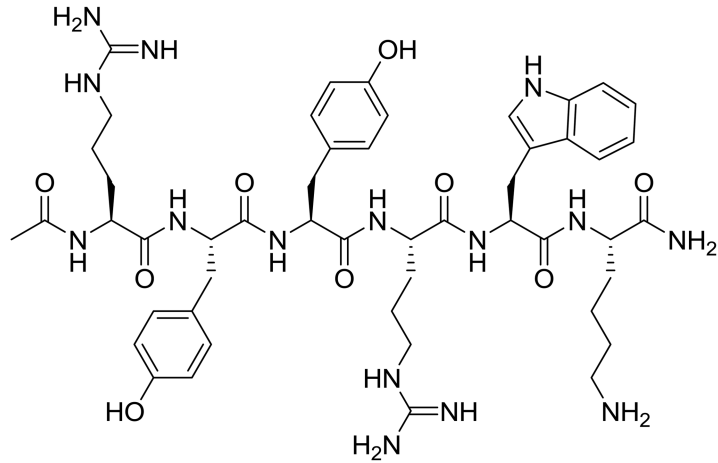
effects in the tail-withdrawal assay and inhibiting locomotor activity. In these studies [Arg^{14}Lys^{15}]N/OFQ displayed approximately 30 fold higher potency then N/OFQ and produced longer lasting effects (*58*). Similar results were obtained in rats comparing the effect of N/OFQ and [Arg^{14}Lys^{15}]N/OFQ on gastrointestinal functions (gastric secretion and emptying, colonic propulsion) (*215*) and on paracetamol-induced analgesia (*217*); in these studies the NOP antagonist UFP-101 was used to demonstrated the involvement of NOP receptor into the effects elicited by [Arg^{14}Lys^{15}]N/OFQ.



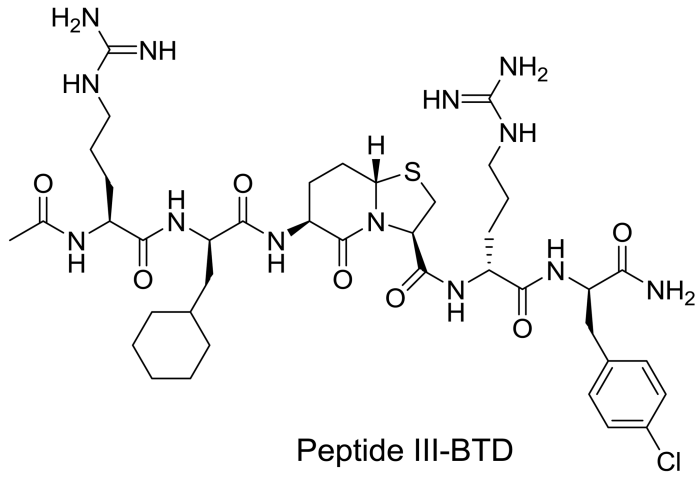


Figure 4. Chemical formula of Ac-RYYRWK-NH$_2$ and peptide III-BTD.

Table I. Pharmacological activity of NOP peptide ligands at human recombinant and rodent native receptors[a,b]

	Human NOP						*Rodent NOP*					
	[^{35}S]GTPγS			*[Ca^{2+}]$_i$*			*mVD*			*rVD*		
	pEC_{50}	*α*	*pA_2/pK_B*	*pEC_{50}*	*α*	*pA_2/pK_B*	*pEC_{50}*	*α*	*pA_2/pK_B*	*pEC_{50}*	*α*	*pA_2/pK_B*
N/OFQ	9.03	1.00	-	9.54	1.00	-	7.45	1.00	-	6.83	1.00	-
N/OFQ(1-13)-NH_2	9.24	1.06	-	9.30	0.97	-	7.41	1.01	-	6.92	0.99	-
[(pF)Phe^4]	9.99	1.15	-	not determined			8.19	1.08	-	7.95	1.03	-
[Arg^{14}Lys^{15}]N/OFQ	9.85	0.89	-	9.56[c]	1.01		8.93	0.99	-	8.13	1.02	-
UFP-112	10.55	1.03	-	9.05	1.04	-	9.24	0.97	-	8.34	1.14	-
[F/G]	8.05	0.67	-	8.03	0.55	-	slight transient effect			6.75	inactive	6.83
UFP-113	9.73	0.79	-	7.97	0.63	-	variable effects			9.10	variable effects	9.22
Ac-RYYRWK-NH_2	8.67	0.57	-	8.68	0.58	-	8.07	0.71	-	8.30	0.97	-
ZP 120	not determined			7.15	0.58	-	8.88	0.76	-	8.80	0.75	-
[Nphe^1]	inactive		7.04[d]	inactive		6.29	inactive		6.04	inactive		6.16
UFP-101	inactive		9.12	inactive		7.66	inactive		7.29	inactive		7.30

[a] [^{35}S]GTPγS, [^{35}S]GTPγS binding in membranes from CHO cells expressing the human NOP. [Ca^{2+}]$_i$, calcium mobilization in CHO cells coexpressing the human NOP and the $G\alpha_{qi5}$ chimeric protein. mVD and rVD, electrically stimulated mouse and rat vas deferens. pEC_{50}, agonist potency. α, agonist efficacy expressed as fraction of N/OFQ maximal effect. pA_2/pK_B, antagonist potency. [(pF)Phe^4], [(pF)Phe^4]N/OFQ(1-13)-NH_2. [F/G], [F/G]N/OFQ(1-13)-NH_2. [Nphe^1], [Nphe^1]N/OFQ(1-13)-NH_2. Inactive, inactive up to 10 μM. [b] Data are taken from the following references: (*31*, *52–54*, *58–60*, *63*, *65*, *66*, *124*, *228*, *229*, *259*, *260*). [c] This is an original result. [d] The assay was performed using Ro 64-6198 as agonist.

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In 2002 Zhang et al. (*56*) demonstrated that the [Aib^{7}] chemical modification increases N/OFQ potency. Thus the chemical modifications [(pF)Phe^{4}], [Arg^{14}Lys^{15}], [Aib^{7}] and C terminal amidation were combined in the same molecule generating the peptide [(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2 (UFP-112) (*60*, *61*). As showed in Table I UFP-112 displayed similar efficacy as N/OFQ but up to 100 fold higher potency in isolated tissues. Thus the above mentioned chemical modifications produced on peptide potency synergistic rather than additive effects (see for a detailed discussion (*62*)). The high potency of UFP-112 has been confirmed in other N/OFQ preparations such as the guinea pig ileum and the mouse lung. In the vas deferens and lung taken from NOP(-/-) mice UFP-112 (as well as N/OFQ) was completely inactive (*61*, *87*) demonstrating that its high potency is associated to high NOP selectivity. Moreover, the degradation half-life of N/OFQ and UFP-112 in mouse plasma and brain homogenates was studied. UFP-112 exhibited significantly longer half-lives compared to the natural peptide. In particular, the plasma $T_{1/2}$ of UFP-112 is about 3-fold longer than that of N/OFQ and this difference was even more pronounced in the mouse brain homogenate (*61*). This peptide was then used in a large series of in vivo studies investigating different biological functions including pain transmission, locomotor activity, food and alcohol intake, cardiovascular parameters, gastric and colonic functions under normal and pathological conditions (*61*, *62*, *218–222*). In all these studies UFP-112 mimicked N/OFQ actions showing values of potency approximately 100 fold higher and eliciting very long lasting effects compared to the natural peptide. The NOP dependence of some of these UFP-112 in vivo actions has been demonstrated with receptor antagonist (J-113397 or UFP-101) and/or with receptor knockout studies. Interestingly in some cases such as stimulation of food intake, decrease in heart rate and blood pressure, and inhibition of colonic propulsion the amount of the effect elicited by UFP-112 was significantly larger than that of N/OFQ (*61*, *62*, *221*). This may likely be the consequence of the prolonged activation of NOP receptors elicited by UFP-112 but not N/OFQ whose in vivo effects are in general short lasting.

Collectively this large body of evidence demonstrates that N/OFQ(1-13)-NH_2, [(pF)Phe^{4}]N/OFQ(1-13)-NH_2, [Arg^{14}Lys^{15}]N/OFQ, and UFP-112 all behave as NOP selective full agonists. Results summarized in Table I together with finding from literature indicated that the following rank order of potency of agonists, UFP-112 > [Arg^{14}Lys^{15}]N/OFQ > [(pF)Phe^{4}]N/OFQ(1-13)-NH_2 > N/OFQ(1-13)-NH_2 = N/OFQ, can be considered as the NOP receptor fingerprint.

The very high selectivity of action of NOP agonists of peptide nature make them valuable research tools. As a matter of fact, the evidence collected using these molecules was useful for increasing our knowledge on the effects of the selective activation of NOP receptor in the periphery in the respiratory, gastrointestinal, genitourinary, cardiovascular, and renal systems as well as in the central nervous system for the control of the response to stress and anxiety levels, pain transmission, regulation of food intake, control of locomotor and memory functions, and drug addiction. On the other hand, the poor pharmacokinetic properties of peptides (short half life, partial or complete inability to cross biological membranes, need of parental route of administration) limit their usefulness to target validation studies. However for some selected indications

peptides might be developed as drugs. For instance, in patients suffering from urinary incontinence due to overactive bladder clean intermittent catheterization is considered the method of choice for the management of the urinary incontinence, limiting the complications and improving the patient prognosis and quality of life. In these patients the intravesical instillation of N/OFQ produced robust beneficial effects both in acute (*149*, *150*) and chronic (10 days) studies (*151*). Obviously the intermittent catheterization may be used for the intravesical delivery of a peptide NOP agonist. Long term clinical studies are now needed to assess the therapeutic value of NOP agonists as innovative drugs to treat this condition. Another indication for which peptide NOP agonists can be evaluated as drugs is spinal analgesia. Administering drugs into the intrathecal space is becoming more popular in the treatment of patients with intractable pain (*223*) and only two drugs are approved for this indication: the gold standard opioid morphine and the N type calcium channel blocker ziconotide (*224*). However ziconotide has a poor tolerability profile while the analgesic effect of morphine displays strong tolerance liability. As mentioned before the spinal administration of N/OFQ in rodents produced opposite results: pronociceptive effects at very low doses (femto to picomole range) and antinociceptive effects at higher doses (nanomole range) (*35*, *110*). Moreover in rodents profound analgesia can not be achieved with spinal N/OFQ due to the appearance of side effects such as hind limb flaccidity. Rather different results have been obtained investigating the effect of spinal N/OFQ in monkeys where the peptide is inactive at low doses and produced in the nanomole range dose dependent and behaviorally selective analgesia (*28*, *116*). However N/OFQ displayed lower potency than morphine and produced shorter lasting effects. On the contrary UFP-112 was even more potent than morphine and produced a similar magnitude of analgesia with a similar duration of action (*222*). The antinociceptive effects of spinal UFP-112 in monkeys were sensitive to J-113397 but not to naltrexone. In addition, intrathecal inactive doses of UFP-112 and morphine produced significant antinociceptive effects when given in combination (*222*). Collectively these non human primate studies suggest that NOP peptide agonist has the potential to be developed as innovative spinal analgesics. Tolerance is one of the major problems with spinal morphine long lasting treatments and it is reasonable to foresee that this will also apply to NOP agonists. In fact tolerance has been described to the effects of spinal N/OFQ in rats (*225*). Importantly literature evidence indicate that that there is no cross-tolerance between N/OFQ and morphine in eliciting spinal antinociception (*225*). This, together with the fact that the shift from morphine to a peptide NOP agonist such as UFP-112 of a patient with a permanent spinal catheter is expected to be a rather simple procedure, makes possible to offer to patients with intractable pain an interesting option: to alternate the two drugs each time tolerance develops to one of the treatments resulting in a continuous pain relief.

Partial Agonists

[F/G]N/OFQ(1-13)-NH_2 was the first N/OFQ related peptide showing reduced efficacy (*52*). Results of Table I demonstrated that the efficacy of this NOP ligand is a fraction of that of N/OFQ at the human NOP receptor.

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In N/OFQ sensitive isolated tissues [F/G]N/OFQ(1-13)-NH_2 either produced a slight and transient agonist effect (mouse vas deferens) or was completely inactive (rat vas deferens). However when assayed in antagonist type experiments [F/G]N/OFQ(1-13)-NH_2 behaved as a competitive antagonist with pA_2 values around 7. Being one of the first synthetic ligands selective for the NOP receptor [F/G]N/OFQ(1-13)-NH_2 has been broadly used and evaluated in vitro and in vivo (reviewed in (*34*)). Both in vitro and in vivo [F/G]N/OFQ(1-13)-NH_2 behaved as a partial or full agonist or as a pure antagonist depending on the preparation or the assay. Just to give few in vivo examples, in mice [F/G]N/OFQ(1-13)-NH_2 mimicked the pronociceptive effect of N/OFQ in the tail withdrawal assay (*226*), behaved as a partial agonist in the locomotor activity test (*166*), while antagonized N/OFQ induced bradycardia and hypotension being per se inactive (*32*). This variable pharmacological activity has been interpreted (*34*) as due to the low efficacy agonist properties of this ligand whose final effect (antagonist, partial or full agonist) strongly depends on the efficiency of the stimulus-response coupling of the preparation under study. This interpretation has been later confirmed experimentally. In fact, The pharmacological activity of [F/G]N/OFQ(1-13)-NH_2 has been manipulated to encompass full and partial agonism along with antagonism using the same cells with NOP receptor density as the only variable (*227*). Interestingly [F/G]N/OFQ(1-13)-NH_2 potency is on average 10 fold lower than N/OFQ in vitro, however, the ratio of potency [F/G]N/OFQ(1-13)-NH_2 / N/OFQ obtained in in vivo studies is near 1 or even less (*34*). This difference can be explained assuming a higher metabolic stability of [F/G]N/OFQ(1-13)-NH_2 as compared to the natural peptide; the pseudopeptide bond between Phe^1 and Gly^2 probably confers such metabolic stability the molecule preventing the action of aminopeptidases. Corroborating this proposal, in some in vivo studies [F/G]N/OFQ(1-13)-NH_2 elicited longer lasting effects than N/OFQ (*34*).

The same chemical modifications used to produce UFP-112 ([(pF)Phe^4], [$Arg^{14}Lys^{15}$], [Aib^7]) have been combined with [F/G] to generate UFP-113 (*60*). As shown in Table I this peptide maintains the same pharmacological activity as [F/G]N/OFQ(1-13)-NH_2 being however approximately 100 fold more potent. Our knowledge related to UFP-113 is limited to in vitro studies but it is highly probable that UFP-113 shares with UFP-112 its in vivo properties i.e. high potency and selectivity associated with long duration of action.

In 1997, Dooley et al. identified from a large peptide combinatorial library fifteen hexapeptides with high affinity for the NOP receptor (*65*). In functional experiments these hexapeptides behaved as potent partial agonists. Table I reports data relative to Ac-RYYRWK-NH_2, the most used Dooley hexapeptide. Ac-RYYRWK-NH_2 displayed high potency with values similar to those of N/OFQ. However Ac-RYYRWK-NH_2 showed reduced efficacy in all the assays with α values in the range 0.57 – 0.75. Ac-RYYRWK-NH_2 has been evaluated in several studies mainly in vitro (reviewed in (*31*)) where, similar to [F/G]N/OFQ(1-13)-NH_2 it behaved as full or partial agonist or even as a pure antagonist. The reasons for the differing pharmacological behavior of Ac-RYYRWK-NH_2 are similar to those already discussed for [F/G]N/OFQ(1-13)-NH_2 and validated using a NOP receptor inducible system (*227*).

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Ac-RYYRWK-NH_2 was used to generate the NOP ligand ZP120 (*66*) by applying the SIP technology (*210*). This approach was indeed successful as demonstrated by the fact that in electrically stimulated mouse vas deferens ZP120 displayed the same efficacy as Ac-RYYRWK-NH_2 but approximately 10 fold higher potency (*66*). Similar results were obtained in the rat vas deferens (Table I). Interestingly at the human NOP investigated in calcium mobilization studies the potency of ZP120 was relatively low. This also applies to other NOP ligands such as UFP-112 and UFP-113 (Table I). All these compounds are characterized by a slow kinetic of interaction with the receptor as suggested by bioassay experiments in isolated tissues (*61*, *66*). Thus, the slow kinetic of action of these ligands may be relevant for the estimation of their potency in the $G\alpha_{qi5}$ NOP receptor calcium assay. In fact, the long time required to get full activation of NOP receptors with these ligands may be incompatible with the rapid kinetics which characterized the calcium transient response (for a detailed discussion of this topic see (*228*)). Thus the calcium mobilization assay clearly underestimates the potency of this panel of NOP ligands.

The effects of ZP120 in the mouse vas deferens are due to selective NOP receptor activation as demonstrated by antagonist (*66*) and knockout (*229*) studies. More importantly ZP120 displayed very high potency and long duration of action in vivo in locomotor activity and tail withdrawal experiments in mice (*66*). The effects of ZP120 in the latter test were no longer present in NOP(-/-) mice (*229*).

In summary [F/G]N/OFQ(1-13)-NH_2, UFP-113, Ac-RYYRWK-NH_2, and ZP120 all behaved as selective partial agonists at NOP receptor showing the following rank order of potency: UFP-113 > ZP120 > Ac-RYYRWK-NH_2 > [F/G]N/OFQ(1-13)-NH_2.

The variable pharmacological activity displayed by these ligands on the different functions controlled by N/OFQ can be exploited to obtain selective actions. For instance after i.v. administration NOP partial agonists produce per se negligible effects on cardiovascular functions (where they behaved as antagonists) while full agonists consistently evoke bradycardia and hypotension (*230*). On the other hand, as far as renal functions are concerned full and partial NOP agonist are able to elicit similar diuretic, in particular aquaretic, effects (*230*). Therefore for those conditions e.g congestive heart failure for which aquaresis is beneficial while cardiovascular depressor effects are unwanted, NOP receptor partial agonists (but not full agonists) can be proposed for drug development. As a matter of fact, the NOP partial agonist ZP120 displayed in rats sodium-potassium-sparing aquaretic activity without showing relevant cardiovascular side effects (*67*). This favorable profile of action of ZP120 has been later confirmed in humans in phase I and II clinical trials (*36*).

Similar considerations can be proposed for other biological actions in which partial agonists mimicked N/OFQ effects. For instance after i.v. administration, [F/G]N/OFQ(1-13)-NH_2 mimicked the inhibitory effects elicited by N/OFQ on the micturition reflex in rats without causing concurrent bradycardia and hypotension (*148*). This suggests that NOP partial agonist should be preferred to full agonists for the development of a peripherally acting drug to treat urinary incontinence due to neurogenic bladder.

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Antagonists

[NPhe^1]N/OFQ(1-13)-NH_2 was the first peptide antagonist reported in literature. The NOP antagonist properties of this ligand were first assessed in the mouse colon where the peptide was able to block the contractile effect of N/OFQ but not of endomorphin-1 showing a competitive type of antagonism and low potency (pA_2 6.0) (*231*). These findings were confirmed and extended in a detailed study (*53*) demonstrating that [NPhe^1]N/OFQ(1-13)-NH_2 binds selectively to recombinant NOP receptors and competitively antagonizes the inhibitory effects of N/OFQ in CHONOP cells on cyclic AMP accumulation (pA_2 6.0), and in electrically stimulated isolated tissues of the mouse, rat and guinea-pig (pA_2 6.0 - 6.4). [NPhe^1]N/OFQ(1-13)-NH_2 was also active in vivo, where it prevents the pronociceptive and antimorphine actions of i.c.v. N/OFQ. Data shown in Table I are in line with these results both in terms of pure antagonist properties and of relatively low potency. These features of [NPhe^1]N/OFQ(1-13)-NH_2 were later confirmed in numerous studies performed in different laboratories both in vitro and in vivo; this large body of evidence has been previously reviewed (*34*, *232*). In order to increase ligand potency the chemical modifications [NPhe^1] and [Arg^{14}Lys^{15}] were combined into the same peptide sequence generating UFP-101 (*63*). As showed in Table I this peptide maintains the same pharmacological activity of [NPhe^1]N/OFQ(1-13)-NH_2 i.e. pure antagonism being however at least 10 fold more potent. The NOP selective antagonist properties of UFP-101 have been confirmed in several in vitro and in vivo studies investigating most of the biological actions of actions of N/OFQ including locomotor activity, pain transmission, neurochemical actions, food intake, cardiovascular, kindney and gastric functions (reviewed in (*64*)). A tritiated version of UFP-101 was also generated and found useful for receptor binding studies at human recombinant and animal native NOP sites (*233*). More recent studies investigated UFP-101 (and N/OFQ) effects in the regulation of the following functions: pain transmission in the brain (*234*, *235*) and spinal cord (*90*, *94*), memory (*236*), food intake (*218*), drug reward (*103*, *237*), hypothalamic-pituitary-adrenal axis responses (*238–240*), anxiety (*241*), depression (*161*, *162*), locomotor activity (*171*, *242*), sexual behaviour (*243*), gastrointestinal (colon (*221*, *244*), stomach (*219*, *220*, *245*), and pancreas (*246*)), respiratory (*87*) and cardiovascular (*247–249*) functions, and inflammation (*180*, *250*).

