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Review

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Dynamic Measurement of Extracellular Opioid Activity: Status Quo, Challenges, and Significance in Rewarded Behaviors

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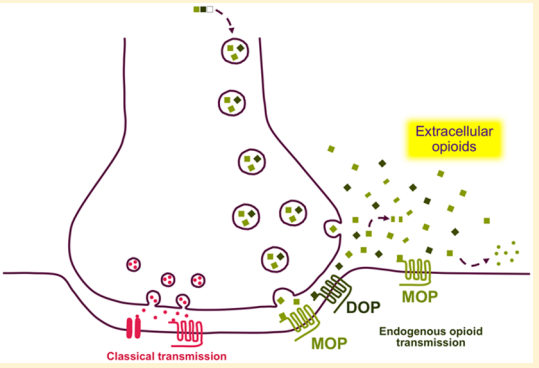
ABSTRACT: Opioid peptides are the endogenous ligands of opioid receptors, which are also the molecular target of naturally occurring and synthetic opiates, such as morphine and heroin. Since their discovery in the 1970s, opioid peptides, which are found widely throughout the central nervous system and the periphery, have been intensely studied because of their involvement in pain and pleasure. Over the years, our understanding of opioid peptides has widened to cover a multitude of functions, including learning and memory, affective state, gastrointestinal transit, feeding, immune function, and metabolism. Unsurprisingly, aberrant opioid activity is implicated in numerous pathologies, including drug addiction, overeating, pain, depression, and obesity. To date, virtually all preclinical and clinical studies aimed at understanding the function of endogenous opioids have relied upon manipulating endogenous opioid fluxes using opioid receptor ligands or genetic manipulations of opioid receptors and endogenous opioids. Difficulties in directly monitoring endogenous opioid fluxes, particularly in the central nervous system, have presented a major obstacle to fully understanding endogenous opioid function. This review summarizes these challenges and offers suggestions for future goals while focusing on the neurobiology of reward, specifically drawing attention to studies that have succeeded in dynamically measuring opioid peptides.



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OPIOID PEPTIDES

Nearly four decades have passed since the discovery of the first opioid, enkephalin, by Hughes and Kosterlitz. This event not only launched the study of endogenous opioid peptides (EOPs) per se, but it was also highly influential in the rapid expansion of the then nascent field of neuropeptide research.[1,2] To this day, EOPs remain an important and popular topic of study among an increasing number of identified endogenous peptides with neuromodulatory ability, and EOPs show enormous promise as potential therapeutic targets. Indeed, the last four decades have revealed that EOPs are at the heart of several physiological and psychological processes considered critically important to adaptiveness and survival, namely, pleasure and pain.[3−5] EOPs also play a role in numerous corollary processes, such as motivation, feeding, anxiety, learning, reproduction, metabolic control, and energy balance. A recent Medline search of the word "opioid" (which includes both natural and synthetic opioid receptor ligands) yielded over 130000 citations with a continuing upward trend (Figure 1). However, virtually none of these citations report dynamic measurements of EOP release in the extracellular space, that is, the site at which they act as neuromodulators. This signifies that almost our entire understanding of the role of EOPs in physiological function and their therapeutic application is based on approaches using proxy and inferential measures of EOP effects, such as administering opioid receptor agonists, blocking

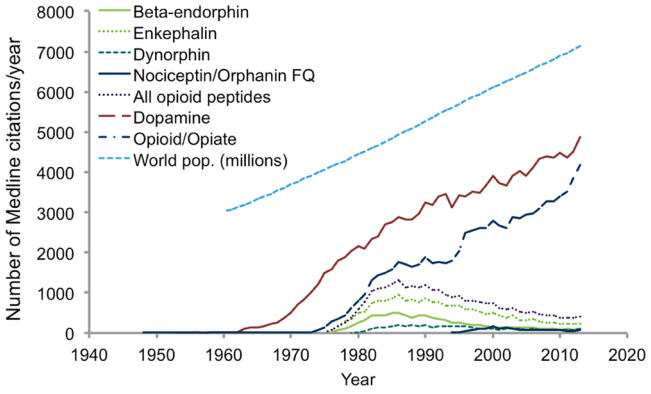

Figure 1. Publication rates in opioid-related fields. The graph shows the number of citations per year returned from Medline searches using the keywords listed. Results for the neurotransmitter dopamine are shown for comparison. Note that the terms "opioid" and "opiate" are often used interchangeably and that the term "endorphin" sometimes incorporates all areas of opioid study, especially during the early days of the field.

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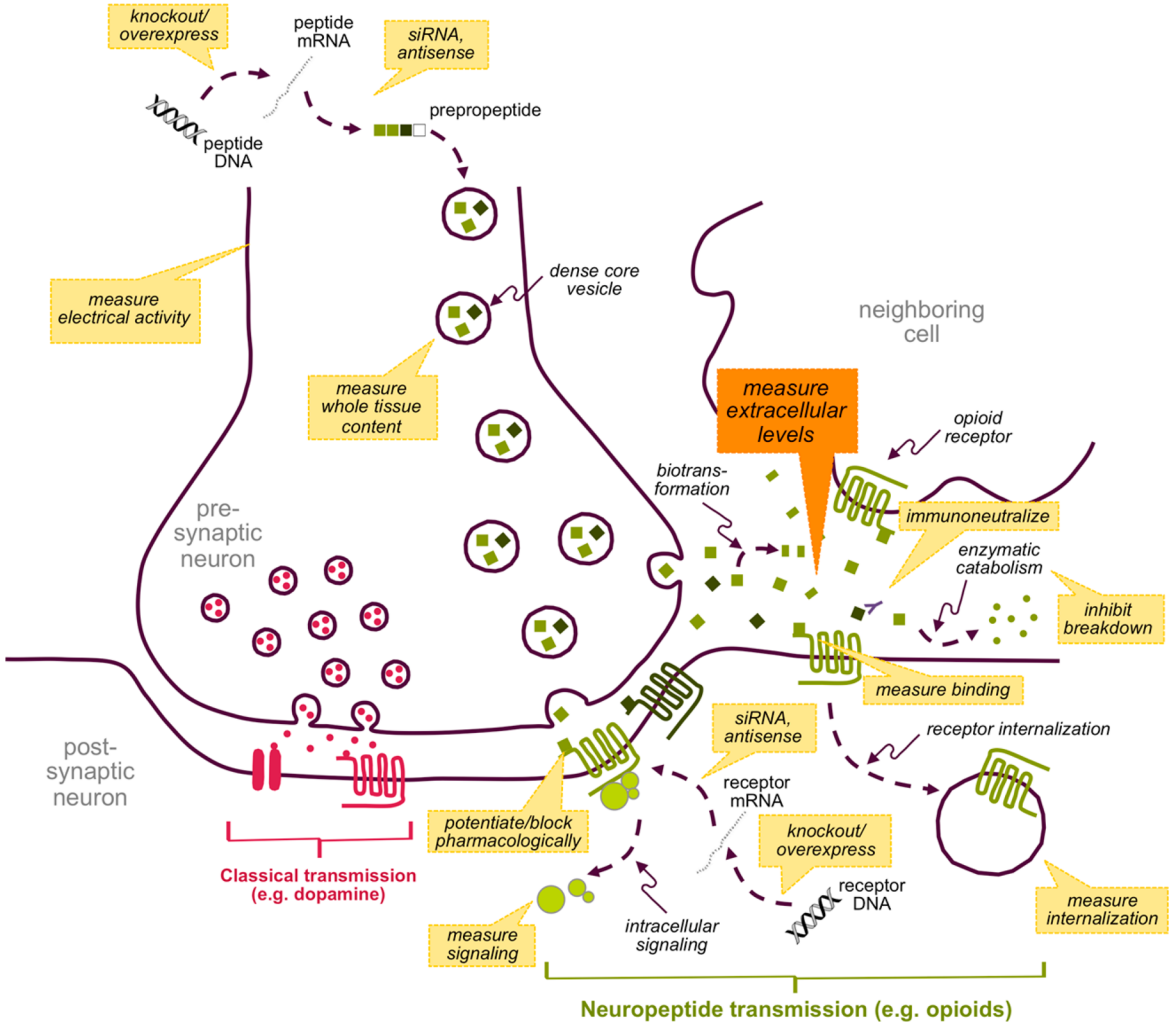