Clearly this large body of evidence firmly demonstrated that the [NPhe^1] modification confers to [NPhe^1]N/OFQ(1-13)-NH_2 and UFP-101 NOP antagonist properties. However, there are also some few results indicating that the elimination of ligand efficacy produced by [NPhe^1] could not be complete. In a cAMP level study performed in intact cells and in cell membranes, sodium and GTP concentrations affected the potency of [NPhe^1]N/OFQ(1-13)-NH_2 in a manner similar to that of agonists (N/OFQ) but not of pure antagonists (J-113397) (*251*). In addition, in [^{35}S]GTPγS binding studies performed in membranes of cells expressing the NOP-G_0 fusoprotein GTP concentrations modulated [NPhe^1]N/OFQ(1-13)-NH_2 and UFP-101 efficacy. In the micromolar range of GTP both ligands behaved as pure antagonists while reducing GTP concentrations produced a clear increase of [NPhe^1]N/OFQ(1-13)-NH_2 and UFP-101 efficacy up

to $\alpha \approx 0.8$ for GTP concentrations in the low nanomolar range (T. Costa, personal communication). Finally, in an elegant elecrophysiological study performed measuring calcium currents in control rat stellate ganglion neurons and in neurons microinjected with a plasmid coding for the NOP receptor it was found that C-24 or Trap-101 behaved as pure antagonists in control neurons and as inverse agonists in transfected neurons. On the contrary, UFP-101 acted as an antagonist in control cells while displayed a partial agonist behaviour in transfected neurons (*252*). It should be underlined that these observations have been made under extreme conditions (very low concentrations of GTP, very high levels of expression of NOP receptors) and this strongly limited their physiological significance. Despite this, the above mentioned evidence suggests that differences may exist in the way the peptide antagonists bind to the NOP receptor compared to antagonists of non peptide nature. These differences should be however subtle since molecular docking studies performed on the NOP receptor crystal structure indicate that the N terminal tetrapeptide of UFP-101 and C-24 occupy the NOP binding pocket in a very similar manner (*106*).

Despite these speculative considerations, there is no doubt that NOP peptide antagonists contributed to increase our knowledge in this field in several important manners. First, pharmacological studies with [NPhe^1]N/OFQ(1-13)-NH_2 and UFP-101 demonstrated the involvement of the NOP receptor into several in vitro and in vivo actions of N/OFQ. In most cases, this has been confirmed with the use of non peptide antagonists such as J-113397, SB 612111 and C-24 and/or with NOP(-/-) mice. Importantly NOP peptide antagonists were also useful for demonstrating the NOP dependence of the effects of synthetic NOP agonists such as UFP-112 (*62*), Ro 64-6198 (*253–255*), or buprenorphine (*237*, *256*). Second, peptide NOP antagonists were useful for investigating the role played by the endogenous N/OFQ-NOP receptor system in controlling biological functions. In fact, [NPhe^1]N/OFQ(1-13)-NH_2 and/or UFP-101 not only blocked N/OFQ actions but produced per se opposite effects on locomotor performance on the rotarod (*98*), stress induced analgesia (*257*), ibotenate induced neurotoxicity (*258*), and mortality in an animal model of sepsis (*180*). Moreover peptide antagonists for the NOP receptor produced antidepressant like effects in the forced swimming, tail suspension and chronic mild stress assays (*95*, *96*, *160*, *162*). Of note in these latter tests N/OFQ was inactive per se but was able to revert the effects of the antagonists. Third, NOP peptide antagonists were instrumental for performing target validation studies with the aim to foresee the possible therapeutic indications of NOP selective antagonists. These studies compared the effects of [NPhe^1]N/OFQ(1-13)-NH_2 and UFP-101 with those elicited by non peptide antagonists (in most cases J-113397, SB 612111, or C-24) and with the phenotype of NOP(-/-) mice. In such way a large body of evidence has been collected indicating that NOP antagonists are worthy of development as innovative drugs for the treatment of Parkinson disease and depression. For such indications the development of orally active brain penetrant non peptide molecules is mandatory for performing clinical investigations aimed at firmly identify their effectiveness in patients and eventually their place in therapy. Limited information also suggests that NOP antagonists may exert beneficial effects in some inflammatory conditions such as ulcerative colitis (*178*, *179*) and

sepsis (*180*). In the latter case peptide antagonists such as UFP-101 can possibly be developed as drugs since it can be easily administrated as intravenous infusion to critically ill patients in intensive care units.

Conclusions

The broad pharmacological spectrum of N/OFQ actions points to a number of potential therapeutic applications of selective NOP receptor ligands. Structure activity studies on N/OFQ sequence allowed to understand its most important residues for NOP receptor occupation and activation and to generate highly potent and selective ligands encompassing pure antagonist (UFP-101), and partial (UFP-113) or full (UFP-112) agonist activities. These compounds together with non peptide molecules identified by industrial laboratories and NOP(-/-) animals allowed the scientific community to collect a rather large body of evidence on the biological functions controlled by the N/OFQ – NOP receptor systems. Thus, based on the available information, NOP receptor agonists are claimed to be potentially useful for treating stress and anxiety, drug addiction, anorexia, and cough. One advantage of NOP receptor agonists over currently used drugs such as benzodiazepines or opioids would be the lack of abuse potential. However, given their broad spectrum of actions, NOP receptor agonists may also be anticipated to have many unwanted effects. This however might not be true for selected indications for which systemic administration is not needed such as urinary incontinence (intravesical route) and intractable pain (spinal route). For such indications peptide NOP agonists are worthy of development. NOP selective partial agonists might be preferable to full agonists for some particular indications such as induction of water diuresis. NOP receptor antagonists could be useful for treating Parkinson's disease and depression, and perhaps as novel antinflammatory agents. Antagonists are expected to have fewer side effects than agonists, as suggested by the fact that NOP(-/-) mice and rats appear to be outwardly normal. For some indications such as sepsis peptide antagonists can be used as drugs.

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Chapter 16

A Review of the NOP (ORL-1)-Nociceptin/ Orphanin FQ System Covering Receptor Structure, Distribution, Role in Analgesia and Reward and Interactions with Other Receptors

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The nociceptin/orphanin FQ receptor (NOP; also known as ORL-1, OP4) was first identified and cloned in the mid-90s using reverse pharmacology; shortly after the endogenous ligand nociceptin/orphanin FQ (N/OFQ) was identified. Since these initial discoveries there have been extensive investigations performed, all aimed toward gaining an understanding of NOP's peripheral, spinal, and supraspinal roles in physiological and pathophysiological processes. This article reviews the molecular biology, in vitro and in vivo pharmacology of the nociceptin system, including the structure-activity relationships associated with the endogenous agonist, as well as the NOP receptor structure and binding-site elucidation studies. As the receptor and its endogenous ligand are widely expressed a cross-species comparison of the anatomical distribution pattern is presented focusing on CNS expression where most literature data is available. While the nociceptin system has been implicated in a wide range of biological actions, the similarities between NOP and the classical opioid receptors have ensured much of this work has centered on the role of NOP in pain transmission, substance abuse, and tolerance; as such this review includes advances from studies in these areas. Where appropriate comparison to the classic opioid system is included and, in addition, literature that is supportive of a central NOP-mediated, regulation of the MOP receptor is presented.

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Finally the status of the first generation of compounds with activity at NOP receptors to enter the clinic is provided.

Introduction

Shortly after the reported cloning of the classical MOP, DOP and KOP opioid receptors, several laboratories cloned a highly homologous receptor that was not activated by the classical endogenous opioid ligands (*1–4*). This receptor was originally named ORL-1 since it's primary sequence was *"Opioid Receptor-Like"*, but it has also been named OP4, the nociceptin receptor, and NOP, the latter stands for nociceptin/orphanin FQ (N/OFQ) peptide receptor and is most consistent with IUPHAR guidelines.

Since it's initial discovery there have been extensive investigations performed, all aimed toward gaining an understanding of NOP's peripheral, spinal, and supraspinal roles in physiological and pathophysiological processes. Robust reviews of the NOP literature have been published regularly during the past 15 years (*5–17*). Small molecule (non-peptide) agonists and antagonists that act upon the NOP receptor have been discovered and five comprehensive reviews of these first generation exogenous NOP ligands have been published (*5*, *9*, *15*, *18*, *19*).

Due to the similarities between NOP and the classical opioid receptors, a significant number of the published NOP manuscripts are related to pain transmission, substance abuse, and tolerance, so the focus of this review is upon the most recent developments in these areas. In addition, literature that is supportive of central NOP-mediated, MOP regulation is presented. Finally, the structure-activity relationships associated with the endogenous agonist, as well as the NOP receptor structure and binding-site elucidation studies are also reviewed here since this latter literature may be useful in the design of novel agonists and antagonists of the NOP receptor. The possible participation of the NOP system in other areas including epilepsy (*20*), gastrointestinal function (*21–24*), circadian rhythms (*25–27*) kainite-related seizures (*28*, *29*), cough (*30–33*), colitis (*34*), cardio-renal functions (*35–37*), and neutrophil chemotaxis (*38*), will not be reviewed here, although the aforementioned citations may be a useful starting point for deeper literature investigation.

Some cellular actions of NOP are similar to those of the MOP, KOP and DOP opioid receptors. For example, agonist binding leads to G protein-coupling (G_i/G_o) and inhibition of adenylyl cyclase causing an intracellular decrease in cAMP levels (*39*, *40*), as well as activation of mitogen-activated protein kinases (MAPK) (*41*, *42*). Moreover, there is an activation of inwardly rectifying K^+ currents in many brain regions and cells (*43–47*). NOP activation also modulates a variety of voltage dependent calcium currents, including N-type (*48*), L-type, and P/Q-types (*49–51*). Activation of this receptor has been reported to further inhibit R-type channels in some *in vitro* neuronal preparations, and has been reported to be a potent inhibitor of T-type channels in rat dorsal root ganglion (DRG) neurons (*49*, *51–53*).

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Further investigation into the mechanism by which NOP activation can inhibit N-type channels has revealed that NOP receptors can form association complexes with N-type channels (*54*). The NOP-N-type calcium channel complex, characterized by C-terminal protein-protein interactions, was detected in expression systems and in DRG neurons. The proposed complex results in an agonist independent, receptor concentration-dependent inhibition of the channel. The authors have suggested that the activity of the N-type channels, and consequently the transmission of pain, may be regulated in part by NOP receptor density on the surface of DRG neurons. It is of related interest that NOP receptor upregulation occurs as a result of nerve injury (*55*, *56*). In follow-on publications, it was demonstrated that these complexes are internalized to vesicular compartments following prolonged exposure to the endogenous NOP agonist, N/OFQ (*57*) and that this internalization may be dependent on the formation of NOP/MOP opioid heterodimers (*58*) although loss of N-type channels from the membrane was not confirmed by electrophysiological experiments (*59*). The formation of these complexes causes decreased N-type calcium channel-mediated calcium entry into cells, occurs selectively for N-type channels and is not observed for MOP opioid receptors (*58*).

Receptor Structure and Endogenous Agonist SAR

The NOP receptor shares a high degree of sequence homology with the classical opioid receptors. The similarity of the NOP receptor sequence to the MOP receptor sequence is approximately 67% and the overall homology between the 4 receptors is approximately 63%. The three dimensional structure of the NOP receptor contains the hallmark features of other G protein-coupled receptors (GPCR), specifically containing 7 helical transmembrane (TM) domains, 3 extracellular (EL1-EL3) and 3 intracellular (IL1-IL3) loops, as well as N-terminal and C-terminal domains, extracellular and intracellular, respectively. The crystal structure of NOP in complex with a spiropiperidine-containing antagonist named compound 24 (C-24) (*60*) reveals potentially important differences when compared to recent published structures of MOP and KOP receptors (*61*). The proline-induced kinks within the seven transmembrane domains of NOP occur in helices 2, 4, 5, 6, and 7 and uniquely alter the local topology near the presumed orthosteric binding pocket since these kinks occur elsewhere in other the classic opioid receptors. As compared to MOP and KOP opioid receptors, the top of transmembrane 5 (near the extracellular face) of NOP is shifted by more than 4 Å, resulting in a gap between transmembrane domains 4 and 5 that uniquely expands the orthosteric binding pocked in NOP. Extracellular loop 2 in NOP is highly acidic due to a significant number of glutamate and aspartate residues contained therein. It is proposed that this region makes up part of the endogenous agonist binding site, since it is electrostatically complementary to the basic residues in the C-terminal portion of the endogenous agonist, nociceptin. These structural elements are likely to be the main determinants of ligand specificity at the NOP receptor (*61*).

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When compared to the other opioid receptors, the second extracellular loop (EL2) of NOP is most similar to the KOP receptor in that it contains seven acidic residues (KOP contains 8). The analogous loop in MOP and DOP receptors is substantially different, containing only two acidic residues each. Close examination of EL2 in both NOP and KOP receptors reveals interesting differences. For the former, 5 of the 7 acidic residues in the loop are aspartic acid and are tightly localized on the N-terminal side of EL2. In the KOP receptor, 6 of the 8 acidic residues in EL2 are glutamic acid and are equally partitioned on the N-terminal and C-terminal sides of the loop, four on each side. These arrangements are illustrated in Fig. 1.

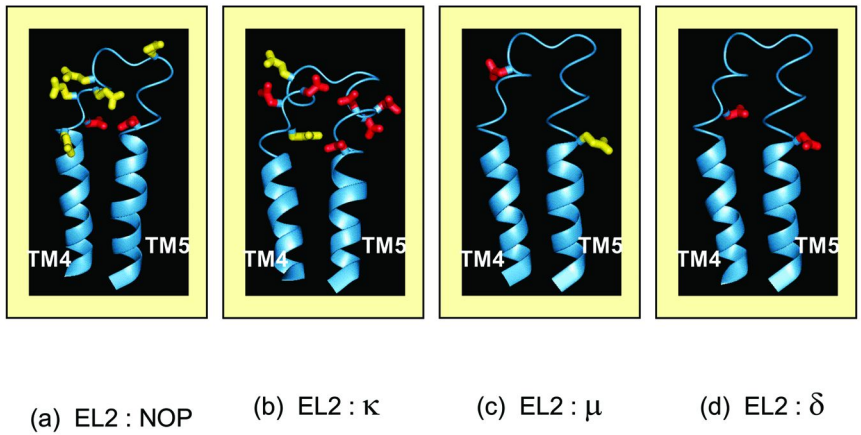

Figure 1. Representation of the second extra-cellular loop (EL2) region of the (a) NOP, (b) KOP, (c) MOP, and (d) DOP receptors. Transmembrane helices 4 and 5 are depicted for illustrative purposes. Aspartic acid is colored yellow and glutamic acid is colored red.

The important role of the extracellular loop domains in opioid receptor binding has been published (*62–65*) and it is clear that the arrangement of acidic residues in EL2 of the NOP receptor is quite unique, and is likely to partially account for the ligand specificity known for this receptor. Receptor binding experiments from several KOP and NOP endogenous agonist hybrid sequences have revealed that binding interactions occur between NOP's unique acidic EL2 environment and the basic residues in the N-terminal "address" portion of the endogenous agonist (*66*, *67*). Photoaffinity labeling experiments provide further evidence that part of the NOP binding site is in the extracellular loop domain, specifically that residues 296-302 of EL3 are near the ligand binding pocket and ILE^{300} is very close to the N-terminus of the endogenous agonist when bound (*68*).

A second zone of the ligand-binding domain on the NOP receptor is the transmembrane (TM) pocket that is typically associated with ligand binding to GPCRs. This domain may be the recognition site for the N-terminal "message" segment of the endogenous agonist. Binding experiments using NOP receptor

sequences, modified via mutagenesis to include residues that are conserved near the TM binding sites of MOP, DOP, KOP receptors (but divergent in NOP) are supportive of this hypothesis (*69*).

Specifically, a 3 consecutive amino acid replacement (Val^{276}-Glu^{277}-Val^{278} → Ile^{276}-His^{277}-Ile^{278}) in TM6 of the NOP receptor improved the NOP affinities of bremazocine and naltrexone, two ligands that have no affinity to the wild type NOP receptor (Table 1). The "I-H-I" sequence that was inserted is a conserved motif from MOP, DOP, and KOP receptors. The same authors also showed that a single mutation, again changing a native residue in NOP to a conserved (MOP, DOP, KOP) residue (Ala^{213}->Lys^{213}) increased the affinity of bremazocine and naltrexone by more than 2-orders of magnitude (Table 1). This residue is situated at the top of TM5, near the C-terminal end of EL2. In these experiments competition binding was performed against 50 pM [^{125}I]-Tyr^{14}-N/OFQ, where N/OFQ refers to nociceptin, the endogenous peptide agonist for the NOP receptor (discussed in a later section of this review).

Table 1. Naltrexone and bremazocine binding affinities to wild type and mutant NOP receptors (*69*)

	TM5 *(A^{213} → K^{213})*	*TM6* *(V^{276}-E^{277}-V^{278} → I^{276}-H^{277}-I^{278})*	*NOP Wildtype*
Bremazocine	K_i= 38 nM	K_i= 150 nM	K_i> 5000 nM
Naltrexone	K_i= 22 nM	K_i= 400 nM	K_i> 5000 nM

Alanine substitution of five conserved residues in the TM pocket, Asp^{130}, Tyr^{131}, Phe^{220}, Phe^{224}, Trp^{276}, followed by N/OFQ receptor binding assays revealed that the most detrimental to N/OFQ binding was Asp^{130}->Ala^{130}, which occurs in TM3 (*70*). No recovery of receptor function was observed when Asp^{130} was further converted to Asn^{130}, leading the authors to conclude that a negative charge in the side chain at this position is critical for ligand binding affinity (*70*). The analogous Asp residue is highly conserved across the entire GPCR family of receptors, where it is thought to be the counter ion for basic amines and ammonium ions contained in the respective ligands. It has likewise been proposed that Asp^{130} of NOP is the critical counter ion for the N-terminal amino group of N/OFQ during binding (*71*).

Molecular modeling of the NOP receptor has been published and many of these models incorporate the known results from mutagenesis experiments to improve accuracy and to aid in the ligand docking process (*71*, *72*). In two of these molecular modeling papers, Asp^{130}, the "FGGF" binding pocket within TM helices 3, 5, 6, 7, and the acidic EL2 are accounted for during the docking of N/OFQ (*71*, *72*). In the third paper, Asp^{130} and Thr^{305} are used as guides to dock a variety of non-peptide NOP agonists, primarily in the "FGGF" binding pocket (*73*).

Shortly after the discovery of the NOP receptor was published, an endogenous agonist was purified and the corresponding sequence of amino acids was published almost simultaneously by two different groups (*39*, *40*), then soon after by a third group (*74*). This 17 amino acid peptide has the primary sequence, "FGGFTGARKSARKLANQ", and is most often referred to as either orphanin FQ (OFQ) (*39*) nociceptin (*40*) or as *N/OFQ*. This peptide is contained within a precursor protein (181 amino acids in length) known as prepronociceptin (ppN/OFQ) which encodes for N/OFQ as well as other biologically active peptides such as nocistatin (*75*, *76*). Within this precursor, N/OFQ is flanked on both sides by pairs of basic amino acids that are known substrates for certain endopeptidase enzymes (*77*). This ppN/OFQ arrangement is another similarity between the NOP and KOP receptor, in that the endogenous KOP agonist dynorphin A (Dyn A) is also embedded in a precursor protein known as preprodynorphin (*78*).

As one approach toward understanding the interactions between N/OFQ and the NOP receptor during binding, NMR experiments have been performed in support of elucidating the solution conformation of N/OFQ (*79*, *80*). In summary, N/OFQ shows little tendency to form ordered conformation in water or other order-inducing solvents such as SDS micelles.

When compared to the other endogenous opiate peptides, the sequence of N/OFQ is most similar to dynorphin A (Dyn A), the endogenous KOP agonist. Fig. 2 illustrates some similarities and differences between N/OFQ and Dyn A, particularly in the "address" segment near the C-terminus. Both peptides are 17 amino acids in length, terminate with "NQ", and have similar N-terminal "message" segments ("YGGF" for Dyn A, "FGGF" for N/OFQ). The "address" segments of these two peptides are highlighted in Fig. 2. Within the segment between positions 5 and 15 in both N/OFQ and Dyn A, there are 11 amino acids. Positions 9 and 13 are conserved basic amino acids in both peptides. At the center of the "5-15" segment (position 10), there is a serine in N/OFQ, or a proline in Dyn A. The inherent conformational constraint in the backbone of the latter would, by definition, create a bend or kink in the Dyn A peptide that is likely to be very different from the N/OFQ peptide.

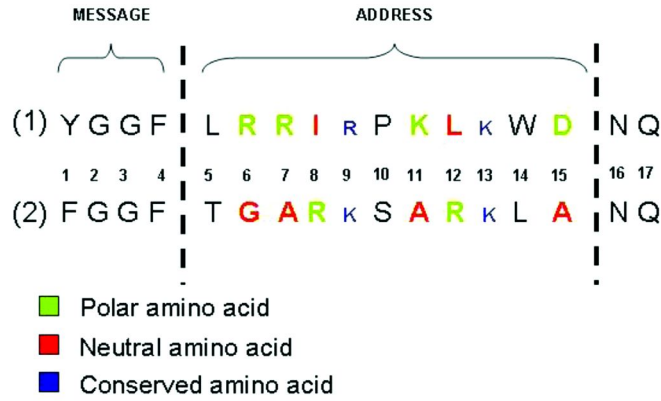

Figure 2. Sequence comparison between (1) dynorphin A and (2) nociceptin.