Figure 2. Schematic overview of EOP transmission highlighting points and strategies (yellow callouts) used in studies of dynamic changes in EOP transmission. This review focuses on the necessity and challenges of directly measuring EOPs in the extracellular space (orange callout).

Table 1. Indirect, Direct and Inferential Approaches for Measuring EOP Activity

index of activity	approach	selectivity	sensitivity	appropriateness for measuring extracellular EOP fluxes	relative financial cost
gene expression	in situ hybridization	excellent	poor	poor	medium
	Northern blotting	excellent	medium	poor	low
	qRT-PCR	excellent	excellent	poor	medium
peptide levels	UV spectrometry (tissue extracts)	poor	poor	poor	medium
	Western blotting (tissue extracts)	good	poor	poor	low
	immunohistochemistry	good	poor	poor	low
	RIA/ELISA (tissue extracts)	good	excellent	poor	medium
	mass spectrometry (tissue extracts)	excellent	excellent	poor	high
	voltammetry/electrochemistry (in vivo)	poor	poor	excellent	high
	RIA/ELISA (with microdialysis)	good	excellent	excellent	high
	mass spectrometry (with microdialysis)	excellent	excellent	excellent	very high
inferential	receptor internalization	good	n/a	poor	low
	antagonist studies	fair	n/a	poor	low
	peptidase inhibition	poor	n/a	poor	low
	receptor phosphorylation	fair	n/a	poor	low
	imaging of opioid receptors	fair	good	fair	high
	immunoneutralization	excellent	n/a	fair	low
	antisense RNA/siRNA	good	good	good	medium
	gene knockout	excellent	good	good	high

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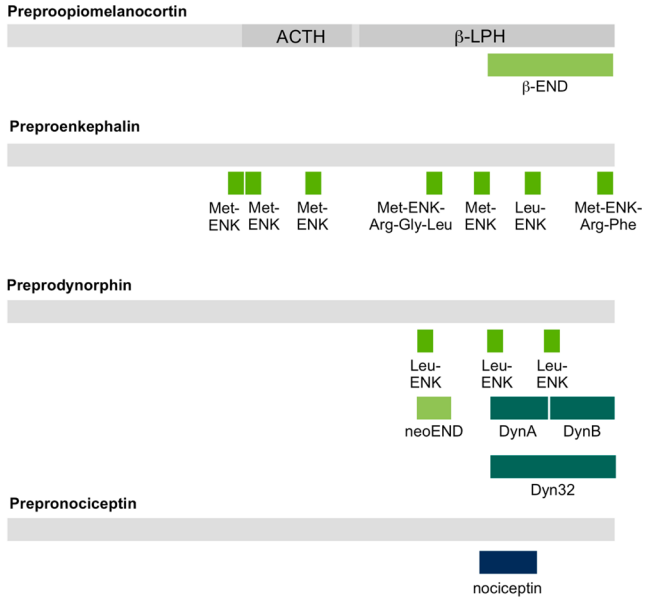
the actions of EOPs through pharmacological and genetic means, or measuring the gene transcription or whole system levels of EOPs (Figure 2, Table 1).

Why is it so important to dynamically measure EOP fluxes? While studying the effects of exogenously applied opioid agonists is useful, ultimately this approach is only generally suited to *inferring* the actual physiological roles for EOPs. Working under the assumption that EOPs act primarily as *extracellular* signaling molecules and transmit their signal by way of dynamic concentration changes, answering two specific questions can provide the most direct insight into the true physiological role of an EOP: First, does the *amount* of the EOP in question *change* during an event of interest? Second, does disturbing this change in EOP concentration have a predictable effect on this event? Notably, these two questions may not always produce answers that are apparently congruent. That is, some receptors exhibit constitutive activity whereby constant amounts of a given signaling molecule are sufficient for maintaining activity.[6] Target cells may alter their response by attenuating or potentiating signaling through the intracellular effector systems of the receptor, rather than relying on input changes produced by the signaling molecule itself. Thus, measuring endogenous levels of signaling molecules, like EOPs, may not only provide important correlative support for EOP function but also be the key to understanding function. Furthermore, there are many outstanding questions regarding the fundamental nature of EOP transmission that remain unanswered, some of which are highlighted below. For example, how far do EOPs travel through tissue to reach their target site? Do they arrive at those sites unchanged? How intransient are EOP-mediated signals? Moreover, there are in fact currently no perfect methodologies for instantaneously and selectively removing the action of endogenous molecules, because pharmacological approaches lack perfect selectivity and genetic approaches are temporally poor, and both approaches can suffer concerns over localization of the action. Thus, achieving insight into moment-by-moment fluxes in EOP transmission in the extracellular space can address many currently unanswered questions.

The issue is compounded further when one considers the fundamental nature of neuropeptides, particularly EOPs. Unlike other extracellular signaling molecules, especially those of the central nervous system, EOPs may exhibit considerable promiscuity in their pharmacological and physiological actions. Figure 3 shows the putative EOPs produced from known opioid peptide precursors, with the exception of endomorphins for which precursors have not yet been identified. This figure highlights that although distinct genes *generally* code for distinct prepropeptides, some EOPs can hypothetically be created from more than one prepropeptide (e.g., Leu-enkephalin from preproenkephalin or preprodynorphin[7]). Furthermore, and perhaps more importantly, in vitro binding and activity studies make a strong case for cross-binding and cross-activation (Figure 4) of enkephalins and endorphins with mu and delta opioid receptors,[8] a situation further complicated by the existence of opioid receptor subtypes.[9,10] Thus, even the development of a perfectly selective mu opioid receptor antagonist may be unable to distinguish between the effects of specific EOPs (e.g., endogenous enkephalins versus endorphins or endomorphins). The same argument applies to the genetic knockout of opioid receptors, regardless of whether it is constitutive, conditional, regionalized, etc. Currently, the approach perhaps closest to revealing distinct physiological



Abbreviations: ACTH: adrenocorticotropic hormone; β-LPH: β-lipotropin; DYN: dynorphin; END: endorphin; ENK: enkephalin

Figure 3. Putative EOPs derived from opioid peptide precursors. Note that endomorphins are omitted because a precursor has yet to be identified. Sequence lengths are shown to approximate scale.

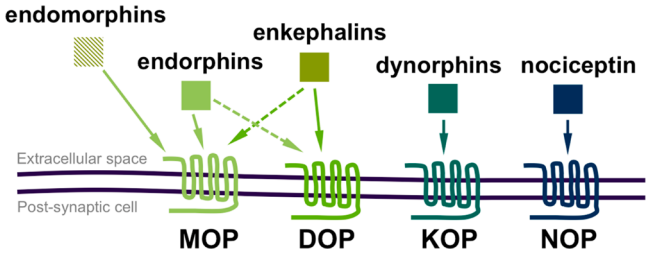


Figure 4. Major opioid receptor targets of EOPs. EOPs shown reflect those of general classes rather than reflecting derivation from the specific EOP propeptides shown in Figure 3.

roles for specific EOPs is the use of animals with null mutations in genes encoding EOP peptides, such as enkephalin or dynorphin knockout mice; however, these methods suffer several caveats regarding the inability to instantaneously, absolutely, and reversibly remove specific EOP tones and other potential endogenous peptide sequences with yet unknown physiological actions. Immunoneutralization, which uses antibodies directed at EOP sequences to block their action, may also provide a means of uncovering specific EOP functions. Use of this technique has yet to be widely reported, but it has been applied to study the role of EOPs in states, such as pain,[11,12] and to investigate the neurochemical effects of blocking EOP fluxes.[13] However, several unanswered questions persist regarding its application, such as how far can antibodies travel through the system under study, how selective are the antibodies for target sequences that could potentially be found in several EOPs, and how does one adequately control for the administration of such a large and possibly foreign protein?

So, why have so few studies measured EOP fluxes? The answer lies almost certainly in their similarity to a multitude of other biologically active and nonactive peptides and their existence at very low levels. Enkephalin holds the distinction of being the first biologically active peptide discovered to be

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secreted from neurons.[2] However, EOPs represent only a fraction of the large class of signaling molecules known as neuropeptides,[1] which are currently defined as "small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrates".[14] Known neuropeptides are encoded by at least 70 different genes in the mammalian genome and vastly outnumber the handful of classically recognized neurotransmitters (e.g., dopamine, GABA, nitric oxide). They have wide ranging modes of function, including the modulation of neurotransmission, autocrine and paracrine effects, and the ability to act as long-range hormones.[15] Estimating the actual number of existing neuropeptides even in just mammalian species is difficult because new ones are continually being discovered. Currently, at least 100 neuropeptides are recognized. Structurally, as their name suggests, the unifying feature of neuropeptides is their peptidergic nature, necessitating highly specific, sensitive, and often specialized assays for their measurement. This contrasts with classical neurotransmitters, such as dopamine and serotonin, which can be readily measured with more generalized techniques, including electrochemistry. An additional problem related to measuring EOPs is the very low levels at which neuropeptides exist in biological systems. For instance, local extracellular GABA concentrations in the brain are many orders of magnitude higher than enkephalins in the brain, and given the generally lower levels of mRNA (identified by in situ hybridization) and peptides (identified by immunohistochemistry), endorphin could exist orders of magnitude lower than enkephalin.

Neuropeptides differ from classical neurotransmitters in several important physiological ways that are particularly relevant to their measurement (see ref 1). Being neuropeptides, EOPs are stored in vesicles before being transported to their release site (Figure 1) and cannot be rapidly replenished. Unlike classical neurotransmitters, such as dopamine and glutamate, which are usually stored in small synaptic vesicles at the synapse, neuropeptides are stored in large dense core vesicles distributed throughout neurons, which can release their contents from any site in response to electrical activity. Thus, signaling by neuropeptides occurs mostly at extrasynaptic sites, though recent studies suggest that many other neurotransmitters, such as dopamine,[16] can also signal through nonsynaptic, that is, dendritic, release. Indeed, consideration of what might constitute a neuropeptide synapse is worthy of special consideration and an area in which direct measurement of EOPs could make significant contributions toward understanding their *modus operandi*, functions, and biological action. For instance, it is unclear how EOPs travel from their release sites to their sites of action. Evidence suggests that neuropeptides, such as EOPs, act as "neurohormones" and travel extensive distances through tissue and fluids. Coupled with the generally long half-life of neuropeptides and high affinity receptors, the release of EOPs may not target specific synapses but rather be intended as a diffuse signal that reverberates across large areas. Thus, the distribution of EOP receptors may be as germane as the distribution of EOP-containing fibers. For instance, one estimation suggests that dynorphin can move 70 μm in 20 s from release sites in the hippocampus.[17] Studies suggest endorphin may move from peripheral sites into the brain and vice versa. For example, β-endorphin administered into the cerebrospinal fluid (CSF) can be found in the paraventricular nucleus of the hypothalamus within 15 min,[18] and β-endorphin administered into the striatum can be found in the CSF.[19] Studies using microprobes suggest that electrical stimulation of the arcuate nucleus (the major source of endorphin-containing neurons) induces a significant spread of endorphin into multiple areas of the brain, such as the midbrain and cerebral cortex, at sites that are very distant from the release sites.[20]

A further issue is that exocytosis of neuropeptide-containing vesicles requires demanding increases in intracellular calcium because the large dense core vesicles are positioned further from membrane bound calcium channels compared with small synaptic vesicles containing classical neurotransmitters. This resulting change in the release latency is only one of many differences in the release mechanisms of neuropeptides (see refs 21 and 22 for discussion). The consequence of these differences is that neuropeptides require a more intense stimulation to trigger release, which adds practical challenges to verifying their function experimentally.