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Of interest is the observation that the polar/neutral character of most of the remaining side chains in this segment are opposites between N/OFQ and Dyn A. Specifically positions 6, 7, 11, and 15 (Gly^6, Ala^7, Ala^{11}, Ala^{15}) in N/OFQ contain chemically neutral side chains that correspond to Arg^6, Arg$_7$, Lys^{11}, Asp^{15} in Dyn A, all of which have polar side chains. Positions 8 and 12 in N/OFQ are both polar Arg residues, and the corresponding positions in Dyn A are occupied by neutral Ile^8 and Leu^{12}, respectively. The apparent "opposing" chemical character of most of the amino acids in the "address" segment of these two peptides, together with the unique proline-induced bend about position 10 in Dyn A, likely contribute to their receptor specificity profiles.

Using a combination of computational approaches and design of conformationally-constrained peptides, an early model of the receptor-bound conformation of N/OFQ was proposed. Specifically, a series of novel N/OFQ analogs, wherein Ala^7, Ala^{11}, Ala^{15} were replaced by the α-helix promoting residue Aib (*81*, *82*), were found to be highly potent NOP agonists with binding affinities and functional efficacies similar to, or better than, N/OFQ itself (*83*). A complementary series of peptides using the extended backbone-promoting residue N-methylalanine at the same positions led to peptides with lower affinity and weaker agonist efficacies than N/OFQ. The authors proposed for the first time that an amphipathic helical conformation in the "address" segment range of 7-15 of the N/OFQ sequence might be representative of the receptor-bound conformation. A subsequent publication from another group confirmed this hypothesis by using leucine residues as helix promoters at position 7, 11, and 15 of the sequence (*84*). Moreover, 2D NMR experiments were reported that showed proton-proton NOE patterns characteristic of a helical structure in the "address" segment of the peptides. The same authors also described a feasible docked model of helical N/OFQ, interacting with the two-part binding site on the NOP receptor. Most recently, a similar proposed model of O/FQ bound to the NOP receptor was proposed based on crystallography and docking experiments (*61*).

A wide variety of N/OFQ analogs have been reported since publication of the sequence of the endogenous peptide. One publication described three series of N/OFQ related peptides which varied in the peptide bond between the first two amino acids to include a regular peptide bond, a pseudopeptide bond and a peptoid bond (*85*). Analogs containing the regular peptide bond acted as potent NOP receptor agonists as expected; utilization of a pseudopeptide bond gave rise to analogs that behaved as antagonists in an assay utilizing inhibition of electrically evoked contraction of mouse vas deferens, while behaved as full agonists in CHO cells expressing the N/OFQ rectors. Finally the majority of the compounds containing the peptoid bond did not display affinity for N/OFQ. The authors concluded that modification of the steric orientation of Phe^1 results in a reduction or elimination of efficacy. A second publication described the incorporation of disulfide bonds into the N/OFQ sequence, although the native sequence was altered to contain two cysteine residues and the C-terminus was truncated (*86*). It was reported that cyclizations at the N-terminal or middle portion of the peptide significantly diminished binding and functional potency, but C-terminal cyclization led to agonists with *in vitro* potencies similar to N/OFQ.

One of the differences between N/OFQ and other classical endogenous opioid agonists is the presence of a Phe at the first position, rather than a Tyrosine. N/OFQ is known to have minimal affinity to other opioid receptors but when Phe^1 is replaced by Tyr^1 in N/OFQ the affinity to the classical opioid receptors is reportedly increased by 10-40 fold (*87–91*). The largest effect was on MOP receptors. This modified N/OFQ peptide was further reported to retain affinity to the NOP receptor comparable to the endogenous agonist, N/OFQ, providing evidence that part of the receptor selectivity profile is derived from position one of the primary N/OFQ sequence. Consistent with it's *in vitro* profile as a NOP agonist, Tyr^1- N/OFQ has also been shown to decrease systemic arterial blood pressure in rat (*87*), and inhibit electrically-induced contractions of isolated guinea pig bronchus and ileum (GPI) (*88*, *92*).

Leu^{14} of the native N/OFQ sequence has been systematically explored using R, K, A, F, Y, and W amino acid substitutions (*93*). The published results indicate that NOP binding affinity for each modified peptide was similar to N/OFQ, indicating significant side chain tolerance at position 14. Peptide truncation studies were used to identify N/OFQ(1-13)-NH_2 as an NOP agonist with similar potency as N/OFQ (*94*). Subsequent SAR series based on N/OFQ(1-13)-NH_2 revealed that basic residues, notably $Arg^{8,12}$ and $Lys^{9,13}$ in truncated peptides are important for functional activity since replacement by Ala leads to inactive peptides (*95*). A series of C-terminally truncated N/OFQ analogs further support this observation (*96*).

Several modifications to the amide bond linking Phe^1-Gly^2 in the peptide N/OFQ(1-13)-NH_2 have been published. Specifically the replacement of CO-NH in the Phe^1-Gly^2 bond with CH_2-NH decreased selectivity for NOP versus MOP, a retro-inverso bond (NH-CO) led to a peptide with no activity at NOP, and CH_2-O caused 3-fold loss in agonist potency (*97*). The reduced amide analog at the same position, [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]nociceptin-(1-13)-$NH_2$ was originally shown to be a selective antagonist of NOP receptor function in isolated GPI and mouse vas deferens (*98*). A substantial and often contradictory body of literature has arisen around this peptide. According to the literature, it is a potent NOP agonist in CHO cells expressing human NOP (*99*), acts as a partial agonist on NOP receptors in rat periaqueductal gray (*100*), is a potent agonist when administered spinally to rats (*101*), is a neutral antagonist in CHO cells overexpressing the expressing human NOP (*102*) and in the isolated rabbit ileum (*103*), and is a full agonist in an *in vivo* mouse model of pain (*104*) yet antagonizes the pronociceptive and antiopioid actions of i.c.v. N/OFQ (*102*). One hypothesis for these differing results is based on differing GTP binding efficacy (*105*) and/or in receptor reserve between the differing systems/species. Regarding receptor reserve and expression levels, substantial evidence for this hypothesis has been obtained using an inducible expression system; in these studies a single peptide active at NOP receptors can present as a full agonist, partial agonist or antagonist in the same cell system when the only variable is NOP expression level (*106*).

Further modifications of Phe^1 in N/OFQ(1-13)-NH_2 include 3′,6′-dimethylphenylalanine (Dmp) at positions 1 and 4 of the sequence (*107*, *108*). The former resulted in a peptide of similar high NOP affinity as N/OFQ(1-13)-NH_2, but also showed enhanced affinity toward MOP, DOP and KOP. The latter decreased

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NOP affinity. In a recent publication, many of these results were combined to prepare several new N/OFQ analogs containing multiple modifications (*109*). Twelve peptides were assayed in CHO cells expressing the human NOP receptor, in electrically-stimulated mouse vas deferens, and in isolated GPI assays. Peptides with a regular amide linking Phe^1-Gly^2 behaved as NOP agonists, while those with the reduced amide at the same position were reportedly partial agonists. In that work, the side chain of Phe^1 was also moved from the α-carbon to the N-terminal amino nitrogen atom thus creating a N-benzylglycine residue. The authors reported that this can lead to peptides that are either partial agonists or neutral antagonists *in vitro*, depending on the presence or absence of (*p* fluoro)Phe^4, respectively (*109*).

Combinatorial libraries of peptides have also been described, from which several new sequences were identified that are not related to N/OFQ, yet still act upon the NOP receptor. One feature that these new sequences share with N/OFQ is the presence of many polar amino acids such as Lys and Arg in the sequence. Several of these peptides contain various β-turn mimetics in the sequences (*110*, *111*). Some of these peptides are NOP selective while others non-selectively bind to MOP, DOP and KOP receptors.

In other work, the peptide sequence "Ac-R-Y-Y-R-I-K-NH_2" was reported to be a high affinity, partial agonist at NOP (*112*, *113*). This peptide was discovered by a combinatorial approach involving randomization of a hexapeptide sequence. Subsequent N- and C-terminal modifications of this sequence led to the discovery of "Ac-RYYRIK-ol", which is described as a high affinity, NOP antagonist, with a K_i similar to wild type N/OFQ (*114*). Using a close analog from this series (Ac-RYYRWK-NH_2) to inform design of a tool molecule to investigate the hypothesis that a peripherally-acting NOP agonist might have therapeutic utility to elicit diuresis, a peripheralized NOP agonist known as ZP120 was created with the sequence is "Ac-RYYRWKKKKKKK-NH_2" (*115*). The rationale for the C-terminal homologation by 6 sequential lysine residues is their propensity to form a helix, thus protecting the N-terminal segment from enzymatic degradation. The pK_i for ZP120 binding to NOP is reportedly 9.6. (*116*). At a dose of 1 nmol/kg/min, ZP120 produced a slight, but significant reduction in mean arterial blood pressure, but had no effect on heart rate (i.v. infusion doses of 0.1, 1.0 and 10 nmol/kg/min) (*116*). An increase in urine output occurred at all three of these doses, and evoked significant reductions in urinary sodium and potassium excretion (*116*). The authors postulate that, due to the partial agonist profile of ZP120, it may not mediate strong NOP receptor internalization to the same extent as full agonists (*117*). The hypothesis is that the partial agonism of ZP120 may render the renal and cardiovascular responses less likely to develop tolerance. Taken together with the improved PK profile of ZP120 over its parent, "Ac-RYYRWK-NH_2", the long lasting effects of this peripherally-restricted peptide are understandable.

Although beyond the scope of this review to compare and contrast all prior homology models of NOP with the recently published crystallographic structure there are several caveats associated with homology modeling techniques that have likely led to inconsistencies. Notably, the positions of the proline residues in the transmembrane domains of the homology template (rhodopsin) are not

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conserved in the NOP, meaning that the kinks in the helices are likely misplaced in many of the computer models. Since the orthosteric site is in the transmembrane domain, inaccuracies in the binding sites of the models are likely, especially if one further considers that the helical tilts in the NOP structure and rhodopsin are also not the same. Second, the creation of homology models requires various computational methods to create extra- and intra-cellular loops, none of which are based on the rhodopsin structure since loops were not solved in the original structure. This makes it highly unlikely that the loop regions of the homology models accurately predict the crystallographically-derived loop conformations of NOP. This is important given the proposed role of the highly acidic extra-cellular loop two of NOP in binding certain ligands which is likely not modeled correctly in most computational NOP structures. A final point worth considering in this general comparison is that while a major achievement, even the recently solved crystal structure of NOP (*61*) is not bound to an agonist and the receptor sequence has engineered modifications in the N- and C-terminal domains that facilitated the crystallography. Hence it is not a purely native system, meaning that computational procedures will still be utilized in the coming years to further model and understand the structural basis of ligand binding and function.

Distribution

N/OFQ and NOP receptors are widely expressed across multiple systems including nervous, gastrointestinal, hepatic, splenic, reproductive and renal and often by multiple cell types within each system (*75*, *76*, *118*). While this review focuses on the expression patterns in the nervous system, it is important to note that NOP is also expressed in non-neuronal cells including epidermis, cells of the immune system and the vascular endothelium; receptor modulation at these sites should therefore be considered when interpreting biological readouts in particular behavioral endpoints (*19*). The distribution of NOP receptors has been studied in newt, mouse, rat, gerbil, guinea pig, dog, monkey and human using a range of techniques including reverse transcriptase polymerase chain reaction, immunohistochemistry, in situ hybridization, radioimmunoassay, autoradiography, GTPγS binding and PET imaging ((*56*, *75*, *119–137*); Table 2). NOP and its endogenous ligand, N/OFQ, are frequently localized to the same area indicating that N/OFQ can function in a local autocrine or paracrine fashion; however this does not rule out more distant signaling via neuronal projections or even endocrine signaling as suggested by the decreased plasma levels of N/OFQ in fibromyalgia sufferers (*138*). While each of the techniques mentioned above have caveats it is important to note that the available antibodies directed against NOP and used in immunohistochemistry studies have not been fully validated using tissues from wild type and knock out animals; as such we have tried to conclude from convergent data where possible.

In the central nervous system, NOP is consistently found in cortex, hippocampus, septum, amygdala, thalamus, hypothalamus and spinal cord across multiple species (Table 2). In the brainstem, NOP is expressed in the substantia

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nigra, raphe nucleus, periaqueductal grey, locus coeruleus, spinal trigeminal nucleus and solitary tract nucleus (Table 2). As compared to MOP, KOP, and DOP receptors, the distribution of NOP between species has been described as being "*more consistent*" (*139*); however notable differences do exist. For example, one study describes significant expression in human striatum (*135*) in contrast to observations in lower species. Furthermore, while the expression pattern may be consistent, the overall level of expression may vary; such is the case in dog where the expression level of receptors in cortex and midbrain is 80% lower that the expression levels in the same areas of the rat brain (*140*, *141*). Spinal cord expression of NOP has been described in multiple species including human, and is commonly described as being present in both the dorsal and ventral horns in addition to the central canal (Table 2). The level of expression is higher in the dorsal horn as compared to the ventral horn except for monkeys where a higher density of signal was noted ventrally (*132*).

In addition to the central nervous system, NOP has been localized to neurons of the peripheral sensory and sympathetic systems including neurons located in the dorsal root ganglia (*126*, *127*), nodose ganglia (*142*), trigeminal ganglia (*123*, *127*), superior cervical ganglia (*126*, *127*) and the lumbar sympathetic ganglia (*127*) (Table 2).

Overall the brain and spinal cord expression pattern of NOP is in line with the physiological roles that have been proposed and are discussed below.

Pain

The role of the N/OFQ-NOP system in the transmission of painful stimuli is highly complex and the pharmacological action of N/OFQ as well as small molecules active at NOP varies with route of administration and dose. Initial reports provided evidence that N/OFQ administration (i.c.v.) produced hyperalgesia in mice (*39*, *40*), but this was subsequently shown to be due to the inhibition of endogenous opioid-mediated, stress-induced analgesia (*143*). At high intrathecal doses, N/OFQ administration reportedly produces analgesia (*144*), anti-hyperalgesia (*145*), and is anti-allodynic (*145*, *146*). Other reports demonstrated a contrasting pharmacology by using much lower dosing concentrations of N/OFQ (*147–149*).

As discussed earlier the NOP receptor is localized along all parts of the pain pathway including; key regions of the brain involved in pain perception, the spinal cord, the DRG, and on the terminals of primary afferent neurons (*126*, *139*, *150–156*). NOP localization has been reported for rat brain and spinal cord (*57*, *75*, *123–128*, *139*, *150–156*), dog brain (*140*, *154*), non-human primate brain (*132*, *133*), and mouse brain (*120–123*, *155*). The distribution of N/OFQ in the adult human brain has also been described (*127*, *128*, *134*, *135*, *137*).

Double label, immunohistochemistry experiments have revealed that the MOP receptor and the NOP receptor are not co-localized on neurons in the DRG, spinal cord, or regions of the brain implicated in pain signaling (*156*). This differential distribution may provide a partial explanation for the differing pharmacology associated with MOP and NOP receptor activation.

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Table 2. Anatomical distribution of N/OFQ and NOP. rtPCR, reverse transcriptase polymerase chain reaction; N/OFQ, nociception; IHC, immunohistochemistry; ISH, in situ hybridization; PET, positron emission topography; RIA, radioimmunoassay

reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
Walthers et al., 2005 (ref (*119*))	newt	multiple	receptor	rtPCR	brain spinal cord lung
Slowe et al., 2001 (ref (*120*))	mice	brain	receptor	autradiography H3N/OFQ	accessory olfactory bulb suprachiasmatic nucleus amygdala ventromedial hypthalamus presubiculum hippocampus cortex hypothalamus
Boom et al., 1999 (ref (*121*))	mouse	multiple	ligand	ISH preproN/OFQ	hippocampus amygdala thalamus septum raphe periaquaductal grey

reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
					hypothalamus
					solitary tract nucleus
					spinal trigeminal nucleus
Narita et al., 1999 (ref (*122*))	mouse	spinal cord	ligand	IHC	superficial dorsal horn
					central canal
Schulz et al., 1996 (ref (*123*))	rat and mice	brain and spinal cord	ligand	IHC	spinal cord dorsal horn
					sensory trigeminal complex
					raphe nuclei
					locus coeruleus
					periaquaductal grey
					amygdala
					habenula
					hypothalamic region
					septal area
Gehlert et al., 2006 (ref (*124*))	rat	brain	receptor	Ro64-6198 GTPγS activation	cortex
					amygdala
					hippocampus
					thalamus

Continued on next page.

Table 2. (Continued). Anatomical distribution of N/OFQ and NOP. rtPCR, reverse transcriptase polymerase chain reaction; N/OFQ, nociception; IHC, immunohistochemistry; ISH, in situ hybridization; PET, positron emission topography; RIA, radioimmunoassay

reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
					hypothalamus
Mollereau et al., 1996 (ref (*75*))	rat	multiple	ligand	northern blot	brain spinal cord ovary (weak)
Foddi and Mennini, 1997 (ref (*125*))	rat	brain	receptor	autoradiography I125N/OFQ	cortex subiculum hippocampus accumbens ponstine nuclei thalamus hypothalamus
Pettersson et al, 2002 (ref (*126*))	rat	peripheral ganglia and spinal cord	receptor, ligand	ISH, IHC, autradiography I125N/OFQ	dorsal root ganglion superior cervical ganglia spinal cord doral and ventral horns central canal
Ma et al., 2005 (ref (*57*))	rat	brain	receptor	ISH	nucleus of the raphe magnus ventrolateral periaquaductal grey

reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
					dorse raphe nucleus
Xie et al., 1999 (ref (*127*))	rat	SCG, lumbar sympathetics	receptor	rtPCR	dorsal root ganglion
					superior cervical ganglia
					lumbar sympathetic ganglia
	human	DRG and SCG	receptor	rtPCR	dorsal root ganglion
					trigeminal ganglia
Neal et al., 2001 (ref (*128*))	rat		receptor, ligand	ISH	spinal cord
					brainstem
					ventral forebrain
					neocortex
	human	brain (embryonic)	receptor, ligand	ISH	thalamus
					internal pallidum
					amygdala
					hypothalamus
					cortex
					reticular nucleus
					hippocampus
					substantia nigra

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Table 2. (Continued). Anatomical distribution of N/OFQ and NOP. rtPCR, reverse transcriptase polymerase chain reaction; N/OFQ, nociception; IHC, immunohistochemistry; ISH, in situ hybridization; PET, positron emission topography; RIA, radioimmunoassay

reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
Kummer and Fishcher, 1997 (ref (*129*))	guinea pig	sympathetic ganglia	receptor, ligand	IHC (N/OFQ) rtPCR (NOP)	N/OFQ - prevertebral ganglia NOP - para and prevertebral gangia
Sim and Childers, 1997 (ref (*130*))	guinea pig	brain	receptor	N/OFQ GTPγS activation	cortex hippocampus hypothalamus
Kim et al., 2002 (ref (*131*))	gerbil	brain and spinal cord	ligand	IHC	hypothalamus hippocampus cerebral peduncle substantia nigra doral raphe periaquaductal grey locus coeruleus trapezoid nucleus
Bridge et al., 2003 (ref (*132*))	monkey	brain and spinal cord	receptor	autradiography I125N/OFQ	neocortex hippocampus amygdala caudate nucleus

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reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
					putamen
					medial thalamic nuclei
					spinal cord
					superior colliculus
Kimura et al, 2011 (ref (*133*))	monkey	whole body	receptor	small molecule PET ligand	cortex
					amygdala
					hippocampus
					anterior cingulate
					putamen
					thalamus
Witta et al., 2004 (ref (*134*))	human	brain and spinal cord	ligand	RIA	periaquaductal grey
					locus coeruleus
					hypothalamus
					septum
					dorsal horn of the spinal cord
					pontine tegmentum
					amygdala
					reticular formation

Continued on next page.

Table 2. (Continued). Anatomical distribution of N/OFQ and NOP. rtPCR, reverse transcriptase polymerase chain reaction; N/OFQ, nociception; IHC, immunohistochemistry; ISH, in situ hybridization; PET, positron emission topography; RIA, radioimmunoassay

reference	*species*	*tissues*	*receptor/ligand*	*technique*	*main sites of expression*
					spinal trigeminal nucleus
Peluso et al., 1998 (ref (*135*))	human	brain	receptor	rtPCR	cortex striatum thalamus hypothalamus
Pike et al., 2011 (ref (*136*))	mouse	brain	receptor	small molecule PET ligand	cortex septum dorsal endopiriform nucleus hippocampus hypothalamus amygdala thalamus
Lohith et al., 2012 (ref (*137*))	human	whole body	receptor	small molecule PET ligand	cortex putamen thalamus

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Central and peripheral adaptive neuronal changes that occur in response to either prolonged inflammation or nerve injury further implicate NOP as an important receptor in pain signaling. For example, following sciatic nerve injury in rat, NOP receptors (both long and short form splice variants, as well as N/OFQ) were upregulated in allodynic animals but were unmodified in non-allodynic rats (*55*, *157*). Both isoforms increased in the ipsilateral lumbar enlargement (100% increase for the long, 50% increase for the short), and 60% increase in long (50% increase for short) was found in the ipsilateral L5-L6 DRG. No changes were observed for the MOP receptor levels (*55*, *157*). In addition, intrathecal administration of N/OFQ in the presence of chronic inflammation downregulates mRNA transcripts of proinflammatory cytokines (*158*).