A further defining feature of neuropeptides, such as EOPs, is their co-existence with other neuropeptides and classical transmitters. Perhaps the best defined examples of this include colocalization of enkephalin or dynorphin with GABA in striatal medium spiny neurons; however, various other examples exist, such as colocalization of enkephalin with serotonin in the caudal raphe nuclei,[23] dynorphin with orexin in the lateral hypothalamus,[24] and GABA in endorphin producing neurons in the arcuate nucleus.[25] It is likely that all co-stored peptides are released together as a cocktail to produce coordinated responses in target cells, which likely explains why co-released peptides generally have harmonious sets of actions. Neuropeptide release is generally prompted by small increases in intracellular Ca^{2+} concentration across the cytoplasm, whereas localized increases in intracellular Ca^{2+} concentrations near the synaptic Ca^{2+} channels in close proximity to the active zone triggers release of small fast-acting transmitters. It is unclear to what extent neuropeptide-containing vesicles are targeted across the cell spatially. It appears neuropeptide containing vesicles are generally targeted to axon terminals, though localization to dendrites also occurs. This may mean that neuropeptide release is generally a more sluggish but sustained event that is only vaguely related to electrical activity and the passage of action potentials.[26] Whereas fast-acting classical transmitters act as the principal messenger to deliver rapid and transient signals at so-called hard wired synapses, neuropeptides, such as EOPs, deliver a slower, prolonged, and diffuse signal. Another delay in their signaling may depend on the distance they must travel to reach their target sites. Furthermore, neuropeptides may directly act to modulate the action of the co-released principal neurotransmitters by activating second messenger systems that modulate signal transduction through the receptors of the principal transmitter. Interactions between cotransmitters may also be evident presynaptically, such as a messenger acting on presynaptic receptors to influence the release of the other (see refs 15 and 27−29 for further discussion of this topic).

The situation is even further complicated by the fact that neuropeptides, such as EOPs, may undergo cleavage once released, yielding "daughter" molecules with inherent biological actions distinct from their parent molecules, a process akin to the conversion of angiotensin I to angiotensin II. These "biotransformations" may produce waves of biologically active molecules, each with distinct cellular effects. Numerous studies show nonopioid receptor mediated actions of EOPs[30] and particular interest has been placed on dynorphin and

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dynorphin-related peptides. For instance, the major form of dynorphin is dynorphin1−17, which is enzymatically cleaved in the striatum to yield various smaller peptides, including Dyn A(1−6), Dyn A(1−7), Dyn A(7−17), Dyn A(8−17), and Dyn A(9−17).[31] Understanding the pharmacological specificity of opioid peptides is likely to contribute immensely to understanding their functional specificity. It is difficult to determine whether and where EOP fragments have biological activity; however, studies of other dynorphin fragments, particularly Dyn A(2−17), suggest that these peptides can act at nonopioid sites, such as at melanocortin and NMDA receptors,[32−34] and influence various biological activities, such as reducing somatic signs of antagonist-precipitated morphine withdrawal[35] (see refs 30, 36, and 37 for reviews). Interest in the biotransformation of EOPs is increasing,[38] and recent studies suggest that EOPs can undergo biotransformation across entirely different classes, for example, dynorphin to enkephalin.[39] Furthermore, the exact mechanisms of EOP biotransformation may depend on the current physiological state, as evidenced in a rodent Parkinson's disease model.[40] To add further complexity, opioid receptors may form homo- and heterodimers, which may represent a largely unrecognized form of signaling, especially at the intracellular level (e.g., refs 41 and 42).

INDIRECT MEASURES OF EOP ACTIVITY

The emphasis of this review is to affirm the importance and necessity of dynamically monitoring EOP fluxes in vivo in order to better understand their physiological function. As yet, very few such studies have achieved this particular goal; however, many studies have used alternative, more indirect, approaches to produce significant contributions to understanding EOP physiology (see ref 3 for recent review). Thus, a brief review of these approaches, along with their advantages and disadvantages, is deserved (Table 2). The major shortcomings of the majority of these methods are a lack of specificity and temporal resolution and a highly invasive nature. While pharmacological and genetic approaches have largely contributed to the *inference* of EOP activities (Table 1, see ref 3), here we discuss the *direct* measurement of EOP levels by addressing two major questions: how does one *access* EOPs for measurement, and how does one *measure* EOPs?

Table 2. Access Approaches for Studying EOPs

approach	invasiveness	effectiveness for measuring extracellular EOPs	appropriateness for measuring extracellular EOPs	relative financial cost
superfusion	very high	poor	very poor	very low
tissue punching	very high	very poor	poor	low
blood/plasma	low	poor	poor	low
cerebrospinal fluid	medium	poor	poor	low
imaging	very low	medium	good	very high
in vivo voltammetry	low	medium	poor	high
push pull	medium	medium	good	medium
microdialysis	medium	very good	very good	high

Most methods applied for accessing and measuring EOPs have been extremely invasive and indirect. One such method is biopsying tissue from an area of interest, which in the case of the central nervous system is almost invariably a terminal procedure. This approach, such as taking brain "punches", provides a single measurement of all EOP levels either locally or globally and, importantly, is unable to distinguish intra- versus extracellular localization, a key issue for a secreted signaling molecule. Given that the majority of measured EOPs are likely to be of intracellular origin, whole tissue EOP measurements probably poorly reflect events occurring in the extracellular space. For instance, systemic alcohol administration stimulates increased extracellular Met-enkephalin in the nucleus accumbens yet decreases whole accumbal content.[43] This limitation should not imply that these biopsy methods are not without utility; for example, there are instances where gaining an *overall* measure of regional EOP levels is desired in order to provide a general view of up- or down-regulation in the system.

An alternative approach that may circumvent the finality of biopsy methods is to take a proxy for EOP activity. Most notably in this respect has been the measurement of EOPs in the cerebrospinal fluid or plasma, which was widely applied in the early days of EOP studies. In fact, much of the lay view of the role of EOPs in the body, such as their relationship with orosensory pleasure[44] and pain,[45] is based on these studies. However, such studies should be considered with a good degree of caution, because exactly what peripheral EOP measures actually reflect is poorly understood. For instance, it is unclear to what extent any particular EOP crosses the blood−brain barrier in its native form (e.g., ref 46; see ref 47). It is also unclear how well peptides penetrate and move through brain tissue from their site of release (see discussion above), despite decades of studies using intracerebroventricular administration of peptides as a means to deliver peptides throughout the brain. Furthermore, EOPs are synthesized in many sites outside of the CNS, such as endorphins from the pituitary gland and enkephalin in the gut.[48] Thus, it is unlikely that EOPs measured outside of the brain closely reflect central EOP fluxes.[49]

Other indirect measures of EOP activity that have been utilized are summarized and exemplified in Figure 1 and Table 1. Proxy measures, such as gene expression through Northern analysis and RT-PCR, provide strong quantitative measures but lack dynamic properties and are even further removed from actual extracellular EOP release. In situ measures, such as in situ hybridization of EOP coding mRNA or immunohistochemical localization of EOPs, can provide significantly more detail regarding specific neuroanatomical and cellular localization; however, these methods often lack a strong quantitative potential. Moreover, there are a multitude of processes that occur during the transcription of peptide genes, as reflected in mRNA levels and the actual release of bioactive peptides. This phenomenon explains the oft poor correlations between gene expression and protein levels (see ref 50), due to points of modulation such as translation at ribosomes in the endoplasmic reticulum, enzymatic cleavage, packaging in the Golgi apparatus, and transportation into storage vesicles. Post-translational processing under the control of changing environmental conditions may also impact the production of specific neuropeptides, such as the splicing of precursor peptides at different signal sites rather than an alteration in gene expression. Furthermore, these approaches provide one-off measurements that require terminal procedures.

Other indirect methods also exist. For instance, some, but not all, opioids and opiates induce internalization of opioid receptors;[51,52] therefore, the degree internalization can be quantified as a proxy for EOP release, but again this approach

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can only provide a single snapshot of activity. Additionally, opioid receptors undergo phosphorylation upon activation (see ref 53), an action inherent to the modulation of signaling processes that could potentially be used as a proxy for changes in EOP fluxes. Upon release, EOPs ultimately undergo enzymatic degradations, and thus manipulations of endogenous catabolic enzyme activity can give rudimentary insight into EOP release and action (see ref 54).

A notable exception to the above is brain imaging techniques, such as PET,[55] that use opioid receptor ligands. This approach has been widely used in human studies (see ref 55) to reveal dynamic changes in EOP activity related to various pharmacological and behavioral manipulations. For instance, changes in EOP tone during behavioral outcomes have been demonstrated during placebo and nocebo effects,[56] exercise,[57] eating disorders,[58] and pain.[59] However, the approach of displacing labeled pharmacological ligands suffers from many of the same issues plaguing pharmacological approaches (see ref 60), including poor receptor selectivity of ligands and an inability to distinguish exactly which EOP is undergoing a change in activity. Furthermore, this approach is somewhat difficult to apply in preclinical studies, though recent advances in brain imaging in rodents, especially in related peptide fields,[61,62] point toward increased application of this approach in the future.

DIRECTLY ACCESSING EXTRACELLULAR EOPS IN VIVO FOR MEASUREMENT

Aside from imaging approaches, the methods for accessing EOPs in vivo that were outlined above are invasive, often terminal, and do not directly access extracellular EOPs. However, there are relatively less invasive methods that are better suited for accessing and monitoring dynamic changes in extracellular EOPs (Table 2). These methods are primarily push−pull perfusion and microdialysis. In essence, these two similar approaches recover molecules from the extracellular space over a physical field typically 500 to 4000 μm in size. In push−pull perfusion, a perfusion medium, such as artificial CSF or Ringers solution, is pumped through one of two cannulae in concentric arrangement, while negative pressure is applied to the other cannula. Consequently, the perfusion medium is recovered from the sampling site after exposure to local analytes of interest. Similar to microdialysis, the push−pull perfusion method samples discrete fields but also has the added advantage of high recovery rates. The major disadvantage of push−pull perfusion is the small yield in terms of actual volume sampled and the risk of local tissue damage due to the physical disturbance brought about by the perfusate. This damage can also be exacerbated if the input positive pressure is not perfectly balanced with the output negative pressure. Even after achieving a balanced pressure, it is unclear to what degree a physical disturbance of the tissue can impact physiological responses, which raises concerns over artifactual findings.

Microdialysis sampling uses a probe fitted with a semipermeable dialysis membrane, which like a push−pull cannula is comprised of an input and output cannula. Though in neither case is a concentric design necessary, modern microdialysis probes are usually constructed in this manner. Also similar to push−pull perfusion, a medium is perfused through the cannulae where analytes diffuse across the microdialysis membrane from the site of interest to be caught in the stream of passing perfusate and ultimately collected for analysis. There are several theoretical considerations in the application of microdialysis, especially in the selection of the dialysis membrane, including flow rates, the physicochemical characteristics of the membrane and the analytes intended for collection, and diffusion characteristics, such as tortuosity, which impact the recovery of analytes (see refs 63−66 for reviews). Importantly, both push−pull perfusion and microdialysis techniques sample analytes from the extracellular space and have the added benefit of being able to introduce molecules of interest, for example, drugs and enzyme inhibitors, to the collection site by incorporation into the perfusion medium. Microdialysis has several notable limitations, such as relatively poor recovery rates (often less than 5% for peptides such as EOPs,[67]) and a difficulty in calibrating probes due to differing tortuosity in the extracellular space compared with in vitro conditions. Furthermore, both methods necessitate a significant physical lesion at the actual site of interest, which brings about a myriad of cellular, neurochemical, and immune responses that must be considered when interpreting data obtained using these techniques (see ref 64). Nonetheless, microdialysis is widely considered the superior technique out of all the approaches currently available for accessing extracellular EOP fluxes (see ref 64 for further discussion).

SEPARATION AND ASSAY METHODS

Neuropeptides, including EOPs, can be quantified by several means, the most common of which are summarized in Table 1. As alluded to above, an ideal method is both highly selective and highly sensitive. Broadly, the methods used for quantifying EOPs can be divided into those using some form of chemical separation of the sample (such as high pressure liquid chromatography, HPLC) as a means to increase assay selectivity and those that detect the target molecule directly. Two of the most common methods of the latter type, which are fundamentally very similar, are enzyme-linked immunosorbant assays (ELISAs) and radioimmunoassays (RIAs). These assays rely on the specificity of antibodies directed at epitopes found within peptide sequences. Indeed, the majority of studies on EOP fluxes have used RIAs to quantify peptide content (Table 3), largely because they provide a reasonably high degree of specificity and sensitivity for relatively low financial outlay. However, ELISAs and RIAs depend so heavily on the specificity of the antibodies forming the heart of the assay that they are vulnerable to artifactual measures due to the cross-reactivity of antibodies against unintended targets. These targets may be chemically similar but functionally unrelated, or they may be both chemically and functionally related, such as biotransformed forms of the peptide of interest that contain the same epitope as the parent compound. Both assays fundamentally depend on the same antibody binding strategy; thus, they generally have similar degrees of specificity. However, ELISAs tend to be less sensitive with a typical limit of detection of 0.1 to 1 fmol, whereas RIAs can detect as little as 0.01 fmol of a compound. However, this increase in sensitivity comes at the cost of greater financial outlay, including the purchase of radiation counters, radioactivity storage facilities, short shelf life of isotopes, waste disposal, and safety equipment. As yet, ELISA and RIA remain among the most common methods employed for detecting extracellular EOPs (Table 3).