Likewise, in the chronic constriction injury (CCI) model in rat, NOP mRNA expression was also significantly increased in three pain processing areas of the brain (nucleus of raphe magnus (NRM), ventrolateral periaqueductal gray (vlPAG), and dorsal raphe nucleus (DRN)) (*56*). NOP mRNA expression levels increased by day seven, post surgery, and remained elevated for two weeks. It is further noteworthy that sciatic nerve axotomy reduced the analgesic effectiveness of morphine in rat, but enhanced the efficacy of the NOP agonist, N/OFQ, in cultured DRG neurons (*159*). This result is in agreement with a previous observation that nerve injury causes a down-regulation of MOP receptor and an upregulation of the NOP receptor (*55*, *160*).

When striatal and cortical neurons are exposed to cilliary neurotrophic factor (CNTF), a factor that is induced upon nerve injury and supports neuronal survival, upregulation of N/OFQ expression results (*161*). Adaptive responses such as those described in the various reports cited here lend further supportive evidence for a possible therapeutic role for NOP receptor agonists in the treatment of neuropathic pain.

Recently, convincing *in vivo* evidence in support of anti-hyperalgesic and anti-allodynic efficacy associated with NOP activation in the periphery has been described using multiple animal species and multiple NOP agonists. The first evidence for a peripheral anti-hyperalgesic effect was published by Ko, et al, who showed that co-administration of N/OFQ (1-30 μg) with capsaicin in the tail of a monkey dose-dependently inhibited thermal nociception (*162*). The same of 30 μg of N/OFQ, when administered distally (in the back) showed no effect. This analgesic effect was antagonized by a small dose (10 μg) of the NOP antagonist, J-113397 (*163*) when administered locally in the tail, but had no effect when administered distally. N/OFQ did not change the base-line thermal pain threshold in the animals when administered into the tail in the absence of capsaicin. Another report described the efficacy of N/OFQ in a rat model of neuropathic pain (*164*). The agonist was administered i.t., i.p., and s.c. and the responses of the neuropathic rats were measured in tactile and thermal allodynia tests. The authors also tested a non-peptide NOP agonist known as Ro64-6198 (*165*) in the same experiments as a comparator. Ro64-6198 had no effect on the baseline pain threshold in naive rats and was anti-allodynic in neuropathic rats following either i.t. or i.pl. administration, but not after s.c. administration. At doses between 2.3 and 23 nmol (i.t.) Ro64-6198 dose-dependently decreased tactile (Von Frey) and thermal (cold water) allodynia in neuropathic rats. Similar results were observed

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following i.pl. administration of Ro64-6198, with the maximum effect occurring at the 30 minute time point at doses of 46 and 69 nmol (Von Frey), and 69 nmol for the cold water test. N/OFQ was shown to be an effective anti-allodynic agent in these models.

Additional supporting evidence for a role of NOP stimulation as a pain signaling modulator was recently published by Courteix, *et al*. (*166*). In two models of neuropathic pain in rats, N/OFQ (i.t.) at doses between 0.1 and 10 μg /rat reduced mechanical hyperalgesia (paw pressure), although the authors noted that the effect could surprisingly be suppressed by naloxone. The same report describes a supra-additive inhibitory effect on mechanical hyperalgesia by the co-administration of N/OFQ and morphine. Finally, in rats, i.t. administration of N/OFQ attenuated the maintenance of secondary mechanical allodynia, but had no effect on the development of the allodynia (*167*). These findings are consistent with our demonstration in monkeys that a small molecule NOP agonist is analgesic when administered either i.t. or epidurally (Figure 3). The effect following epidural administration may be via action on the dorsal roots or alternatively by the compound penetrating the blood-brain-barrier and reaching spinal circuits.

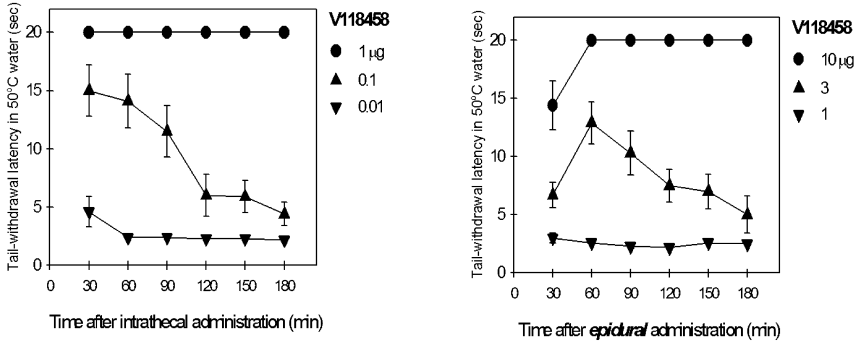

Figure 3. The analgesic effect of a Purdue ORL1 agonist (V118458) following intrathecal and epidural administration to rhesus monkeys.

The hypothesis that peripherally restricted NOP agonists might be effective agents for the treatment of chronic pain (including neuropathic pain) is noteworthy. Similarly, the increasing number of publications that report no changes in baseline pain thresholds of normal animals is also of interest. A similar observation was made in man following a 200 pmol injection of N/OFQ directly into tender trapezius muscle (*168*).

Reward

It is widely known that the endogenous opioid system plays a major role in mediating the rewarding and reinforcing properties of exogenous opiates and many other drugs of abuse (*169–172*). Moreover, there is substantial evidence

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that opioid receptor activation can modulate the activity of mesolimbic dopamine neurons and that this action underlies the motivational effects of these drugs (*173–176*). As previously discussed, NOP and its endogenous agonist, N/OFQ, are for the most part, densely and widely distributed throughout the regions of the brain known as the brain reward system, which includes the nucleus accumbens (NA) (*130*, *134*, *177*, *178*). The systemic administration of MOP or DOP agonists is known to stimulate the release of dopamine in the NA (*179*, *180*), whereas KOP activation decreases basal dopamine levels in this brain region (*179*, *181*). A recent microdialysis study showed that the central administration of N/OFQ (i.c.v. doses of 2, 5, and 10 nmol/rat) resulted in no elevation or depression in basal dopamine levels in the NA. Moreover, doses of 5 and 10 nmol/rat (i.c.v.) resulted in an inhibition of morphine-induced elevation of dopamine in this brain structure (morphine was dosed i.p. at 5 and 10 mg/kg) (*182*). It is noteworthy that the authors indicated the inability to perform the experiment at higher doses (>10 nmol) of N/OFQ due to impaired motor control and loss of their righting reflex. Centrally-mediated side effects have also been observed and reported for the non-peptide NOP agonist Ro64-6198 (*183*).

The previously described results are in agreement with a previous report showing that i.c.v. doses of N/OFQ (16-160 nmol) also inhibited the morphine-mediated increase in dopamine levels in the NA (*184*). *In vivo* behavioral support for this biochemical result has been published using conditioned place preference experiments (*185–187*). One such report (*185*) demonstrated that i.c.v. administration of N/OFQ at either 250, 500, or 1000 ng/rat produced no place preference or aversion on its own, and further showed that at 500 or 1000 ng/rat the place preference produced by morphine (3 mg/kg s.c.) could be abolished. Similar NOP-mediated inhibitory effects have been demonstrated in place preference experiments using a variety of other drugs of abuse including methamphetamine (*188*), cocaine (*189*, *190*), and ethanol (*191*, *193*). Moreover, the non-peptide NOP agonist, Ro64-6198, has also been proven to exert similar inhibitory effects on morphine conditioned place preference (*194*).

Central NOP activation prevents ethanol-induced gastric lesions in the rat, possibly by accelerating gastric emptying and increasing blood flow to the stomach (*195*). Taken together with the NOP-mediated inhibition of ethanol reward, a role for NOP agonists as a treatment of alcoholism can be imagined *(for reviews see refs* (*192*, *193*, *196*)*)*.

Finally, central NOP stimulation appears to play an anxiolytic role which may have further implications regarding anxiety-related behavior that leads to substance abuse. Specifically, ppN/OFQ knockout mice, which lack N/OFQ as well as other biologically active peptides such as nocistatin, exhibit increased anxiety behavior in the light-dark box and plus maze models, and both N/OFQ and Ro64-6198 are anxiolytic in various rodent models (*197–200*).

Taken together, NOP activation in the brain reduces anxiety behavior that often leads to substance abuse, has no effect on basal dopamine levels in the NA but inhibits dopamine overflow mediated by abused substances, inhibits place preference of abused substances but causes no place preference or aversion on its own, and inhibits ethanol-induced GI erosion in rats. Assuming that a similar profile could be achieved in humans at doses below those that may otherwise cause

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the locomotor and other deficits that have been observed in some animals, then central NOP agonism may be an attractive mechanism for managing substance abuse in man.

NOP as a Supraspinal Regulator of MOP Receptor Action

As noted previously, many early publications in the N/OFQ and NOP field provide evidence that activation of NOP receptors in the brain can inhibit opioid-mediated analgesia (*12*, *13*, *201–205*). Moreover, NOP activation has an inhibitory effect on the rewarding properties of many drugs of abuse, including MOP agonists. A growing body of evidence is supportive of a more generalized regulatory role for NOP against the actions of the MOP receptor, possibly contributing to the development of MOP-agonist tolerance in patients being treated with classical opiates (*206*, *207*).

Consistent with expectations for a regulatory system, N/OFQ is upregulated in the CNS in response to long-term administration of the MOP-agonist, morphine (*208*). Moreover, N/OFQ and NOP are co-localized with MOP receptors in some cells in the pain pathway (*209–211*) and co-administration of N/OFQ can alter the analgesic efficacy of morphine in both a positive and a negative direction depending on site of administration *(for review see ref* (*212*)*)*.

Homologous MOP desensitization, or loss of function, has been suggested to be the underlying reason for tolerance to MOP agonists (*213*), and the GRK-mediated phosphorylation of the MOP receptor in response to agonist occupation occurs in a conventional way, presumably resulting in phosphorylation of Ser^{375} (*214*, *215*). Recent evidence suggests that N/OFQ, acting upon NOP, potentiates DAMGO-induced MOP receptor phoshorylation via activation of PKC, GRK2 and GRK3 in BE(2)-C human neuroblastoma cells (*214*). DAMGO challenge in N/OFQ pre-treated cells increased MOP phosphorylation 50% more than what occurred in vehicle-treated cells. Selective inhibition of these three kinases blocked the N/OFQ -induced MOP receptor heterologous desensitization (*216*).

It has been suggested that N/OFQ may work as a regulatory peptide, similar to cholecystokinin (CCK), which is known to be involved in analgesia, as well antagonizing the analgesic effects induced by opiates (*217–219*). In a rat model of neuropathic pain there is an increase in spinal CCK and a reduction in the potency of spinal morphine (*220*). This is presumed to be part of the mechanism of reduced opioid sensitivity in neuropathic pain in man. In contrast, CCK has been shown to enhance the anti-hyperalgesic effects of spinally administered N/OFQ (*220*). It is of further significance that both CCK and NOP receptors are up-regulated in the spinal cord in animal models of nerve injury (*55*, *221*), while MOP receptors are down-regulated (*159*, *160*).

In addition to the MOP receptor inhibitory role associated with the NOP receptor (heterologous desensitization), a physically associated NOP- MOP heterodimer has also been reported to occur in HEK 293 cells (*222*) and in DRG neurons (*58*) that exhibits a similar MOP-inhibitory pharmacology. In the studies using HEK cells, treatment with the MOP agonist, DAMGO, desensitized

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MOP but not NOP receptors. Contrastingly, pretreatment of the cells with the NOP agonist, N/OFQ, impaired the potency by which DAMGO inhibits cAMP production. The heterodimer thus displayed a cross desensitization of the MOP receptor and the formation of the complex apparently depends upon direct interaction between the C-termini of the two receptors.

Considered as a whole, there is substantial albeit emerging evidence that is supportive of a MOP receptor regulatory role for the NOP system in some brain regions and this will likely be a very important area of research in the future. As one example, recently published results using buprenorphine, a mixed MOP agonist and ORL-1 agonist (although potency at MOP receptors is much greater than potency at ORL-1 receptors) (*223*) in a rodent model of analgesia led to intriguing possible insight into the anti-MOP effects of NOP stimulation (*224*). It was proposed that the well-known bell-shaped analgesic dose-response curve associated with buprenorphine might be due to the induction of the regulatory NOP system at higher doses. Of interest was the observation that, in NOP knockout mice, the analgesic effect of buprenorphine was not bell shaped but instead was dose-dependent. This may be suggestive of a new approach toward the use of classical opiates as analgesics. In particular, the simultaneous administration of a NOP antagonist and a MOP agonist, either as two substances or a single compound, may allow one to achieve MOP-mediated analgesia at lower doses than normally given, and therefore a coincident reduction of side effects. Since NOP knockout mice do not develop full tolerance to MOP agonists (*225*), this mixed pharmacology approach may offer the additional benefit of avoiding the need for dose escalation over time in patients using opiates, since they too may not develop tolerance as a result of the NOP antagonism. A very recently published paper has indicated that some of these findings may be species dependent and may not be relevant in monkeys (*226*); in addition these concepts remain to be proven in man.

Summary & Conclusions

Despite the sequence, structural and signal transduction similarities between NOP and the classical opioid receptors, there are also several intriguing differences. Many of these have been summarized and discussed in this review. Agonism of NOP receptors located supraspinally may prove to be a useful mechanism for the treatment of several important human conditions, but presumed on-target side effects that have been reported at high concentrations in some species may turn out to be an insurmountable limitation if they also occur in humans. On the other hand, a combination treatment composed of a NOP antagonist and a MOP agonist may modulate known MOP tolerance while potentiating MOP analgesia. This strategy may also alleviate some of the known side effects associated with MOP agonists since lower doses might maintain analgesic effectiveness. Of course, this hypothesis remains to be proven in man and evidence that NOP antagonism might differentially potentiate the analgesic and side effect dose responses needs to be collected. While the targeting of spinally located NOP receptors may also provide analgesic efficacy in man this

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again remains to be proven and a systemically administered CNS penetrant compound would likely have both spinal and supraspinal effects.

Finally, there is growing evidence from multiple species, multiple behavioral models/endpoints, and multiple NOP ligands, that potent anti-hyperalgesia and/or anti-allodynia might be achieved via the direct activation of NOP receptors located in the periphery. At this time, no restricted, long-lasting, non-peptide NOP agonist has been published, but such a molecule might be predicted to have much reduced adverse, on-target-side effects while providing a novel mechanistic approach toward the treatment of nociceptive and neuropathic pain conditions in man. Consistent with this concept is the robust demonstration for a role of NOP on peripheral neurons in bladder disorders (*227–229*); in these studies N/OFQ was instilled intravesicularlly in patients with refactory detrusor hyperreflexia and produced an increase in mean bladder capacity and volume threshold for the appearance of detrusor hyperreflexia. This effect was reproduced in patients with neurogenic detrusor activity due to spinal cord injury (*228*, *229*), was also shown to persist with daily administrations (*229*) and at the same dose had no effect on bladder parameters in normal volunteers. The authors suggest the effect is mediated via an inhibitory effect of NOP receptors located on c-fibers in the bladder (*227–229*).

We move ever closer to testing the above hypotheses in the clinic; currently at least two small molecule agonists have been tested in clinical trials directed towards demonstration of efficacy against human disorders. JNJ-19385899 (*230*) has advanced for anxiety and SCH486757 for cough (*231*). In addition, the NOP antagonist JTC-801 has also advanced into the clinic, and this will shed further light on the clinical relevance of the NOP receptor in humans. These combined with availability of competitive PET ligands (*133*, *136*, *137*, *232*) will not only allow pharmacodymic and efficacy readouts to be obtained but offer the potential to determine the site of action of these antagonists and agonists.

As these first generation NOP ligands are characterized in the clinic, basic preclinical research in multiple laboratories continues further exploration of the role of NOP in physiology and pathophysiology and attempts to design novel NOP ligands with appropriate pharmacological and pharmaceutical profiles. Each new NOP-related publication provides another clue in the ongoing effort to understand this unique and interesting "opioid-like" receptor. Perhaps in the near future our insights into the interplay between NOP, pain, reward, anxiety, and the classical opioid receptors will be sufficient to facilitate the design of a new generation of human therapeutics that act, at least in part, via the NOP receptor.

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Chapter 17

Pharmacology of Mixed NOP/Mu Ligands

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Activation of NOP receptors produces a complex pharmacology leading to either antinociceptive activity or inhibition of opiate antinociception, depending upon the route of administration. Systemic administration of NOP receptor agonists does not induce antinociception in most acute pain models in rodents. However, activation of NOP receptors does block opiate-mediated reward and reduces opiate tolerance development. Compounds have been synthesized to test the hypothesis that mixed NOP/mu ligands could have antinociceptive activity, with reduced addiction liability and reduced tolerance development. We discuss the background and rationale for developing mixed NOP/mu compounds and review the pharmacology of compounds with varying affinity and efficacy at NOP and mu opioid receptors. Among these novel compounds are those that have potent antinociceptive activity with reduced reward and other compounds that are devoid of antinociceptive activity, but attenuate morphine CPP. These results suggest that mixed NOP/mu compounds have potential clinical value as analgesics and/or as treatments for drug abuse.

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N/OFQ: A Complex Pharmacology

Shortly after the discovery of nociceptin/orphanin FQ (N/OFQ), it became clear that the pharmacology of this neuropeptide was not similar to the classical opioid peptides. In particular, the initial two papers describing the discovery of this neuropeptide both demonstrated modulation of nociceptive information, i.e., a decrease in hotplate and tail flick latency subsequent to intracerebroventricular (i.c.v.) administration of this peptide in mice (*1*, *2*). This nociceptive action of N/OFQ was in contrast to endogenous and exogenous opiates that have antinociceptive activity and produce an increase in tail flick and hot plate latencies. Subsequent studies showed that the biological actions of N/OFQ were quite complex, particularly with respect to its effect on opiate actions

Although i.c.v. administration of N/OFQ into mice resulted in apparent nociceptive activity, similar experiments in rats could not reproduce this phenomenon (*3*). Grandy and co-workers determined that acute i.c.v. injections into mice caused stress-induced analgesia, which could be reversed by the administration of N/OFQ (*4*, *5*). N/OFQ apparently does not regulate nociceptive activity, it blocks stress-induced analgesia, and therefore is ineffective in rats, in which an indwelling cannula allowed administration of the N/OFQ in the absence of a stress response. In fact, N/OFQ blocks stress-induced analgesia, as well as analgesia induced by activation of mu, delta, and kappa opioid receptors (*5*). Therefore, rather than being pronociceptive in its own right, N/OFQ was thought of as being "anti-opiate". However, in the presence of an opiate antagonist, and therefore in the absence of an endogenous opioid tone, i.c.v. administration of N/OFQ reveals hyperalgesia (*6*). Another striking example of the complicated in vivo actions of N/OFQ is the observation that, when administered intrathecally (i.t.), N/OFQ potentiates morphine analgesia, and has antinociceptive activity, rather than the nociceptive or anti-opiate activity when administered i.c.v. (*7*, *8*). Therefore, the "antiopiate" actions of N/OFQ, particularly with respect to pain responses, are dependent upon route of administration, dose, and time course of action and the state of opioid activation in the brain.

In addition to the opposing actions of N/OFQ due to differences in route of administration, N/OFQ was found to induce a pronociceptive response at very low doses (amol to fmol) after intraplantar or i.t. administration, due to stimulation of substance P release (*9*, *10*). However, at higher doses (nmol, i.t.) this effect disappeared and N/OFQ blocked substance P-induced pain response (*11*). Furthermore, Rossi et al., reported that N/OFQ, when administered i.c.v., initially was pronociceptive, but then over time, this developed into a naloxone-reversible antinociceptive action (*12*). In addition, this group also reported that two N/OFQ N-terminal fragments N/OFQ (*1–7*) and N/OFQ (*1–11*) both had naloxone-reversible antinociceptive activity, despite the fact that neither peptide has high affinity for the NOP receptor or any of the classical opiate receptors (*12*, *13*).

Because the pronociceptive/antinociceptive actions of N/OFQ are dependent upon the site of administration, the effect on nociception of peripherally administered small molecule agonists and antagonists was not obvious. With the development of the first selective small molecule agonist, Ro 64-6198

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(*14*, *15*), and selective antagonists J113397 (*16*, *17*) and JTC-801 (*18*, *19*), the determination of the effect of systemic administration of these types of compounds was possible. Ro 64-6198, when administered intraperitoneally (i.p.), has no effect on tail flick latency in mice (*14*, *20*). Subsequent studies confirmed the lack of effect on tail flick, but found a moderate reduction in hot plate latency (*21*). Additional studies with other NOP agonists, including SR16835 (full NOP agonist, and approximately 8-fold selective for NOP over mu opioid receptors) suggest that there is no profound effect of selective NOP agonists on acute nociception in rodents (*22*). An early hypothesis was that NOP antagonists might have antinociceptive activity by blocking the nociceptive actions of endogenous N/OFQ. This has also produced controversial results. The first non-peptide NOP receptor antagonist reported, J-113397, has no antinociceptive activity when administered systemically (*23*). However, JTC-801, a less selective NOP receptor antagonist has naloxone insensitive antinociceptive activity in a variety of acute and chronic pain models (*19*, *24*, *25*). This compound was advanced to Phase I and Phase II clinical trials by Japan Tobacco, prior to being discontinued. It is not entirely clear how this compound works, particularly since the higher affinity and more selective antagonist SB 612111, like J113397, is devoid of acute antinociceptive activity in rodents (*26*, *27*). Generally, it is thought that neither selective NOP agonists nor antagonists have significant antinociceptive activity in acute pain models when administered systemically to rodents (*28*). It should be noted, however, that peptides might be different. High affinity and selective NOP receptor antagonist peptides, such as UFP-101, but not small molecule antagonists, have direct antinociceptive activity when administered i.c.v. (*29*, *30*). It is not clear why peptide and small molecule antagonists behave differently in this regard. Furthermore, although Ro 64-6198 is not an analgesic in many pain models in rodents, it appears to be a powerful analgesic in non-human primates (*31*).