Virtually all alternatives to the ELISA and RIA methods for measuring neuropeptides, such as EOPs, use sample separation steps, including reversed phase HPLC or capillary electrophoresis, typically with gradient elution on C18 columns, which separate peptides based on size due to differences in the

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Table 3. Summary of Major Reward and Addiction Related Studies Reporting Direct Measurement of Dynamic Changes in Extracellular EOPs

stimulus	stimulus	opioid	collection site	route of administration	opioid assay	finding	ref
behavioral	lateral hypothalamus self-stimulation or heroin self-administration	β-endorphin	nucleus accumbens	n/a	ELISA	no increase during LHSS, but increase during extinction and aversive footshock; increase also observed during extinction of heroin self-administration	95
behavioral	morphine-induced conditioned place preference	Met-enkephalin	nucleus accumbens	systemic	RIA	increase in morphine-paired area; decrease in vehicle-paired area; no equivalent changes in cholecystokinin	84
behavioral	palatable food	Met-enkephalin, Leu-enkephalin, dynorphin	striatum	oral	HPLC-MS	increased release, correlating with latency to eat palatable food	96
behavioral and pharmacological	cocaine	β-endorphin	nucleus accumbens	systemic (experimenter and self-administered)	ELISA/fMRI	increase to experimenter-administered cocaine, attenuated by 6-hydroxydopamine lesion of ventral tegmental area or systemic administration of D1 antagonist; increased by local perfusion of dopamine, blocked by D1 antagonist; self-administered cocaine also increased β-end; fMRI signal in nucleus accumbens and arcuate nucleus increased during experimenter-administered cocaine	81
pharmacological	alcohol	β-endorphin	nucleus accumbens	systemic	RIA	increase in alcohol preferring but not nonalcohol rats	92
pharmacological	alcohol	β-endorphin	ventral tegmental area/midbrain	systemic	RIA	increase (low doses only)	93
pharmacological	Δ^9-tetrahydrocannabinol	β-endorphin	nucleus accumbens and ventral tegmental area	systemic	ELISA	increased, stronger in ventral tegmental area than nucleus accumbens	89
pharmacological	amphetamine	β-endorphin	nucleus accumbens	systemic	RIA	increased, acute tolerance	83
pharmacological	cocaine	β-endorphin	nucleus accumbens	systemic	RIA	increased, no acute tolerance	83
pharmacological	ethanol	β-endorphin	nucleus accumbens	systemic	RIA	increased, no acute tolerance	83
pharmacological	nicotine	β-endorphin	nucleus accumbens	systemic	RIA	no effect	83
pharmacological	alcohol	dynorphin 1−8	central amygdala	systemic	RIA	increase, attenuated by CRH-2 receptor antagonist	91
pharmacological	alcohol	dynorphin 1−8	ventral tegmental area/midbrain	systemic	RIA	increase, delayed response at low doses only	93
pharmacological	morphine	dynorphin B	striatum, substantia nigra	systemic	RIA	increase in substantia nigra but not neostriatum after systemic administration; increased by local perfusion of substantia nigra; perfusion of neostriatum increased in ipsilateral substantia nigra but no local effect; naloxone blocked effect of systemic morphine on substantia nigra; naloxone alone, systemically or intracerebrally, no effect in neostriatum or substantia nigra	86
pharmacological	heroin	Met- and Leu-enkephalin primarily	globus pallidus/ventral pallidum	systemic	RIA	increase, short-term tolerance	88
pharmacological	morphine	Met- and Leu-enkephalin primarily	globus pallidus/ventral pallidum, nucleus accumbens, caudate nucleus	systemic	RIA	increase at mid to lower doses in the globus pallidus/ventral pallidum only; no acute tolerance.	87
pharmacological	morphine, morphine-6-glucuronide, SNC80	Met- and Leu-enkephalin primarily	globus pallidus	local	RIA	low concentrations of morphine or morphine-6-glucuronide enhance, reversed by β-funaltrexamine; stimulatory effect observed with low concentrations of SNC80, blocked by naltrindole; high concentration of morphine little or no effect; morphine-6-glucuronide suppressed release, reversed by β-funaltrexamine; high concentration SNC80 suppressed release, not blocked by naltrindole, but attenuated by β-funaltrexamine	85
pharmacological	alcohol	Met-enkephalin	nucleus accumbens	systemic	RIA	increase (lower dose only)	94
pharmacological	alcohol	Met-enkephalin	ventral tegmental area/midbrain	systemic	RIA	no effect	93

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Table 3. continued

stimulus	stimulus	opioid	collection site	route of administration	opioid assay	finding	ref
pharmacological	chronic morphine	Met-enkephalin	periaqueductal gray	systemic	RIA	chronically increased by morphine; no effect of naloxone-precipitated withdrawal	84
pharmacological	Δ^9-tetrahydrocannabinol	Met-enkephalin	nucleus accumbens	systemic	RIA	increased	90
pharmacological	ethanol	Met-enkephalin	nucleus accumbens, prefrontal cortex and caudate putamen	systemic	RIA	increased	43

amount of charge on the various peptides in the sample (Table 1). Following separation, several detection methods of various selectivity and sensitivity may be applied. Perhaps the simplest of these is the measurement of UV absorption, which while lacking the selectivity to identify particular peptides in complex mixtures, may be effective for detecting peptides in simple mixtures, such as those prepared by investigators for the in vitro calibration of microdialysis probes. Fluorescence detection following derivatization with agents such as naphthalene-2,3-dicarboxyaldehyde/cyanide can detect peptides in the nanomolar range but lack specificity. Electrochemical detection may also be applied, either for peptides naturally containing electrochemically active groups, such as tyrosine, tryptophan, methionine, and cysteine, or peptides coupled with specific agents, such as copper. For instance, enkephalin and endorphins found in push–pull and microdialysis perfusates have been measured by HPLC coupled to electrochemical detection.[68,69]

However, the approach offering the greatest selectivity to confidently measure EOPs is mass spectrometry. This approach has recently undergone a great deal of development in studies aimed directly at measuring specific and known EOPs (see ref 70 for review) and discovering previously unknown peptides (e.g., refs 71 and 72; see ref 73). A major advantage of mass spectrometry is its ability to identify and quantify numerous peptides simultaneously. However, mass spectrometry is an expensive technique, requiring considerable investments in training and machinery. Nonetheless, several groups have recently focused on developing capillary liquid chromatography coupled to mass spectrometry to detect neuropeptides, especially EOPs, in microdialysates,[72] and these efforts have begun to produce significant results, as introduced in the next section.

REWARD-RELATED STUDIES OF EOP FLUXES

The discussion above introduced the necessity and means of studying dynamic EOP release in living systems. Here, focus is placed on studies that have achieved the direct and dynamic measurement of EOP fluxes in the extracellular space at presumed release sites (though see discussion above) relevant to reward and addiction. This analysis covers the majority of EOP flux studies, which is unsurprising given the interest in their roles in affective and addiction processes. On the other hand, studies have directly identified various stimuli capable of physiologically inducing EOP peptide release, including kainic acid-induced seizures,[74] amygdala kindling,[75] high potassium pulses,[76] and veratridine perfusion.[76] While little is known about the physiological stimuli that activate EOP release, there is a wealth of studies inferring the functional release of EOPs in response to a physiological stimulus, such as termination of stress,[77] sympathetic activation,[78] placebo-induced analgesia,[79] and inflammation.[80] The publication rate of reward-related studies *directly* measuring EOP release has averaged little more than just one article per year since the first published study (Table 3), which testifies to the technical challenges related to this topic. Notably, there has been a recent increase in related studies, particularly those focusing on technical developments for measuring EOPs in vivo. Although such studies are not the primary focus of this text (see refs 63 and 70), they are important for bringing us closer to consistently and readily measuring EOPs in vivo and aiding in the interpretation of these findings.

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Pharmacologically Induced Changes in Extracellular EOP Fluxes. Table 3 summarizes the major studies on direct EOP measurements as they relate to reward, reinforcement, and addiction processes. Study of the table reveals several general points. First, very few studies have been published in this area despite considerable interest in EOP actions, as reflected in Figure 1. Not surprisingly, as a point of comparison, several-fold more studies have been published in the area of dopamine research. However, if one considers all EOPs, there have been nearly as many published studies in this area as for dopamine. Considering that virtually none of these studies have directly measured EOP release, this becomes quite an impressive statistic. Indeed, it is difficult to imagine that such interest in dopamine-related studies would exist if there were not so many relatively easy and financially viable methods of measuring endogenous dopamine fluxes.

Second, Table 3 shows that most EOP flux studies thus far have addressed changes in EOP fluxes in response to pharmacological stimulations, specifically drugs of abuse. Notably, all of the studies shown in this table used microdialysis as a means for accessing extracellular EOPs. This reflects at least two issues. First, there is a strong interest in understanding how drugs of abuse act. Accordingly, studies at this slowly emerging stage of the field have focused on rudimentary questions related to how abused drugs impact EOP release. Furthermore, pharmacological challenges are generally more potent and persistent in their actions on neurochemical changes when compared with behavioral stimulations, especially in the case of neuropeptides that are generally sluggish to release, and thus are a logical place to start when faced with the challenges of measuring their release.

Thus, far, these pharmacological studies have covered cocaine,[81,82] amphetamine,[83] morphine,[84−87] heroin,[88] nicotine,[83] tetrahydrocannabinol (THC),[89,90] and especially alcohol.[43,83,91−94] The EOPs measured include endorphins,[81,83,89,92,93,95] enkephalins,[43,84,85,87,88,90,93,94,96] and dynorphins.[86,91,93] As yet, no studies have addressed nociceptin, despite a predicted involvement in reward and addiction,[5] especially in responses to ethanol.[97] An overarching theme found in the pharmacological studies summarized in Table 3 is a general increase in extracellular EOPs in response to drug administration. A notable exception to this is nicotine, which thus far has been found to have no effects on the extracellular levels of any EOPs.[83] However, nicotine exhibits a narrow window of dose-dependent effects, quickly covering a range of diverse psychological and physiological effects as the dose increases.[98] One effect is the prompt switch from being a mildly rewarding to a strongly aversive stimulus.[99] The limited number of studies on the topic of the effects of abused drugs suggests a rapid tolerance to the stimulatory effects of abused drugs, as reported for heroin-induced enkephalin release in the globus pallidus.[88] Furthermore, almost all studies have focused on striatal regions (both dorsal and ventral), a natural choice given their high EOP content and predicted role in reward and addiction-related behaviors.

Uncovering the actions of exogenously applied drugs on EOP release is undoubtedly of great merit. By way of comparison, revealing that rewarding stimuli elicit dopamine release in the ventral striatum formed the cornerstone of the neuropsychopharmacology of rewarded behaviors nearly three decades ago,[100] and this finding has driven research and considerable discussion ever since. Over time, efforts in the dopamine field have shifted more toward monitoring and interpreting endogenous dopamine fluxes during behaviors relevant to reinforcement and reward, which have been highly instrumental in forming current conceptualizations, such as Schultz's reward prediction error function of dopamine[101] and Robinson and Berridge's incentive sensitization theory of addiction.[102] Like the neuropeptide field, microdialysis for the capture, recovery, and monitoring of dopamine has been the primary approach; however, the renaissance of in vivo voltammetry[103] has opened new avenues based on its ability to monitor dopaminergic activity with subsecond resolution.

An Extinction-Related Change in Extracellular EOP Flux. Table 3 also details the few published works on *behaviorally related* EOP fluxes. Such research is particularly interesting because it helps uncover the physiological function of EOPs and has overcome some of the practical challenges associated with measuring EOPs. Given this, these limited data warrant specific comment.

One study that reveals particular insight into the function EOPs was reported by Zangen and Shalev.[95] The authors used an ELISA to measure extracellular β-endorphin sampled from the nucleus accumbens using microdialysis during lateral hypothalamic self-stimulation (LHSS). LHSS is a potent operant reinforcer driven by activation of mesolimbic dopamine tracts as they pass through the medial forebrain bundle. Though it is unclear to what degrees motivational versus hedonic processes underlie LHSS[104] and given that endorphin has long been considered a mediator of pleasure, one might expect LHSS to increase extracellular endorphin in the nucleus accumbens. Rather, the authors reported a striking increase in accumbal extracellular endorphin during *extinction* of LHSS, at a time when animals attempt to self-stimulate but are not actually administered electrical current. These authors showed similar findings for extinction of heroin self-administration. This finding is interesting viewed in the light of the pharmacological studies presented in Table 3 that show rewarding drugs tend to increase extracellular levels of all EOPs in the brain, even dynorphins, which are usually considered mediators of negative affect.[105] Zangen and Shalev speculate endogenous endorphins may be underlying appetitive motivational processes driving reward seeking behaviors. Thus, general opioid receptor antagonists, such as naloxone and naltrexone, may be useful for treating disorders of appetitive behaviors, such as obesity[106] and alcoholism.[107] Notably, naltrexone (Depade, Revia, Vivitrol) is currently FDA-approved for treating alcoholism and opiate addiction.

A Drug-Conditioned Change in Extracellular EOP Flux. The increased extracellular β-endorphin levels in the nucleus accumbens accompanied by extinction of LHSS is of further interest viewed in the light of studies by Roques' group, who measured extracellular Met-enkephalin in the nucleus accumbens during exposure to a morphine-paired environment.[84] Although Roques and colleagues did not assess extracellular Met-enkephalin levels in the accumbens in response to morphine, it is highly likely that morphine stimulates Met-enkephalin release in this region based on studies showing similar effects of opiates in the globus pallidus and ventral pallidum.[85,88] In their study, Roques and colleagues used Pavlovian conditioning to associate an environment with distinct sensory cues to morphine administration in rats in a paradigm akin to that of conditioned place preference. Rats were administered vehicle in a second environment with different sensory cues. The rats then underwent microdialysis of the nucleus accumbens to recover enkephalin while being