Effect of N/OFQ on Opioid Tolerance and Reward

One characteristic of extended treatment with opioid analgesics is the development of tolerance. NOP receptor agonists and antagonists have effects on opioid tolerance development in rodents. It was originally demonstrated that N/OFQ, administered i.c.v. prior to chronic morphine treatment attenuated the development of tolerance (*32*). Conversely, other studies have suggested N/OFQ induces tolerance, since tolerance is decreased in NOP receptor (*33*) and ppN/OFQ knock out mice (*34*), whereas enhanced expression of NOP receptors in the spinal cord induces morphine tolerance and dependence (*35*). Also, co-administration of the NOP receptor antagonist J-113397 along with morphine is able to block tolerance development to morphine antinociception (*34*). After tolerance has developed, administration of the NOP receptor antagonist SB612111 reversed tolerance and thereby increased the potency of morphine, a property it does not have acutely prior to tolerance development (*27*).

It is possible that chronic morphine induces an increase in NOP receptors or N/OFQ tone, thereby reducing morphine potency, functionally expressed as

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tolerance development. This would explain a decrease in tolerance in knockout animals. If N/OFQ is administered concurrently with morphine, it might prevent an increase in NOP receptor tone, perhaps by receptor desensitization or down regulation, and thus block tolerance development. This is consistent with decreased tolerance development with the mixed NOP/mu partial agonist SR16435 (*36*). Once the morphine-induced increase in NOP receptor tone occurs, in tolerant animals, then an NOP receptor antagonist would block tolerance acutely. Therefore, perhaps paradoxically, NOP receptor agonists and antagonists can both alleviate morphine tolerance, if administered prior to or subsequent to tolerance development respectively.

The effect of N/OFQ and NOP receptor agonists are less complicated with respect to reward. There is a high density of NOP receptors and N/OFQ in areas implicated in drug abuse and reward (*37*, *38*), and because of the obvious relationship to opiates, N/OFQ has been tested repeatedly in drug abuse models alone and in the presence of abused drugs. I.c.v. administration of N/OFQ does not produce either conditioned place preference (CPP) or conditioned place aversion (CPA) in mice and rats (*39*, *40*), indicating that, by itself, N/OFQ does not induce reward or aversion. Subsequent microdialysis experiments demonstrated that i.c.v. and intra-ventral tegmental area (VTA) administration of N/OFQ slightly reduced extracellular dopamine levels in the nucleus accumbens (NAc) (*41*, *42*). Furthermore, i.c.v. injections of N/OFQ blocked cocaine-induced increases in extracellular dopamine in the NAc (*43*). Recently, we have shown that retrodialyzed N/OFQ into the NAc blocks cocaine-induced increases in extracellular dopamine in the same region (*44*). The profound effect of N/OFQ on extracellular dopamine has led a number of investigators to characterize the effect of N/OFQ and small molecule agonists on conditioned place preference and self-administration of a large number of abused drugs. N/OFQ administered i.c.v. blocks CPP induced by morphine (*40*, *45*, *46*), cocaine (*40*, *47*), amphetamine (*48*, *49*), and alcohol (*50*, *51*). It also blocks alcohol (*52*), but not heroin (*53*), self-administration. Systemic administration of small molecule agonists Ro 64-6198, Ro65-6570, and SR 16835 also attenuate opiate and alcohol CPP, and these effects can be blocked by the NOP receptor antagonists, SB612111 and J113397 (*22*, *51*, *54*, *55*). Collectively, these findings indicate that NOP receptor agonists can block reward/reinforcement of drugs of abuse from different classes.

The fact that small molecule NOP receptor agonists, administered systemically to rodents, are not nociceptive, and the observations that N/OFQ blocks both tolerance development and reward associated with morphine, led to the hypothesis that mixed NOP/mu agonists might maintain analgesic activity, but have reduced tolerance development and reduced addiction liability. This hypothesis was tested in experiments described below.

Potential Sites of Interaction between the N/OFQ and Opiate Systems

N/OFQ and NOP receptors are distributed widely in the brain and spinal cord and there are many places in which this receptor system might interact with

opiate receptor systems, in either an opposing or synergistic way, to lead to the complex pharmacology of NOP receptor agonists and antagonists. The apparent paradox in which N/OFQ is pronociceptive or blocks opiate antinociception when administrered i.c.v. but is antinociceptive when administered i.t. can potentially be explained by the differential interaction of the N/OFQ/NOP system with the supraspinal versus spinal nociception pathways. When administered i.c.v., N/OFQ induces an anti-opiate and pronociceptive effect by inhibiting analgesia-producing periaqueductal gray (PAG) neurons that project to the rostral ventromedial medulla (RVM). These are downstream from the opioid-sensitive neurons, and by hyperpolarizing the PAG neurons N/OFQ interrupts the descending nocieptive pathway (*56*). Opiates and N/OFQ also have an effect on the nucleus raphe magnus (NRM), a brainstem region that modulates pain transmission at the spinal cord. This brain region has been demonstrated to be a supraspinal site mediating mu-opiate analgesia (*57*, *58*). In the NRM, primary cells induce an analgesic response (*59*). These cells contain kappa opioid receptors, which hyperpolarize and thereby inhbit cellular activity and are disinhibited by mu receptor inhibition of GABA interneurons. Secondary cells induce hyperalgesia, but these cells are inhibited by mu opioid receptor activation. Mu opiates therefore are analgesic by blocking hyperalgesic secondary cells and facilitating analgesic primary cells. N/OFQ inhibits both cell types, thereby blocking an opioid analgesic response under normal conditions and reducing hyperalgesia during opioid abstinence (*60*).

In the spinal cord, the situation is different. NOP receptors and N/OFQ are abundant in the spinal cord and dorsal root ganglia. However, due to problematic immunohistochemistry the exact localization with respect to opioid receptors is not well understood (*37*). Nevertheless, receptor activation in the spinal cord leads to a clear antinociceptive response in a manner similar to opioid receptor activation and it is very likely that NOP receptor activation acts similarly to mu or more likely delta opiates in its ability to reduce mechanical pain (*61*), as will be discussed below.

N/OFQ and NOP receptors are also highly expressed in many areas of the reward cuircuitry in cells poised to oppose mu-mediated reward (*37*, *38*). Mu opioid receptors on GABA interneurons in the VTA disinhibit dopamine neurons projecting to the NAc (*62*). NOP receptors are found on 91% of tyrosine hydroxylase-positive cells in the VTA (*63*). Since NOP receptor activation hyperpolarizes cells, this identifies a direct mechanism by which N/OFQ could attenuate mu mediated cellular activation and ultimately reward. NOP receptors are also found in the NAc, and as we described above, direct reverse dialysis of N/OFQ attenuates cocaine-induced dopamine release, and most probably would have a similar affect on morphine-induced dopamine release (*44*). Finally, NOP receptors are found in the lateral hypothalamus, a regions recently discovered to be involved in the reward process through the neuropeptide hypocretin/orexin. In this important brain region, hypocretin release has been demonstrated to be required for morphine CPP (*64*, *65*), and furthermore N/OFQ has been shown to hyperpolarize every hypocretin-containing neuron (*66*). Therefore, N/OFQ can work in the VTA, the NAc, and the hypothalamus to attenuate morphine's rewarding activity.

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Mixed NOP/ Opiate Agonists as Analgesics with Reduced Abuse Liability

Buprenorphine

Lutfy et al. made the initial observation that a compound acting at both mu and NOP receptors could have interesting properties with respect to its antinociceptive activity (*67*). Both in vitro and in vivo, buprenorphine has been characterized as a mu partial agonist and delta and kappa antagonist (*68–72*). Due to low mu efficacy, and potentially kappa antagonist activity, buprenorphine has reduced addiction liability and abstinence symptoms relative to other opiate agonists (*73*, *74*). Nevertheless, buprenorphine induces CPP under appropriate conditions (*75*, *76*) and has some addiction liability in humans. Buprenorphine also binds with moderate affinity to NOP receptors, with a Ki in the range of 100-200 nM, nearly 2 or more orders of magnitude lower affinity than it has for the three opioid receptors (*69*, *77*). Buprenorphine also has some agonist activity at NOP receptors in vitro, although this is dependent on the assay. It has been reported to have very low to moderate agonist activity in [^{35}S]GTPγS binding assays (*69*, *72*), and up to full agonist activity in a reporter gene assay (*78*), or for stimulation of ERK phosphorylation (*67*). Because it is a partial agonist at mu opiate receptors, buprenorphine has a ceiling effect with respect to both antinociceptive activity and respiratory depression (*71*, *73*, *79*). In fact, the antinociceptive activity of buprenorphine, and other partial agonists, is dependent upon the painful stimulus such that at lower light intensity in the tail flick test, or lower water temperatures in the case of the warm water tail flick assay, buprenorphine can be fully efficacious in reducing nociceptive responses (*80*). However, at moderate to high temperatures it will not reach a 100% maximal possible effect (MPE) and under the appropriate conditions, buprenorphine can display an inverted U shaped dose response curve (*67*, *73*). Lutfy et al. discovered that pretreatment with the NOP receptor antagonist, J-113379, blocked the downward portion of the inverted U, thus potentiating buprenorphine's activity at the higher doses (*67*). Furthermore, measurement of buprenorphine's antinociceptive activity in NOP receptor knock out mice produced the same fully efficacious dose response. These results strongly suggested that the NOP agonist activity of buprenorphine can attenuate the mu agonist activity of the compound.

SR Compounds

Based upon the ability of NOP agonists to attenuate reward and tolerance development, we began a program to design NOP/mu receptor agonists, with the hypothesis that the NOP agonist activity would block the mu-mediated reward and tolerance development, within the same molecule. The initial compound tested was SR16435 (Figure 1). This compound has high affinity and partial agonist activity at both mu and NOP receptors (see Tables I and II). SR16435 has naloxone reversible antinociceptive activity, and as predicted, it also has reduced tolerance development (*36*, *81*). Furthermore, for SR16435 and other mixed NOP/mu agonists including buprenorphine, the NOP portion

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attenuates the mu-mediated antinociception, since co-administration of the NOP receptor antagonist, SB612111, potentiates the antinociceptive activity of these compounds (*81*) (Figure 2a). However, SR16435, similar to morphine, induces CPP (*36*) (Figure 2b). These studies demonstrated that partial agonism at NOP was sufficicent to attenuate mu-mediated antinociception by SR16435, but not to attenuate the mu-mediated reward. Attenuation of opiate reward may therefore require higher efficacy than inhibition of alcohol reward, since buprenorphine, a weak NOP partial agonist, apparently attenuated alcohol drinking through NOP receptor activation (*82*).

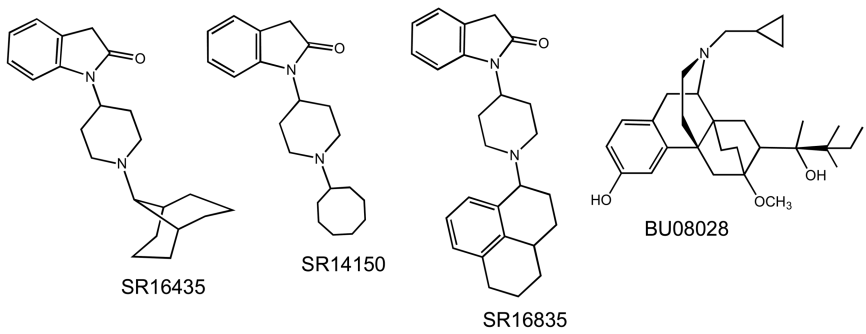

Figure 1. Structure of mixed NOP/mu compounds

Table I. Binding affinities of mixed NOP/mu compounds

	Receptor Binding Ki (nM)		
	NOP	*Mu*	*Kappa*
N/OFQ	0.2 ± 0.04	133 ± 30	247 ± 3.4
Morphine	>10,000	1.1 ± 0.1	46.9 ± 14.5
Buprenorphine	77.4 ± 16	1.5 ± 0.8	2.50 ± 1.2
SR16435	7.49 ± 0.78	2.70 ± 0.05	31.7 ± 4.8
SR14150	1.39 ± 0.42	29.9 ± 2.1	42.7 ± 1.0
SR16835	11.4 ± 0.9	79.9 ± 3.9	681.3 ± 61.6
BU08028	8.46 ± 1.31	2.14 ± 0.79	5.63 ± 1.30

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Table II. Functional activity of mixed NOP/mu compounds

	[35S] GTPγS NOP		*[35S] GTPγS Mu*	
	EC_{50} (nM)	*% Stim*	*EC_{50} (nM)*	*% Stim*
N/OFQ	4.0 ± 0.1	100	>10,000	
Morphine		0	5.2 ± 1.6	93 ± 2.8
Buprenorphine	251 ± 94	15.5 ± 5.8	10.2 ± 2.2	28.7 ± 1.0
SR16435	28.7 ± 0.6	45.0 ± 5	29.5 ± 10	30 ± 0
SR14150	20.8 ± 3.1	54 ± 10.9	99 ± 12	23.4 ± 3.2
SR16835	46.1 ± 20.5	106.6 ±7.4	129 ± 48	18 ±1.6
BU08028	78.6 ± 49	48 ± 13	6.03 ± 2.1	21.1 ± 8.7

Because SR16435 displayed significant antinociceptive activity, but did not have diminished mu-mediated reward, we examined compounds from our compound library with higher NOP efficacy and with decreased mu efficacy to investigate if this profile could potentially result in attenuation/blockade of mu-mediated reward. The compounds examined were SR14150 and SR16835 (Figure 1). As previously shown (*22*, *72*), and seen in Tables I and II, SR14150 is a NOP partial agonist with high affinity and reasonable selectivity for this receptor compared to its activity at mu-opioid receptors, at which it is a weak partial agonist. SR16835 is a full agonist at the NOP receptor, a low efficacy agonist at the mu-opioid receptor and has modest selectivity for NOP receptors. These two compounds were tested for antinociceptive activity in mice, after subcutaneous administration using the tail flick assay. SR14150 had potent antinociceptive activity (ED_{50} less than 10 mg/kg at 30 min) while SR16835 was ineffective. Interestingly, although SR14150 has relatively low affinity and low efficacy at the mu receptor, the antinociceptive activity was completely blocked by naloxone, indicating that it was a mu-mediated effect (*22*) (Figure 3A). Similar to SR16435, and other NOP/mu agonists, the NOP agonist activity of SR14150 attenuates its mu-mediated antinociception, since co-administration of the NOP receptor antagonist, SB612111, potentiates SR14150-induced antinociception (*22*) (Figure 3B). The remaining important question was whether increased NOP agonist activity in a mixed NOP/mu agonist could also attenuate mu-mediated reward. As seen in Figure 4, SR14150, in contrast to morphine, does not produce CPP, indicating that its NOP-mediated agonist activity attenuates any mu-mediated rewarding effects. Overall, a compound with a profile like SR14150 could potentially be used as an analgesic with low abuse liability (*22*).

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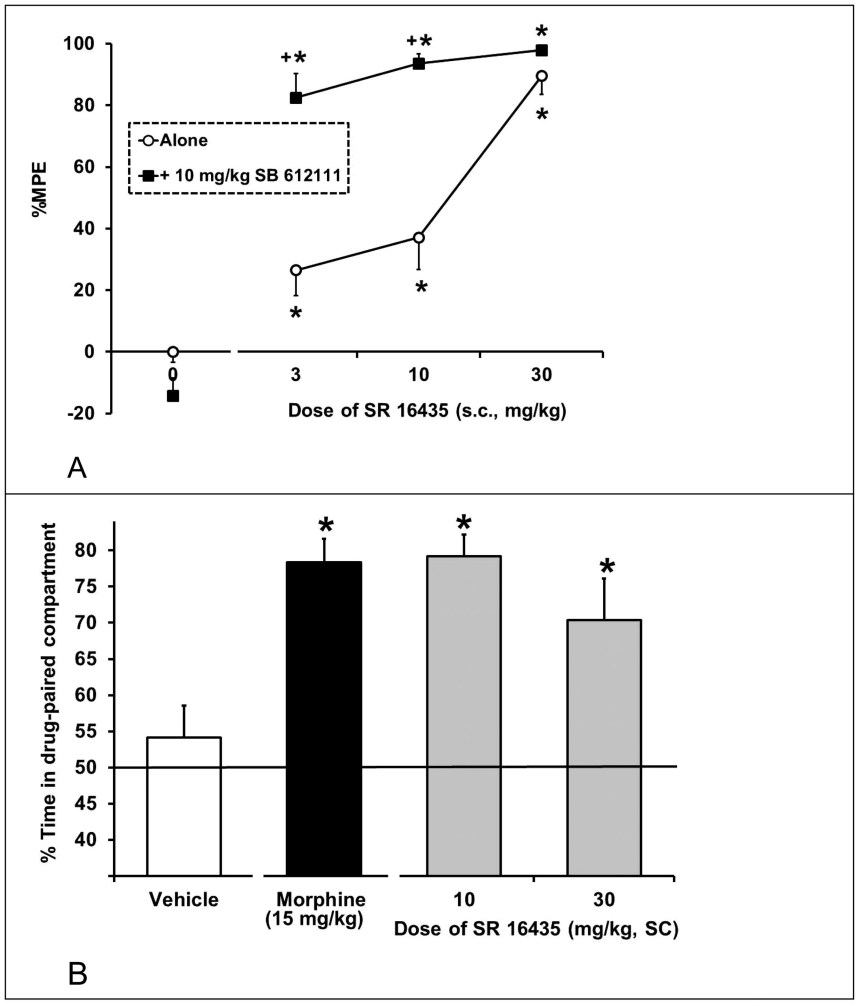

Figure 2. A. Antinociceptive activity of SR 16345 alone and potentiation by the NOP antagonist SB 612111, B. SR16435 produces CPP similar to morphine, despite the presence of NOP agonist activity . An asterisk (*) signifies a difference from vehicle controls, whereas a plus sign (+) signifies a difference from morphine alone (P<0.05). Reproduced with permission from [(81), 2A and (36), 2B]. Copyright 8/29/2009 and 2/2007, both from The American Society for Pharmacology and Experimental Therapeutics

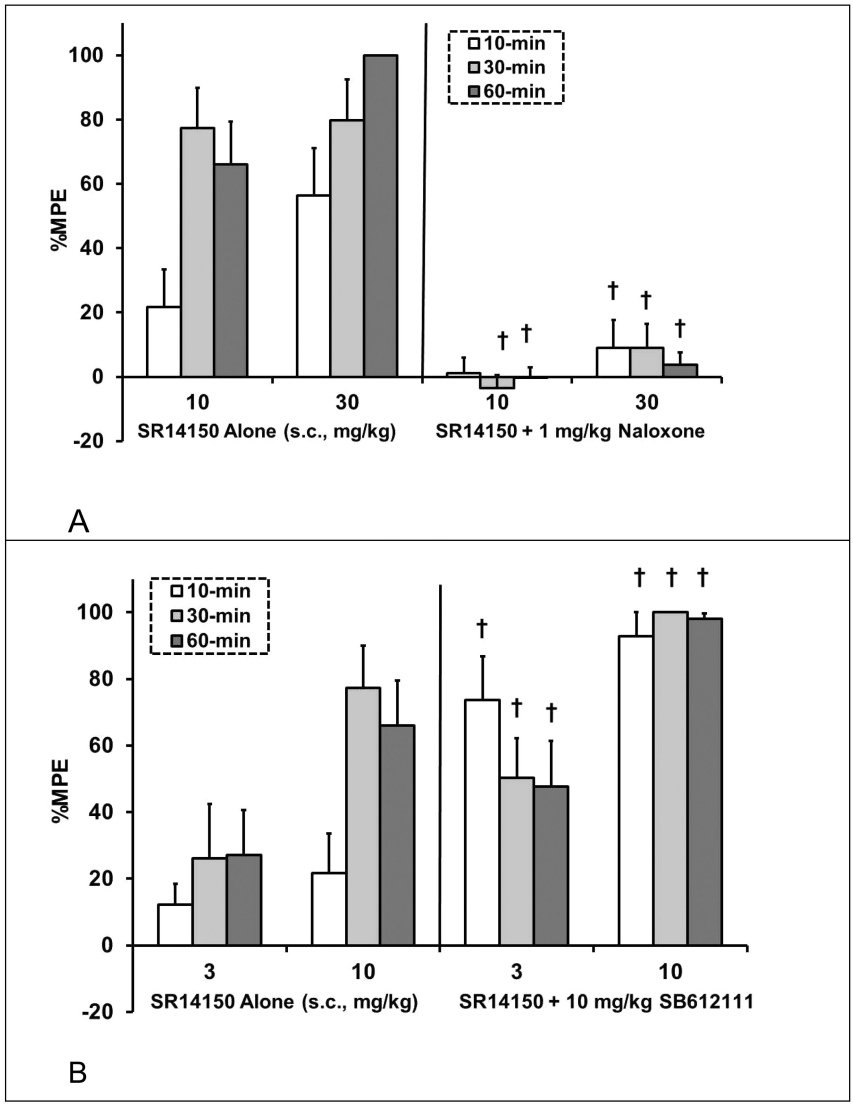

Figure 3. SR14150 produces antinociception that is (A) completely blocked by 1 mg/kg naloxone, and (B) potentiated by SB612111. Reproduced with permission from (22). Copyright 09/24/2009 from The American Society for Pharmacology and Experimental Therapeutics

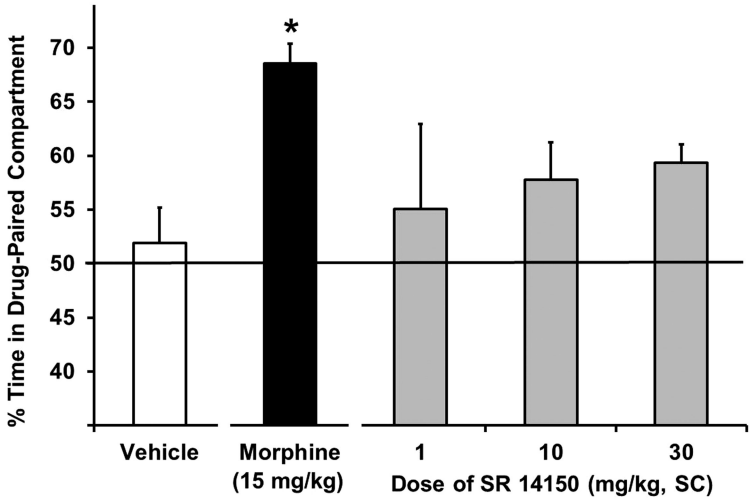

Figure 4. SR14150 does not produce CPP at any of the doses that produced antinociception in mice. An Asterisk (*) signifies a difference from vehicle controls. Reproduced with permission from (22). Copyright 09/24/2009 from The American Society for Pharmacology and Experimental Therapeutics

Chronic Pain Studies

SR14150 and SR16835 have also been tested in models of chronic pain, and the results seem to be somewhat different than in acute pain models (*83*). In spinal nerve-ligated (SNL) mice, SR14150 and SR16835 were tested for antinociceptive activity using the tail flick assay, and for tactile anti-allodynia using von Frey filaments. In SNL mice, similar to normal mice, SR14150 but not SR16835 had naloxone-reversible antinociceptive activity in the tail flick test (Figure 5A and 6A). However, both compounds had anti-allodynic activity in the von Frey test, which was blocked by the NOP receptor antagonist SB612111, but not by naloxone (*83*) (Figure 5B, 6B). One hypothesis to explain this phenomenon is that the NOP system is upregulated in a chronic pain condition, as described in several studies (*84–86*). If NOP receptors are increased in the spinal cord, where N/OFQ has antinociceptive activity, or in brain regions leading to antinociception rather than opiate inhibition, then NOP agonists might have antinociceptive or antiallodynic activity after systemic administration. As an alternate hypothesis, systemic administration of NOP agonists might work on mechanical but not thermal hyperalgesia in response to nerve injury. A very similar phenomenon has been described for delta opioid receptor agonists. The mu opioid receptor is expressed in peptidergic pain fibers, while the delta opioid receptor is expressed on myelinated and nonpeptidergic afferents. Furthermore, the selective mu agonist DAMGO is effective for heat but not mechanical pain, while the delta selective agonist SNC80 is effective for mechanical but not thermal pain (*61*). NOP receptors might localize with the delta opioid receptors and thereby

attenuate mechanical but not thermal pain. We are currently exploring these two hypotheses. Whatever the mechanism, these results suggest that selective NOP agonists could be effective for the treatment of chronic or neuropathic pain.

Buprenorphine Analogs

The above SR compounds were designed based on a non-morphinan NOP receptor-selective scaffold (see (*87*)). As discussed above, morphinan-based buprenorphine also binds to the NOP receptor, albeit with low affinity and efficacy (*69*, *77*). Because of its high mu affinity and partial agonist activity, buprenorphine also has significant antinociceptive activity, reduced opiate side effects, and favorable pharmacokinetics and blood brain barrier penetration. A buprenorphine analog with increased affinity and efficacy at NOP receptors, while maintaining its favorable profile at the opioid receptors, might produce a compound that has the antinociceptive efficacy of buprenorphine, but lower addiction liability and less tolerance development.

BU08028 is the first compound which has a Ki of less than 10 nM at all four receptors in the opioid receptor family (*88*). As seen in Table I, BU08028 had a Ki of 8 nM at NOP receptors, with a modest increase in efficacy at NOP receptors, compared to buprenorphine. This compound has potent, long lasting, antinociceptive activity, but surprisingly, has a steeper dose response curve than buprenorphine, and in contrast to buprenorphine, reached 100% MPE (Figure 7). The NOP agonist activity in BU08028, as defined as inhibition of mu-mediated antinociception, was evident since the antinociceptive activity was potentiated by SB612111 (Figure 8), however, it developed tolerance over the course of several days, in a manner similar to morphine. Furthermore, it produces a CPP after a single training session, greater than buprenorphine and equivalent to morphine. Apparently, higher NOP receptor-mediated efficacy is needed in a compound such as BU08028 in order to have attenuated mu-mediated rewarding effects.

Other NOP/mu Compounds

Additional compounds with high affinity at NOP and other opiate receptors have been reported. For instance, TH-030418 is a thiene etorphine derivative and has very high affinity at all four receptors (*89*, *90*). This is a potent and long lasting analgesic that does not produce an abstinence syndrome in mice, and also did not induce CPP. However, its efficacy at the opioid and NOP receptors was not reported so it is difficult to compare to other mixed compounds discussed above. Furthermore, the long lasting nature of the compound makes the CPP experiment problematic, so the apparent non-rewarding nature of the compound should be considered more carefully (*76*).

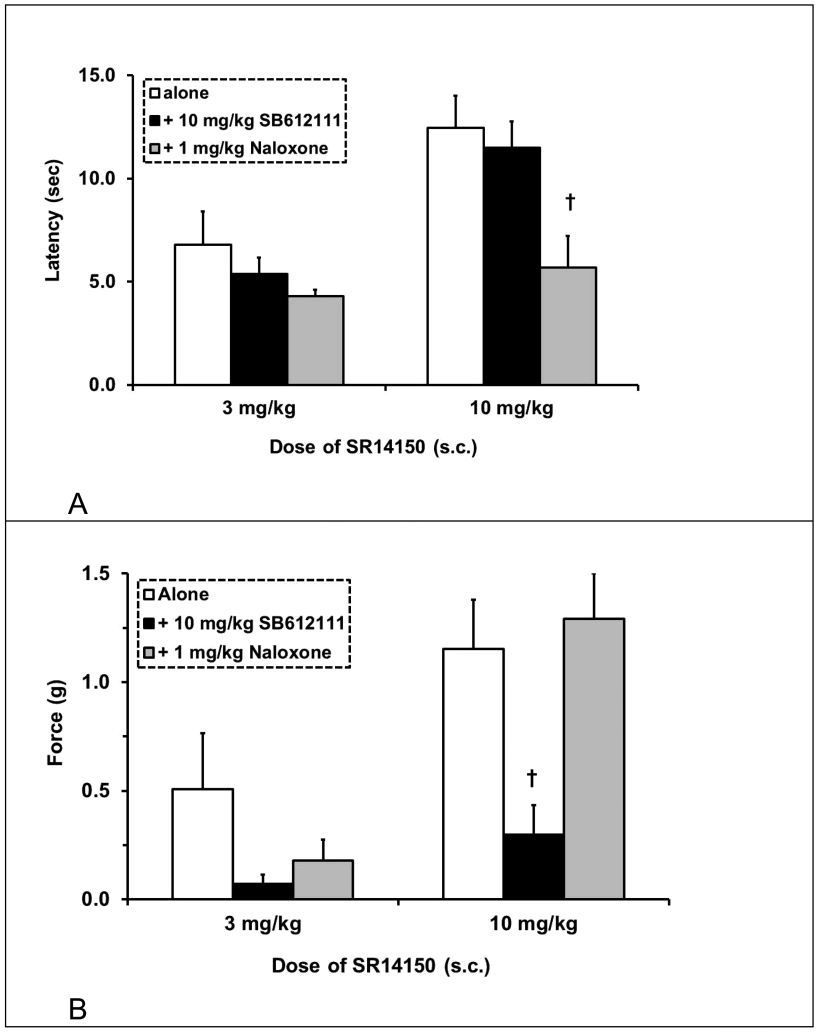

Figure 5. Antinociception and anti-allodynia, induced by SR14150 in SNL mice. A. SR14150 has antinociceptive activity that is blocked by naloxone pretreatment. B. SR14150 has anti-allodynic activity that is blocked by SB612111 and not naloxone pretreatment. A dagger (†) signifies a difference from SR14150 alone ($P<0.05$). Reproduced with permission from (83). Copyright 03/17/2009 from The American Society for Pharmacology and Experimental Therapeutics

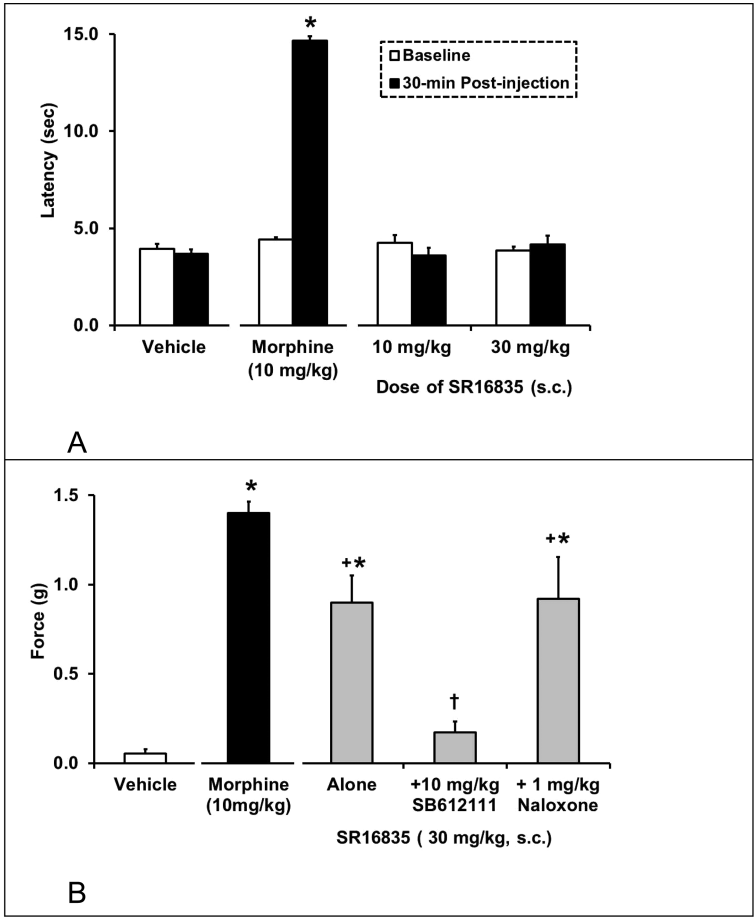

Figure 6. Antinociception and anti-allodynia induced by SR16835 in SNL mice. A. SR16835 does not have antinociceptive activity in SNL mice. B. SR16835 has anti-allodynic activity that is blocked by SB612111 but not naloxone pretreatment. An Asterisk (*) signifies a difference from vehicle controls, a plus sign (+) signifies a difference from morphine alone, whereas a dagger (†) signifies a difference from SR16835 alone (P<0.05). Reproduced with permission from (83). Copyright 03/17/2009 from The American Society for Pharmacology and Experimental Therapeutics

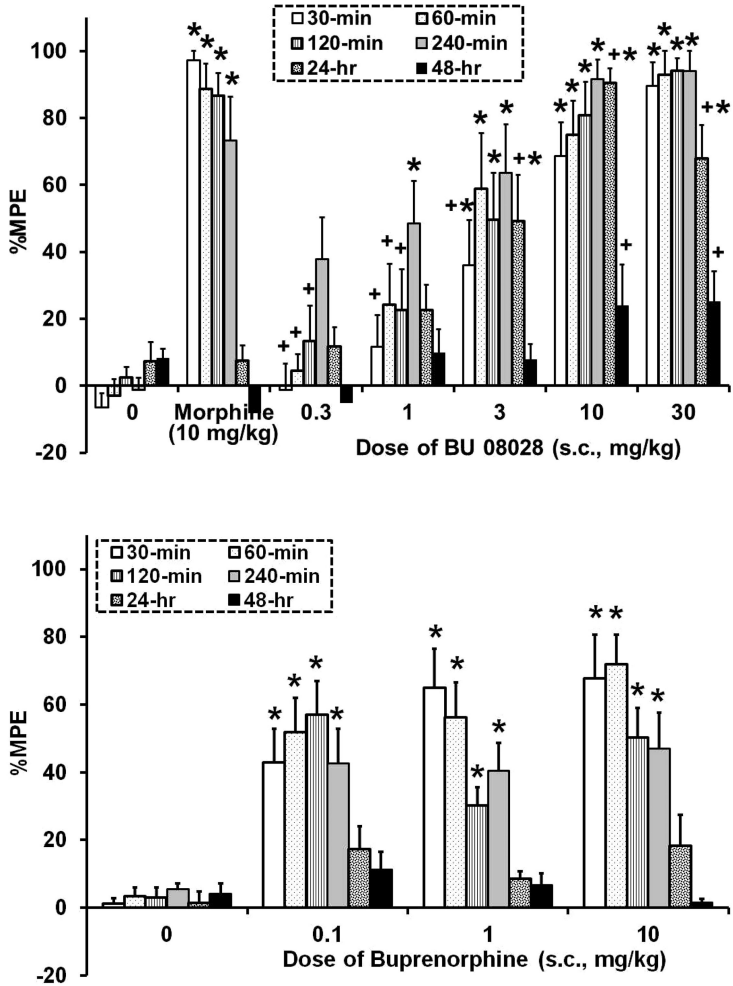

Figure 7. Antinociceptive activity of (A) BU08028 and (B) buprenorphine. BU08028 has full antinociceptive activity whereas buprenorphine never attains 100% MPE. An Asterisk (*) signifies a difference from vehicle controls, a plus sign (+) signifies a difference from morphine alone (P<0.05). Reproduced with permission from (88). Copyright 08/24/2011 from The American Society for Pharmacology and Experimental Therapeutics

Grunenthal GmbH has filed numerous patents on NOP/mu agonists and these patents have shown sporadic in vitro results. Because none of this work has been peer reviewed, it is difficult to identify individual compounds with promising in vitro profiles and in vivo activities. More recently Grunenthal, together with Forest Labs, has taken GRT 6005 into Phase IIb clinical trials. This compound has equal affinity and equal and full efficacy at NOP and mu opiate receptors. Although there is no animal data available in the literature, this compound has successfully completed initial proof-of-concept studies in nociceptive and neuropathic pain with initial Phase II clinical trials.

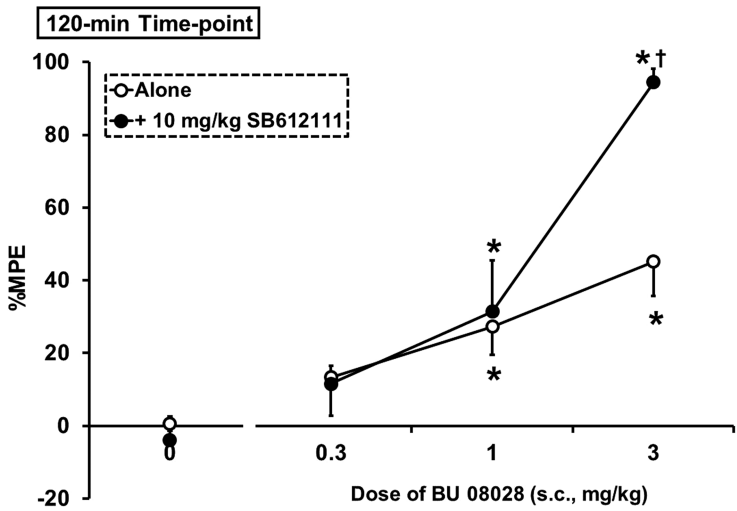

Figure 8. Antinociceptive activity of BU08028 in the presence and absence of SB612111. BU08028 has potent antinociceptive activity in the tail flick test. Nevertheless, its activity is potentiated by SB612111, indicating that NOP receptor activation still attenuates the mu-mediated antinociceptive response. An Asterisk (*) signifies a difference from vehicle controls, whereas a dagger (†) signifies a difference from BU08028 alone (P<0.05). Reproduced with permission from (88). Copyright 08/24/2011 from The American Society for Pharmacology and Experimental Therapeutics

Mixed NOP/Opiate Agonists as Drug Abuse Medications

In addition to its use as an analgesic, buprenorphine has a long history of use as a drug abuse medication. Although buprenorphine is more prominently used as a replacement therapy for opiate narcotic abuse, it can also block cocaine and alcohol self-administration in rodents, monkeys, and humans (*91–94*). Interestingly, in Sardinian alcohol preferring rats, buprenorphine potentiates alcohol consumption at low doses but attenuates alcohol consumption at higher doses. Ciccocioppo et al. determined that the increased alcohol consumption was blocked by naloxone but the inhibition of alcohol consumption, seen with higher doses of buprenorphine, was blocked by i.c.v. administration of the NOP receptor antagonist UFP-101 (*82*). Therefore, just like with its antinociceptive activity, low doses of buprenorphine have mu-mediated effects (increased drinking) while at high doses it has NOP-mediated activity, in this case attenuation of drinking. Not only do these studies demonstrate mixed NOP/mu activity for buprenorphine, they validate the hypothesis that in mixed NOP/mu compounds, the NOP agonist activity can block reward and have potential as drug abuse medication. This hypothesis was more directly studied using selective NOP receptor agonists. Systemic administration of Ro 64-6198 can block both acquisition and reinstatement of morphine and ethanol CPP (*51*, *55*, *95*). Interestingly, Ro 64-6198 increased, rather than attenuated, ethanol self-administration in a two bottle choice paradigm (*96*). Furthermore, this activity was blocked by naloxone, indicating that at high concentrations, Ro 64-6198 appeared to have mu-receptor mediated activity.

Although it has been demonstrated that i.c.v. administered N/OFQ induces neither CPP or CPA (*39*, *40*) some studies have indicated that N/OFQ induces a decrease in extracellular dopamine in the NAc, suggesting that a small molecule full-agonist treatment for drug abuse might be weakly dysphoric (*41*, *42*). Therefore, a compound with a small amount of mu agonist activity may afford better compliance, if the drug ever makes it into people. In this regard, SR16835, the NOP full agonist/mu partial agonist attenuates morphine CPP, an effect reversed by the NOP receptor antagonist SB612111, indicating that the blockade of morphine CPP is due to activation of NOP receptors (Figure 9) (*22*). Interestingly, this compound is not efficacious in blocking the acquisition of cocaine CPP (unpublished observation), even though i.c.v. administered N/OFQ blocks cocaine CPP. Nevertheless, SR16835 blocks reinstatement of cocaine CPP, suggesting that such a compound could have beneficial effects on cocaine relapse (Khroyan et al, unpublished observation). Since drug abuse medications are not going to be taken prophylactically prior to the original addiction, attenuation of relapse would be the logical target as a medication. Therefore, there is reason to believe that a full NOP agonist, like SR16835, with or without concurrent mu-receptor agonist activity, could be beneficial for prevention of relapse to opiates, cocaine, alcohol, and potentially other drugs of abuse. In fact, other NOP/opiate receptor profiles could also be useful drug abuse medications. Kappa antagonists have been demonstrated to attenuate relapse of cocaine, and alcohol, probably due to inhibition of endogenous dynorphin, which mediates some of the negative psychological aspects of withdrawal (*97*, *98*). Therefore, a NOP

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agonist/kappa antagonist could potentially be very beneficial for prevention of relapse to a variety of abused drugs. Such compounds are under development.

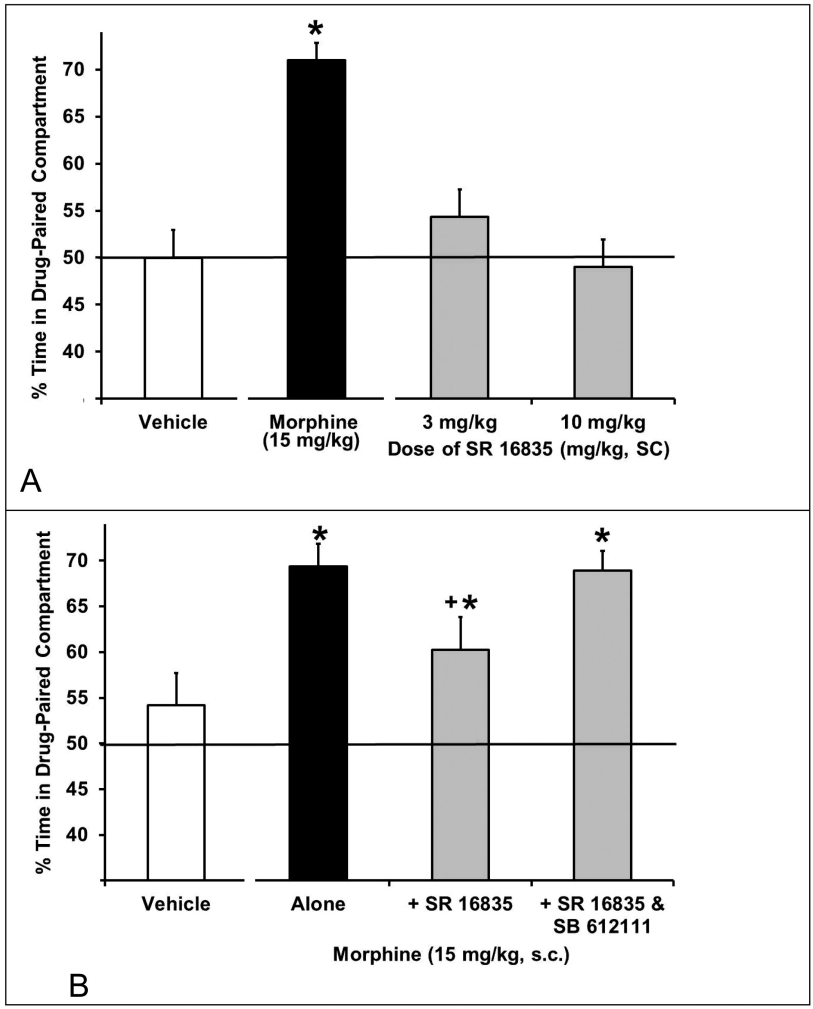

Figure 9. A. SR16835, the full and moderately selective NOP agonist does not induce CPP on its own. B. SR16835 attenuates morphine CPP and this effect is reversed by SB612111, demonstrating that SR16835 is acting through the NOP receptor. An asterisk (*) signifies a difference from vehicle controls, whereas a plus sign (+) signifies a difference from morphine alone (P<0.05). Reproduced with permission from (22). Copyright 09/24/2009 from The American Society for Pharmacology and Experimental Therapeutics

Conclusions

The ultimate goal of this research examining mixed NOP/opiate compounds is the potential development of medications that can be used clinically as analgesics with reduced addiction liability and tolerance development or as drug abuse medications. The NOP receptor, originally called ORL1 among several other names, was identified in 1994, shortly after the cloning of the first opiate receptor, the delta receptor. In the 18 subsequent years, many different groups, both within pharmaceutical companies and academic institutions, have invested in the discovery of NOP peptide and small molecule agonists and antagonists, all with the ultimate goal of developing useful pharmaceutical agents. A small number of compounds have progressed to clinical trials. The NOP receptor antagonist JTC-801 was examined as an analgesic, however, testing never progressed on from Phase II trials. Schering Plough progressed SCH 486757, a selective NOP receptor agonist, into human trials for cough. In phase Ib/II trials, this compound showed a small effect at certain time points, but this is a difficult target since even patients on placebo recover well over the duration of the trial. As discussed above, the NOP/mu full agonist, GRT 6005, was successful in Phase I and Phase IIa and is currently being examined in Phase IIb clinical trials for pain. Considering the preclinical and newly derived clinical data on NOP/mu agonists, there is high promise that such compounds could find their way into the clinic in the foreseeable future.

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Chapter 18

Pharmacological Investigation of NOP-Related Ligands as Analgesics without Abuse Liability

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Most widely used analgesic drugs for the treatment of moderate to severe pain are mu-opioid receptor agonists such as morphine. However, analgesic value of these drugs is compromised due to unwanted side effects including respiratory depression, abuse liability, itch, and tolerance to analgesia. Nociceptin/orphanin FQ receptor (NOP) is emerging as a potential analgesic target without abuse liability. Analgesic properties of NOP-related agonists have been investigated in rodents and monkeys. In rodents, spinal injection of NOP agonists produces antinociception against diverse pain modalities and also potentiates morphine-induced antinociception. In monkeys, both spinal and systemic administration of NOP agonists produce morphine-comparable antinociceptive effects against acute nociception, capsaicin-induced allodynia, and carrageenan-induced hyperalgesia. More importantly, NOP agonists do not produce respiratory depression, itch scratching, and reinforcing effects at the antinociceptive doses. Interestingly, spinal or systemic administration of NOP agonists can potentiate mu-opioid receptor mediated antinociception and widen the therapeutic window in monkeys. Therefore, NOP agonists have a promising analgesic value when injected alone or in combination with mu opioid analgesics. These studies further support the therapeutic potential of NOP-related ligands including selective NOP agonists and bifunctional NOP/MOP agonists as effective analgesics in order to achieve strong pain relief without concerns over abuse and safety.

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Current Status of Using Opioid Analgesics

Opioids are the most effective and widely used analgesic drugs for the management of moderate to severe pain. The most clinically used opioids are mu-opioid receptor (MOP) agonists such as morphine (*1*). However, MOP-mediated analgesia is often accompanied by undesirable side effects such as nausea, vomiting, constipation, and respiratory depression (*1*, *2*). Importantly, MOP agonists possess the risk of being abused, which is a serious public health concern (*3*, *4*). MOP agonists are also administered spinally to provide pain relief and are commonly used to treat obstetric, postoperative and cancer-related pain. Although application of spinal opioids has become one the most significant breakthroughs in pain management, it is also associated with unwanted side effects including pruritus, nausea, urinary retention, and hypotension (*5–7*).

Some patients develop tolerance to opioid analgesia in which greater doses of opioids are required to produce effective pain relief (*8*, *9*). Paradoxically, during rapid dose escalation, unexpected development of opioid-induced hyperalgesia may occur that is unassociated with the original pain (*10–12*). Due to these side effects, quality of opioid-induced analgesia is compromised and the value of opioids as effective analgesic drugs is reduced. Hence, there is a crucial need to identify novel analgesic targets that can provide effective opioid-like analgesia but fewer side effects and reduced abuse liability.

Background of Nociceptin/Orphanin FQ Peptide (NOP) Receptors

Discovery of the NOP Receptor and Its Structure Homology with Classical Opioids

In 1994, different research groups simultaneously characterized an orphan G protein-coupled receptor which showed high structural homology with the classical opioid receptors MOP, KOP (kappa) and DOP (delta) but did not bind to the classical opioids. It was termed as an opioid receptor-like 1 receptor (ORL-1) (*13–15*). One year later, based on the application of 'reverse pharmacology', the ligand for this receptor was identified as a 17 amino acid neuropeptide which showed structural homology with opioid peptides, particularly dynorphin A and was called nociceptin or orphanin FQ (N/OFQ) (*16*, *17*). The receptor is now referred to as N/OFQ peptide receptor or NOP. Despite the structural and localization similarities, N/OFQ does not activate MOP, KOP or DOP receptors. Also, classical opioids have extremely low binding affinity for NOP (*16*, *17*). These differences in ligand selectivity likely arise due to small number of residues that vary between NOP and other opioid receptors and subsequent changes in the structure of the binding pocket of NOP (*18*). Hence, the NOP receptor is currently classified as a non-opioid member of the opioid receptor family by International Union of Basic and Clinical Pharmacology.

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In Vitro Actions of NOP Receptors

At the cellular level, activation of NOP receptors on the membrane has effects that are similar to those of other classical opioid receptors. Activation of NOP receptors causes inhibition of cAMP production, closure of the voltage-sensitive Ca++ and increase in the outward K+ conductance in neurons, events that lead to reduction in neuronal excitability and attenuated neurotransmitter release (*16*, *17*). Naloxone, a non-selective opioid antagonist, does not block N/OFQ-induced intracellular events supporting the evidence that physiological functions of this peptide are not mediated by classical opioid receptors (*19*, *20*).

Abuse Liability of MOP versus NOP Receptor Agonists

Lack of Reinforcing/Rewarding Properties of NOP Agonists

Neuroanatomical and immunohistochemical studies have shown that N/OFQ and NOP receptors are widely distributed in various corticomesolimbic structures involved in regulation of reward and motivational effects of drugs of abuse (*21*, *22*). Hence, potential rewarding and/or reinforcing effects of N/OFQ were examined in animal models. Supraspinal administration of N/OFQ did not produce conditioned place preference or conditioned place aversion, indicating that NOP activation does not have rewarding properties (*23*). Similar studies were also carried out with the non-peptidic highly selective NOP agonists Ro 64-6198 and Ro 65-6570. When injected systemically, both compounds did not produce conditioned place preference in agreement with the previous findings that NOP agonists are devoid of motivational effects (*24*, *25*). These findings are in sharp contrast with the inherent addictive properties of MOP agonists like morphine and heroin which produce conditioned place preference across different animal species (*26–28*) and are addictive in humans (*29*, *30*)

Another procedure commonly used to determine reinforcing properties of a drug is intravenous self-administration. Laboratory animals readily self-administer drugs such as MOP agonists remifentanil and heroin, which have reinforcing properties and abuse potential in animals (*31–33*) as well as humans (*34*, *35*). A recent study showed that rats that were trained to respond in order to self-administer remifentanil, did not respond when presented with SCH221510, another highly potent and selective NOP agonist (*36*). Reinforcing effects of Ro 64-6198 were also investigated in nonhuman primates under the intravenous self-administration procedure. Over a wide dose range (0.03 – 30 μg/kg/injection), Ro 64-6198 was not reinforcing in monkeys that were initially trained to self-administer the MOP agonist alfentanil or the psychostimulant cocaine (figure 1) (*37*). Taken together, these findings suggest that NOP agonists are devoid of rewarding and/or reinforcing properties and may lack abuse liability in humans.

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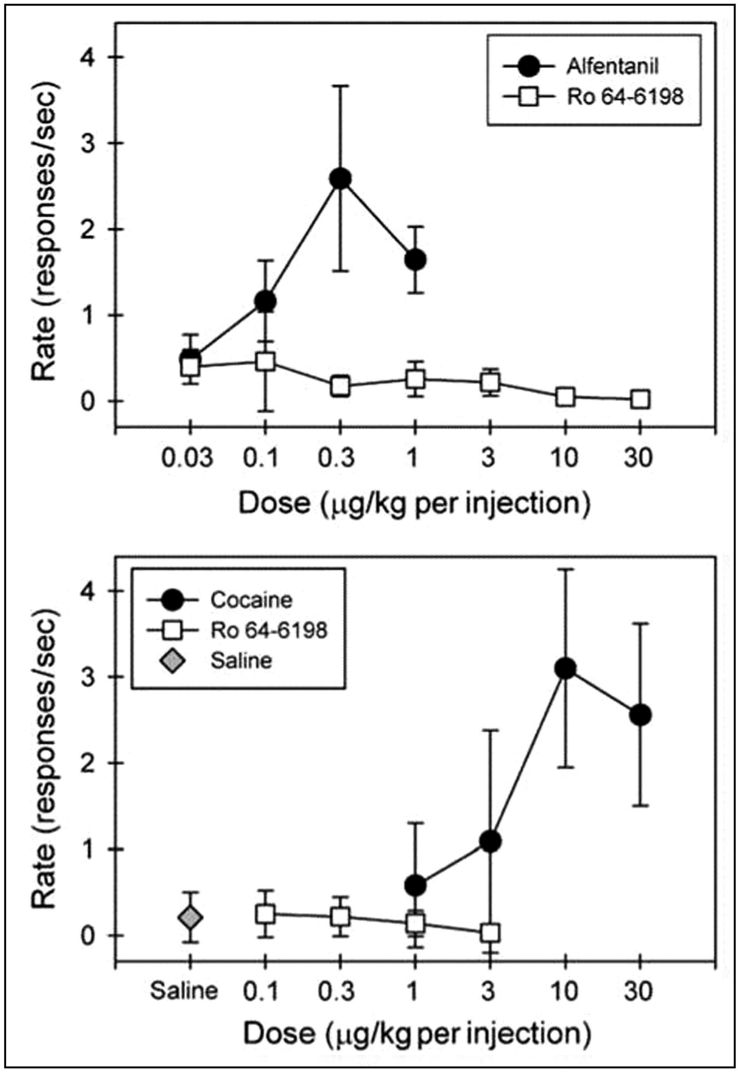

Figure 1. Ko et al showed that Ro 64-6198 is not self-administered over a large dose range including doses required to produce antinociception. Comparison is made with established reinforcers alfentanil and cocaine. Reprinted with permission from reference (37). Copyright 2009 American College of Neuropsychopharmacology (ACNP).

Ability of NOP Agonists To Block Rewarding/ Reinforcing Properties of Drugs of Abuse

Activation of NOP receptors is shown to attenuate dopamine release in the mesolimbic pathway induced by cocaine or morphine (*38–40*). Several studies in rodents have also reported that NOP agonists are able to block rewarding effects of opioids and psychostimulants. For instance, supraspinal injection of N/OFQ blocked morphine, cocaine, ethanol and methamphetamine-induced conditioned place preference in rodents (*41–43*). Similarly, systemically administered Ro 64-6198 blocked rewarding effects of morphine (*44*), ethanol-induced conditioned place preference (*45*) and ethanol self-administration in rodents (*46*) whereas Ro 65-6570 blocked rewarding effects of opiates and cocaine (*25*). Additionally, enhanced rewarding effects of cocaine and morphine were observed in the NOP receptor knockout mice and rats, respectively (*47*, *48*). Hence, pharmacological or genetic blockade of NOP made these animals more susceptible to the rewarding effects of drugs of abuse. In nonhuman primates, Ro 64-6198 seemed to be weak in blocking reinforcing effects of the MOP agonist remifentanil because responding for reminfetanil was only disrupted at substantially large doses of Ro 64-6198 that also produced sedation (*49*). However, the potential anti-reinforcing effects of NOP agonists on other reinforcers such as cocaine and sucrose have not been thoroughly investigated in nonhuman primates.

One of the clinically available anti-addiction treatment options is the partial MOP agonist buprenorphine (*50*, *51*). However, buprenorphine is classified as a schedule III compound by the Drug Enforcement Agency. In other words, buprenorphine possesses the risk of being abused (*52*). Another existing treatment for drug addiction is the MOP antagonist naltrexone. However, it is known to produce aversive and dysphoric effects in both humans and animals (*53–55*). On the other hand, NOP agonists did not produce conditioned place aversion in animal studies (*23*, *24*) but in fact showed prominent anxiolytic and anti-stress actions (*56*, *57*). Overall, these preclinical findings suggest that NOP-related ligands may represent a viable alternative for the treatment of drug addiction.

The NOP system has generated widespread interest among pain researchers since the time of its discovery. Findings from rodent studies suggest that NOP receptor activation can modulate nociception differentially depending on the site of drug administration. For example, supraspinal injection of N/OFQ produced pronociceptive or anti-opioid effects (*16*, *17*, *58*). However, spinal administration of NOP agonists at high doses was antinociceptive, but increased pain sensitivity at ultra-low doses (*59–61*). Interestingly, in nonhuman primates, systemic and spinal administration of NOP agonists produced antinociceptive effects (*37*, *62*, *63*). Hence, NOP-related ligands hold great potential as effective analgesic drugs without abuse liability. In this review, we will discuss the recent findings with NOP-related ligands in both rodent and nonhuman primate models of pain, and the therapeutic potential of NOP-related ligands as effective analgesics without MOP-associated side effects.

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Potential of NOP Agonists as Spinal Analgesics

Spinal Analgesia in Humans and Current Challenges

First study using intrathecal morphine in humans was reported in 1979 (*64*). Since then, spinal analgesia is a common procedure for obstetric and postoperative analgesia. Spinal analgesia is also used for the management of cancer pain. Cancer patients often opt for spinal analgesia when systemically administered analgesic drugs are no longer effective (*65*, *66*) and over 50% patients report pain relief with spinal analgesia (*67–69*). Spinal analgesia is also used when patients develop tolerance to the analgesia induced by systemic opioids. The most commonly delivered drugs for spinal analgesia are MOP agonists such as morphine. Side effects associated with spinal administration of MOP agonists are commonly documented. Some of these side effects include pruritus, sedation, nausea and urinary retention which greatly compromise the quality of analgesia in patients (*5*). In addition, there are contradictory reports on the effectiveness of opioids in treating neuropathic pain. (*70*, *71*). Paradoxically, opioid-induced hyperalgesia is reported in some patients (*72*). Long-term intrathecal opioid treatment may also result in development of tolerance to analgesia (*73*, *74*). Despite the undesired side effects and challenges derived from MOP agonists, they are currently the most commonly used spinal analgesics that are clinically available. Nevertheless, more research is warranted to identify novel molecular targets that can be safely administered as effective spinal analgesics or have the ability to potentiate MOP-mediated analgesia in absence of the undesired side effects.

Studies with Spinal Administration of MOP Agonists in Rodents and Nonhuman Primates

Studies using either acute or chronic intrathecal administration of MOP agonists in rodent models of pain have been extensively carried out in order to understand the neurobiological events and mechanisms underlying modulation of pain sensitivity by opioids. For example, spinal delivery of morphine was shown to elevate tail withdrawal latency in response to acute noxious thermal stimulus (*75*, *76*), attenuate mechanical allodynia in rats with peripheral nerve injury (*77*, *78*) and reduce sensitivity to thermal, mechanical and cold stimuli following acute paw inflammation in rodents (*79*). On the other hand, long-term exposure to intrathecal administration of MOP agonists was shown to induce tolerance development (*80–82*) and opioid-induced hyperalgesia in rodents (*80*, *83*, *84*).

Studies in nonhuman primates show that spinal administration of morphine produces prolonged antinociception in a dose dependent manner (*85*, *86*). One of the side effects of intrathecal morphine also documented in nonhuman primates is itch scratching that is mediated by MOP receptor activation at doses that produce antinociception (*86*). Monkeys showed different susceptibility to intrathecal morphine-induced itch similar to what is reported in the clinical settings (*87*, *88*). Itch is the most common side effect associated with spinal administration of MOP

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agonists and can sometimes be a serious symptom. Hence, research identifying analgesic agents that do not induce itch responses is necessary and the nonhuman primate could serve as a surrogate species for humans in preclinical studies of spinal analgesics without itch/ pruritus (*62*, *86*).

Studies with Spinal Administration of NOP Agonists in Rodents

There is large evidence that spinal administration of NOP agonists has antinociceptive effects in rodents under both acute and chronic pain conditions. In acute pain assays, intrathecally delivered N/OFQ increased tail withdrawal latency from thermal nociceptive stimulus in rodents without producing sedation or motor dysfunction (*59*, *60*). Similarly, it increased paw withdrawal latency in the hot plate assay in mice (*89*). Intrathecal N/OFQ reduced thermal sensitivity from carrageenan-induced paw inflammation and diminished formalin-induced phase 2 flinching in rats (*90–92*). Studies in rodent models of nerve injury-induced pain also showed that intrathecal administration of N/OFQ reduced thermal, mechanical and cold sensitivity in response to partial sciatic nerve injury, chronic constriction of the sciatic nerve and spinal nerve ligation (*93–95*). UFP-112 ([(pF)Phe4Aib7Arg14Lys15]N/OFQ-NH2), which is a chemically modified N/OFQ peptide with increased agonist potency and decreased susceptibility to degradation by peptidases, was antinociceptive in the mouse tail flick assay following intrathecal administration (*96*, *97*). Ro 64-6198 also produced anti-allodynic effects against thermal and mechanical stimuli when given intrathecally in rats with spinal nerve ligation (*98*). Together, peptidic and non-peptidic NOP agonists were able to block pain behaviors in rodents in response to acute noxious stimulus or chronic pain conditions.

Rodent studies have also revealed neuroadaptive changes underlying spinally driven antinociceptive effects of NOP agonists. Increased levels of mRNA and protein for the NOP receptor and precursor of the N/OFQ peptide are reported in various regions of brain, dorsal root ganglia and superficial laminae of spinal cord in response to peripheral nerve injury (*99–101*). Similar increase in NOP receptor expression is also shown in superficial laminae of spinal cord in rats after the injection of Complete Freund's Adjuvant, an inducer of inflammatory pain (*102*). These neurobiological changes in the N/OFQ-NOP system under the conditions of chronic pain are thought to drive antinociceptive effects of NOP agonists.

Studies with Spinal Administration of NOP Agonists in Nonhuman Primates

The pharmacological profile of intrathecally administered NOP agonists is also investigated in nonhuman primates (*62*, *63*, *103*). Intrathecal injection of N/OFQ produced significant thermal antinociception for 2-3 hours over a wide dose range from 10 nmol to 1 μmol, manifested as elevated tail withdrawal latencies from noxious thermal stimulus. The magnitude of antinociceptive effects was similar to that of clinically available MOP agonists such as morphine and fentanyl. NOP antagonist J-113397, but not the classic opioid antagonist naltrexone, blocked antinociception induced by intrathecally injected N/OFQ, indicating a NOP receptor-mediated antinociception. Importantly, intrathecal

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N/OFQ did not induce scratching at antinociceptive doses unlike the MOP agonists in nonhuman primates, indicating that the NOP receptor is a viable target as the spinal analgesic devoid of itch side effect (*63*, *103*).

In order to display distinct actions on modulation of nociceptive thresholds, effects of intrathecally injected ultra-low (femtomole) doses of N/OFQ were compared in the same animals with substance P, a pronociceptive/hyperalgesic agent, as well as DAMGO, a highly specific and potent MOP agonist known to produce antinociception. As expected, substance P produced thermal hyperalgesia by reducing the tail withdrawal latencies from non-noxious thermal stimuli. On the other hand, at ultra-low doses, N/OFQ did not produce hyperalgesia in monkeys unlike its hyperalgesic actions in rodents at ultra-low doses. DAMGO induced antinociception against noxious thermal stimulus but also elicited profound scratching. On the contrary, N/OFQ did not produce antinociception or scratching at ultra-low doses. Overall, these studies showed that intrathecally administered N/OFQ is safe over a wide dose range and has the potential to induce analgesia without observable side effects in nonhuman primates (*63*).

Effects of spinal administration of NOP agonist UFP-112 were also investigated in nonhuman primates in assays of acute thermal nociception and capsaicin-induced allodynia (*62*). Intrathecal administration of UFP-112 (1–10 nmol) produced antinociception against acute thermal noxious stimulus and capsaicin-induced thermal allodynia in a dose dependent manner. Intrathecal UFP-112-induced acute antinociception was fully reversed by J-113397, demonstrating that the antinociceptive action of UFP-112 in monkeys was due to selective NOP receptor activation. Additionally, UFP-112 (3-10 nmol) significantly attenuated capsaicin-induced thermal allodynia (figure 2). These effects were comparable to intrathecal morphine (10-100 nmol) in terms of the magnitude and duration (4 -5 h) of antinociception and antiallodynia. In fact, UFP-112 was more potent than morphine under the conditions of capsaicin-induced thermal allodynia (figure 2). Capsaicin is a natural irritant found in hot chili peppers that evokes pain sensation by activating Transient Receptor Potential Vanilloid 1. Transient Receptor Potential Vanilloid channels are implicated in transmission of noxious stimuli in tissue injury-induced thermal hyperalgesia, diabetic neuropathy and neurogenic inflammatory responses associated with many disease states (*104*, *105*). Capsaicin-induced allodynia has been previously utilized as a pain model in both monkeys (*106*) and humans (*107*, *108*) to study analgesic compounds. Since the capsaicin-sensitive nerve fibers are linked to a number of pain modalities, effects of intrathecal UFP-112 against capsaicin-induced allodynia can suggest a prominent clinical value. Importantly, unlike intrathecal morphine, UFP-112 did not produce scratching responses in monkeys. It will be interesting to further conduct pharmacokinetic studies comparing the levels of UFP-112 and morphine in cerebrospinal fluid following their intrathecal administration. These findings demonstrated that like the intrathecal morphine, UFP-112 produced antinociception in two primate pain modalities with the similar magnitude and long duration of action. Together, the preclinical studies in nonhuman primates strongly indicate that NOP agonists have the potential to be spinal analgesics devoid of the itch side effect and are promising candidates for the future clinical studies.

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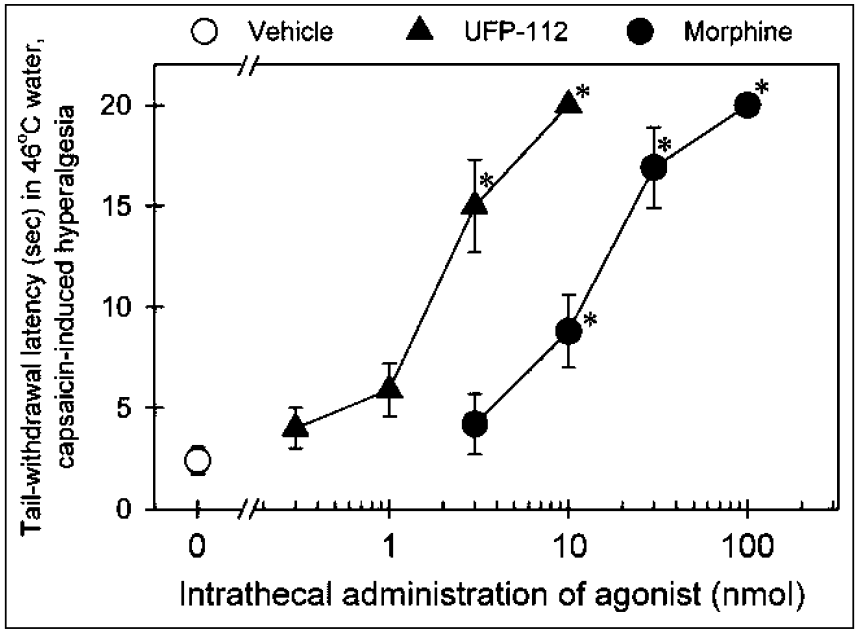

Figure 2. Hu et al showed that Blockade of capsaicin-induced thermal allodynia by intrathecal injection of morphine and UFP-112. UFP-112 is more potent than morphine for its antiallodynic effects. Reprinted with permission from reference (62). Copyright 2010 Elsevier.

NOP Receptor Agonists as Systemic Analgesics

Studies with Systemic Administration of NOP Agonists in Rodents

In rodents, fewer studies have investigated antinociceptive effects of systemically administered NOP agonists. Systemic injection of Ro 64-6198 did not change the nociceptive threshold in rats (*57*) and decreased heat sensitivity of the paw in mouse hot plate assay (*109*). In rats with chronic constriction of the sciatic nerve, Ro 64-6198 failed to produce antiallodynic effects following subcutaneous administration (*98*). However, following subcutaneous injection, peptide NOP agonist Syn1020 was anti-allodynic in this pain model (*110*). Hence, findings with systemic administration of NOP agonists in rodent pain models are not as consistent as their spinal actions and need further investigation.

Studies with Systemic Administration of NOP Agonists in Nonhuman Primates

Effects of systemic administration of Ro 64-6198 were investigated in nonhuman primates against three pain modalities, including acute thermal nociception, capsaicin-induced thermal allodynia, and carrageenan-induced thermal hyperalgesia (*37*, *111*). Following subcutaneous injection, Ro 64-6198

(0.001–0.06 mg/kg) produced significant thermal antinociception that was mediated by NOP receptors but independent of MOP receptors. Ro 64-6198 also attenuated capsaicin-induced thermal allodynia and carrageenan-induced thermal hyperalgesia. In both assays, effects and potency of Ro 64-6198 were shown to be comparable to that of the MOP agonist alfentanil. Importantly, intramuscular injection of Ro 64-6198 did not elicit scratching and respiratory depression at antinociceptive doses, unlike alfentanil. As mentioned previously, Ro 64-6198 did not produce reinforcing effects in these monkeys over a wide dose range. Taken together, these studies provide functional evidence that NOP agonists have a therapeutic value as systemic analgesics without the ability of inducing scratching, respiratory depression and abuse liability.

Antinociceptive effects of NOP agonists seem to vary between rodents and monkeys. Although in rodents only intrathecal administration of NOP agonists is implicated in antinociception, NOP agonists are antinociceptive in monkeys irrespective of the route of administration. It is possible that in rodents, pronociceptive actions mediated by supraspinal NOP counteract antinociception induced by spinal and peripheral NOP receptors, following systemic administration of NOP agonists. In monkeys, however, effects of supraspinal administration of NOP agonists have not been studied given that systemic and spinal routes of drug administration are the most commonly used routes for analgesics in humans. In the future, it would be interesting to see the modulation of physiological responses in monkeys following supraspinal administration of NOP agonists in order to gain better understanding of how supraspinal activation of NOP may regulate antinociceptive effects after systemic or spinal administration. Anatomical studies reveal that there are differences between rodents and primates in terms of the distribution of N/OFQ and its receptors (*112*, *113*). For example, reasonable expression of NOP mRNA and NOP-radioligand binding was detected in striatum and cerebellar cortex of primates in contrast with lack of expression reported in rodents (*114–116*). As a result, degree of physiological outcome from activating supraspinal, spinal and peripheral NOP receptors together following systemic administration of NOP agonists may vary across species. These species differences can be further investigated with the functional determination of neuronal expression of NOP receptors in primates and rodents.

Bifunctional NOP/MOP Ligands

Potentiation of Antinociception with Co-Activation of NOP and MOP Receptors in Rodents

Rodent studies show that activation of spinal NOP receptors can potentiate MOP-mediated antinociception. In the rat tail flick assay, intrathecal administration of N/OFQ potentiated morphine-induced antinociception without affecting the motor function (*59*). Also, when Ro 64-6198 was systemically co-administered at subthreshold doses with morphine, the combination enhanced the attenuation of heat sensitivity in the hot plate test in mice (*109*). In rats with diabetic neuropathy, systemic injection of morphine with intrathecal injection of

N/OFQ together resulted in strong potentiation of analgesia (*117*). In neuropathic rats with chronic constriction of the sciatic nerve, isobolographic analysis showed that intrathecal co-administration of morphine and N/OFQ suppressed mechanical hyperalgesia in a superadditive manner (*117*). Together, these studies indicated that co-activation of NOP and MOP receptors produced synergistic antinociception in rodent models of acute and chronic pain.

Potentiation of Antinociception with Co-Activation of NOP and MOP Receptors in Nonhuman Primates

Previous findings have shown that in primates, there are two independent components – NOP and MOP - that can equally contribute to analgesia. Further studies were carried out to determine if co-activation of NOP and MOP receptors could potentiate antinociception in nonhuman primates. When N/OFQ was combined with a single intrathecal dose of morphine, it dose-dependently potentiated intrathecal morphine-induced antinociception against noxious thermal stimuli of higher intensity. Interestingly, addition of intrathecal N/OFQ did not attenuate intrathecal morphine-induced scratching responses, suggesting that addition of N/OFQ to morphine did not produce motor-related side effects in monkeys (*63*).

In another study, significant blockade of capsaicin-induced thermal allodynia was achieved following spinal administration of a mixture that contained inactive doses of UFP-112 and morphine. This combination effect was attenuated with co-administration of J-113397 and naltrexone but not when both antagonists were administered alone (*62*). Hence, activation of both NOP and MOP concurrently to a small degree contributed to a profound relief of allodynia. More interestingly, although the combination of inactive doses of morphine and UFP-112 produced significant antiallodynic effects, it did not elicit scratching responses in monkeys (*62*), indicating that the therapeutic outcome can be achieved with a wider therapeutic window by activating two receptor reservoirs at the spinal cord level.

Recently, a study was conducted in monkeys in order to understand the roles of NOP and MOP receptors in regulating buprenorphine-induced physiological responses in assays measuring analgesia, respiratory depression and itch (*118*). Pharmacological studies indicate that buprenorphine is a partial agonist at MOP receptors (*119–121*) and that NOP receptor knockout mice or NOP receptor antagonists potentiate antinociception produced by buprenorphine (*122–124*). Hence in rodents, MOP-mediated antinociceptive action of buprenorphine is compromised by concomitant activation of NOP receptors. However, in vitro pharmacological studies indicate that buprenorphine has extremely low binding affinity at NOP as compared to MOP and it is much less potent in activating NOP receptors (*120*). In monkeys, buprenorphine-induced antinociception is mediated by MOP receptors and not altered by NOP antagonists. When NOP agonists Ro 64-6198 and SCH221510 were systemically co-administered with buprenorphine, synergistic antinociceptive effects were obtained. In other words, activation of NOP receptors did not attenuate but instead potentiated buprenorphine's antinociception mediated by MOP receptors in primates (figure 3). When ratio of NOP agonists combined with that buprenorphine was increased,

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the mixture produced full antinociception without respiratory depression or scratching responses (figure 3). Together, these preclinical studies strongly suggest that simultaneous activation of NOP and MOP receptors to a small degree can produce analgesia with minimum side effects at the systemic level in nonhuman primates and may prove to be a promising therapeutic strategy to achieve optimum analgesia.

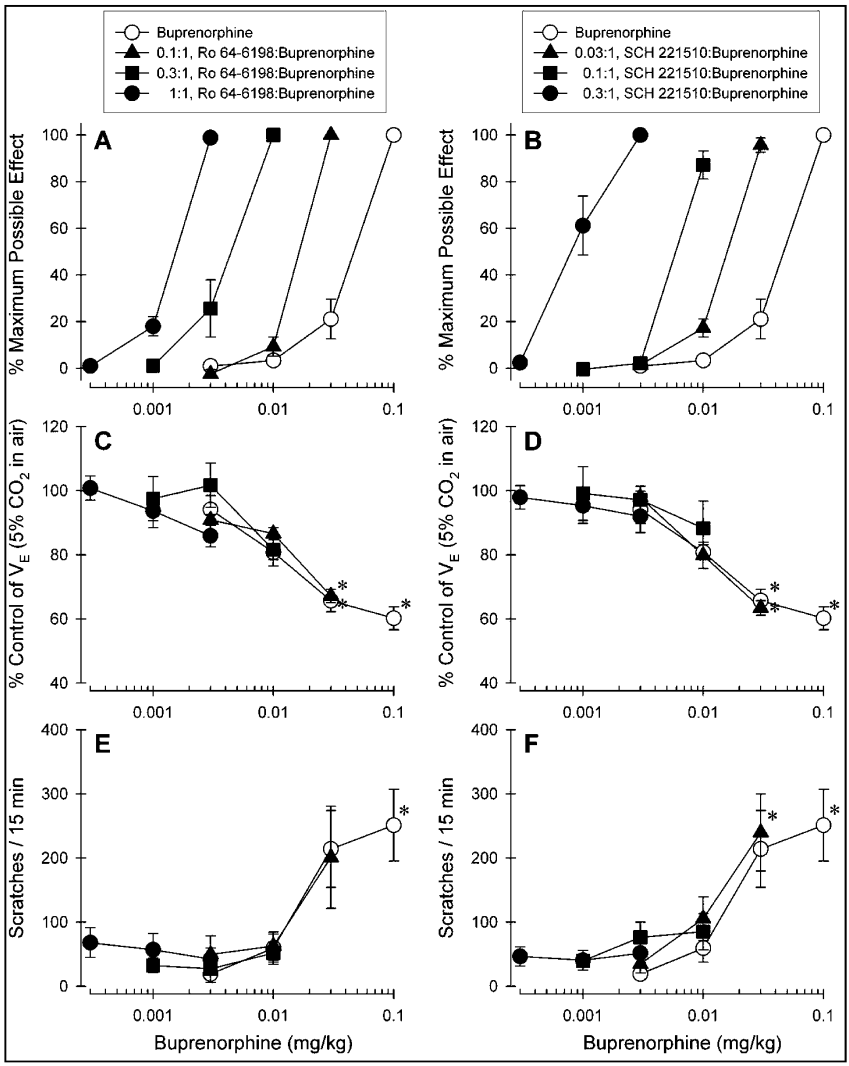

Figure 3. Cremeans et al showed that NOP agonists dose-dependently potentiated buprenorphine-induced antinociception without producing respiratory depression and scratching responses in monkeys. Reprinted with permission from reference (118). Copyright 2012 American Society for Pharmacology and Experimental Therapeutics.

Therapeutic Potential of NOP/MOP Co-Activation

There is solid functional evidence that combination of analgesic drugs targeting NOP and MOP receptors may have the potential to improve efficacy through either additive or synergistic interactions. In primates, NOP agonists produced antinociception in absence of respiratory depression, abuse liability, and itch pruritus. If NOP receptors have side effects yet to be described, adding them to MOP agonists may allow doses for both drugs to be lowered, resulting in enhanced analgesia and reduced side effects. Co-activation of NOP and MOP receptors is particularly important in the context of analgesic tolerance development. With compounds that can activate both NOP and MOP receptors, less receptor pool is utilized to achieve analgesia and more receptors available for the subsequent treatment, causing slower development to analgesic tolerance. Investigation of tolerance development to the analgesic effects of NOP/MOP co-activation in comparison with that of selective agonists is therefore required. Collectively, bifunctional NOP/MOP agonists that simultaneously activate NOP and MOP receptors may be valuable analgesics because; (1) co-activation of NOP and MOP receptors can provide a wider therapeutic window due to their potentiated antinociception and reduced side effect profile, (2) NOP receptor activation is suggested to have anti-addiction property, because of which, the bifunctional ligands have reduced risk of being abused and, (3) reduced or slower development of tolerance to the analgesic function.

Studies with Bifunctional NOP/MOP Agonists

A series of bifunctional agonists that bind to NOP and MOP receptors with different degrees of affinity and efficacy were synthesized and tested in rodent models of acute or chronic neuropathic pain. Two chapters in this book (by Drs. L. Toll and N. Zaveri) discuss in details the exciting research and development of bifunctional NOP/MOP ligands from the chemical and pharmacological perspectives. For example, SR14150 is a partial agonist at both NOP and MOP receptors (*125*). In mice, systemically administered SR14150 produced naloxone-reversible antinociception in an acute thermal nociception assay (*126*) and with spinal nerve ligation, SR14150 displayed potent antiallodynic activity which was blocked by NOP antagonist SB-612111 (*127*). Recently, the effects of intrathecally injected bifunctional NOP/MOP agonists were determined in mice with chronic constriction of the sciatic nerve and acute paw inflammation (*128*). Bifunctional NOP/MOP agonists, SR16435 and BU08028, which show partial agonism at both NOP and MOP receptors, were more potent at blocking pain behaviors than selective MOP or NOP agonists morphine and SCH221510, respectively. The antiallodynic and antihyperalgesic effects of both bifunctional agonists were comparable with those of the selective agonists. Full blockade of antiallodynic activity of these bifunctional agonists was only achieved following spinal co-administration of NOP and MOP antagonists but not when the antagonists were administered alone. These data suggest that at the level of spinal cord, both NOP and MOP receptors independently contribute to antinociception in mice. Overall, the rodent studies indicate that bifunctional ligands with

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partial agonist activity at both NOP and MOP receptors are effective in blocking allodynia under experimental conditions of neuropathic pain, presumably at the level of spinal cord.

Rewarding properties of bifunctional NOP/MOP partial agonists were investigated using the conditioned place preference paradigm. For instance, SR14150 did not produce conditioned place preference in mice. However, reinforcing effects of these bifunctional ligands were not determined using other assays as drug self-administration. It is proposed that by modulating the selectivity of a bifunctional ligand between NOP and MOP receptors while still maintaining partial agonism at both receptors, it is possible to achieve optimum analgesia without the risk of abuse liability (*126*, *129*, *130*)

In monkeys, antinociceptive properties of a bifunctional NOP/MOP agonist peptide were recently determined. [Dmt1]N/OFQ(1-13)-NH2 is a novel bifunctional peptide with full agonist activity at NOP and MOP receptors. Intrathecal administration of this peptide in monkeys demonstrated robust and long lasting antinociceptive effects against acute thermal nociception (*131*). At lower doses, that produced antinociception, no scratching was observed whereas higher doses induced scratching. It is important to conduct antagonist studies to determine the relative contribution of NOP versus MOP in the antinociceptive effects of bifunctional NOP/MOP ligands, and to investigate what types of bifunctional NOP/MOP ligands do or do not have reinforcing effects as measured by the drug self-administration assay.

It is also valuable to determine the rate and degree of tolerance development to analgesia induced by bifunctional NOP/MOP agonists in primates. Overall, the preclinical findings from rodents and primates strongly support the therapeutic potential of bifunctional NOP/MOP agonists as effective analgesics. Most certainly, additional efforts are required to establish the pharmacological profiles of diverse bifunctional NOP/MOP ligands in primates and determine their effects following acute and chronic administration

Conclusion

Taken together, the pharmacological studies strongly suggest that agonists which bind to NOP receptors represent a promising profile as spinal analgesics. In addition, nonpeptidic NOP agonists can also provide effective analgesia when delivered systemically in primates. Analgesia mediated by NOP receptors is independent of MOP receptor activation and MOP-associated side effects such as respiratory depression and pruritus. More importantly, NOP agonists may provide analgesia without abuse liability. Potential utility of NOP-related ligands as primary or secondary analgesic drugs in humans warrants synthesis of highly potent compounds that bind to NOP receptors. Recent discovery of crystal structure of human NOP receptors (*18*) has opened up avenues to design such ligands. These studies reveal atomic details of ligand-receptor recognition and point out conformational differences in the binding pocket of NOP versus MOP or KOP receptors (*132*, *133*). Understanding such differences can improve our knowledge of structural requirements for NOP ligand selectivity and facilitate

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the optimization of bifunctional therapeutics that co-activate NOP and MOP receptors. Pharmacokinetics and functional anatomy of such NOP-related ligands can be investigated using molecular imaging studies in humans with the help of positron emission tomography which will provide further understanding of affinity, biodistribution and duration of action for these compounds.

In conclusion, NOP receptors hold a significant clinical value as analgesic targets with reduced abuse liability. Ligands that bind to NOP receptors can be especially effective in patients unresponsive to treatment with MOP agonists or have developed analgesic tolerance to these drugs. Administration of bifunctional NOP/MOP agonists further provides a promising strategy to gain improved analgesic efficacy and slower tolerance development. With such exciting therapeutic possibilities, NOP receptors create a novel chapter in the research and development surrounding opioid-associated analgesia.

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M. C. (Holden) Ko (Ph.D., University of Michigan) is currently a Professor in the Department of Physiology and Pharmacology at Wake Forest University School of Medicine. He has been a Research Associate Professor in the Department of Pharmacology at the University of Michigan from 2008 to 2012. By using animal models with translational values, his research focuses on the drug discovery at the preclinical stage, especially research and development of novel drugs that have promising therapeutic profiles in primates. He has published over 60 peer-reviewed PubMed articles and received several awards including the Wyeth-Ayerst Psychopharmacologist Award from the American Psychological Association.

Stephen M. Husbands

S. M. Husbands (Ph.D., University of Strathclyde) joined the Department of Pharmacy and Pharmacology of the University of Bath in 2000. Now a Professor in Medicinal Chemistry and Fellow of the Royal Society of Chemistry, he has published over 90 peer reviewed papers and has a long-standing interest in the design, synthesis and application of opioid ligands. Recently his work has focussed on the development of 'selectively promiscuous' ligands, ligands that bind to multiple receptors but with defined efficacy at each. He is particularly interested in these ligands as potential drug abuse treatment agents and as strong analgesics with an improved safety profile.

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