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confined to one of the two environments. Samples were measured using a RIA. Numerous studies, including that of Roques and colleagues, have shown that morphine induces conditioned place preferences in rodents,[108] an action believed to reflect its rewarding and possibly incentive motivational properties. However, the notable aspect of their study was that rats showed a rapid and transient *increase* in extracellular enkephalin when confined to the morphine-paired environment and a rapid and transient *decrease* when confined to the vehicle-paired environment. Moreover, no such effects were observed for cholecystokinin, a neuropeptide that has been implicated in sensitization to psychostimulants.[109] First, these findings show that rapid and tangible changes in extracellular enkephalins are indeed measurable. Second, such enkephalin release occurs in response to a reward-conditioned cue (i.e., a CS+), which like dopamine recapitulates the effect of the unconditioned stimulus,[110] in this case morphine. However, it is important to note that the measurements of extracellular enkephalin fluxes made in this study were not made during the expression of an actual conditioned place preference. Thus, it is difficult to state that such changes actually drive the expression of reward-seeking behavior. The authors suggest that the increase may "reflect an "emotional state" of the animals in relation to the expectation of drug reward". Moreover, exposure to a neutral environment (essentially a CS−) decreases extracellular enkephalin, suggesting that the rats were contrasting the two sets of environmental cues. Such a response is somewhat reminiscent of the effect of omission of a reward on dopamine release following exposure to a reward-conditioned CS+;[101] however, the decrease in extracellular enkephalin release occurred in response to a CS− in the Roques study. Not unexpectedly, changes in extracellular enkephalin may reflect changes in dopamine given that enkephalin in the nucleus accumbens is localized in medium spiny neurons immediately downstream of dopamine cells. However, most studies suggest that enkephalin-containing cells in this region are coupled with D2 dopamine receptors, which are inhibitory in nature. Thus, any change in dopamine activity might be expected to manifest as an opposite change in enkephalin, which is not the case in the study of Roques and colleagues.

Feeding-Related Changes in Extracellular EOP Fluxes. An area of significant topical interest is that of feeding, particularly the motivational and hedonic aspects. Indeed, much of our understanding of the relationship between motivational and reward based processes per se, even as they pertain to abused drugs and other rewarding stimuli, is rooted in studies directed at food reward.[111] Such studies are not only of academic interest but are of increasing importance in their own right, as overeating and obesity become an increasing public health concern. Set on this background, a recent study by Berridge's group in collaboration with Kennedy's group is particularly notable.[96] In their study, these researchers monitored endogenous extracellular enkephalins and dynorphins in the dorsal striatum recovered by microdialysis and assayed by mass spectrometry during consumption of a palatable candy reward. They show that consumption of the candy was accompanied by increases in extracellular Leu- and Met-enkephalins and a mild decrease in extracellular dynorphin. Furthermore, a correlation was found between the latency to begin eating the candy and the relative increase in enkephalins such that the faster rats started eating, the greater the increase in enkephalin levels. The authors suggest this correlation might reflect a motivational "eat now" command, which they further tested by pharmacologically activating mu-opioid receptors specifically in the striatum. In contrast to the ventral striatum (the nucleus accumbens) where direct activation of mu-opioid receptors increases hedonic "liking" of palatable rewards, no such effect was observed when mu-opioid receptors in the dorsal striatum were activated. Given the large body of literature predicting that increased EOP tones underscore hedonic liking by acting in the ventral striatum,[112] direct evidence for this notion has yet to be reported. Generating these data will likely require further technical innovations because the ventral striatum is considerably smaller than the dorsal striatum. The ventral striatum is also one of only a handful of brain regions with a β-endorphin input. Thus, it would be interesting to compare the feeding effects of β-endorphin and enkephalins in the nucleus accumbens. Taken together, the studies of Berridge and Kennedy somewhat define the frontier of dynamically measuring extracellular EOP fluxes and promise to enormously expand our understanding of EOPs in reward-related processes.

■ CONCLUDING COMMENTS

The discussion above outlines the complexities and challenges of measuring EOPs in vivo and provides a brief overview of the status quo. As it stands, our understanding of EOP fluxes and their physiological relevance continues to be a work in progress. Headway in preclinical studies has been slow but constant. Clinical studies using imaging approaches are furlongs ahead, though such studies are poorly suited for discerning changes in specific EOPs. Imaging approaches are also generally very difficult to apply to preclinical models, especially for correlation with behavioral changes. Thus, it is unsurprising and somewhat concerning that some of the most fundamental questions regarding the nature of EOP transmission have yet to be addressed. For instance, the study by Berridge and Kennedy outlined above[96] draws attention to the previously under-appreciated roles of EOPs in motivational processes that contrast with the more traditional understanding of EOPs in hedonic processes.[112] Recent studies have also suggested roles for EOPs in the amygdala in incentive learning that are entirely dissociable from their roles in motivational and reward processes.[113] Thus, EOP action may be embroiled in many, if not most, of the psychological processes underlying motivated and rewarded behavior. Furthermore, the role of in vivo biotransformation of EOPs in these processes is virtually unknown.

Other questions remain. As highlighted in Table 3, most of the studies on EOP transmission to date suggest an increased release following exposure to abused drugs. This change is generally interpreted as being the neurochemical basis of the elevated affective state induced by these drugs. It is easy to grasp why some drugs, such as cocaine and alcohol, might lead to such an increase, particularly because their dopamine-modulating effects do not appear to underpin their subjective effects (see ref 114 for discussion). However, it is difficult to understand why opiates, such as heroin and morphine, may also have similar effects given that these molecules directly mimic the actions of EOPs and would be expected to be auto-inhibitory to their release.[115]

Additional questions that remain unaddressed include how EOP release is influenced by changes in homeostatic states, such as hunger, thirst, and drug withdrawal. For instance, endorphin is produced from the larger pro-opiomelanocortin propeptide (Figure 3), which contains multiple peptides central

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to appetite and metabolic control. Furthermore, the general localization of endorphin-synthesizing neurons next to fenestrated areas of the blood–brain barrier ideally places them to sense changes in peripheral metabolic and hormonal states. This distribution contrasts strongly with those of enkephalin, dynorphin, and nociceptin, which are found relatively more widely distributed throughout the brain. Despite this, endorphins and enkephalins are predicted to activate similar opioid receptors, namely, mu- and delta-opioid receptors. What is the physiological relevance of this overlap? Why are their abundances and distributions so different? Is there an adaptive purpose to this apparent redundancy in the system?

There are many remaining questions of fundamental importance to EOPs and neuropeptides that relate to their pharmacological and biophysical properties. As introduced above, it is becoming increasingly apparent that neuropeptides should be viewed as short and long ranging "brain hormones" that are cleaved from large precursor peptides to form pharmacological action sets with prolonged and wide-ranging actions. To what degree are peptides derived from the same propeptide related? Generally, EOP biotransformation has received a good degree of attention in neuropeptide studies, yet what exactly determines these biotransformations at a temporal and spatial level, how tightly is this process regulated, and perhaps most importantly, what is the functional outcome? Also introduced above, in vivo methods coupled to mass spectrometric analysis promise to expand our understanding of the nature of these biotransformations; however, the road to truly grasping their physiological function is likely to be long and arduous.

Other questions awaiting answers include how wide ranging are the pharmacological actions of EOPs, under what conditions are they released, what is their specific relationship with classical neurotransmission, how are they perturbed by disease states, how much redundancy exists in the system, what is the relationship between neuronal and glial derived EOPs, and can a consistent physiological role be ascribed to a given EOP across all areas in which it is found?

Finally, addiction is characterized by major plastic changes that reflect weakening, strengthening, and possibly rewiring of neural circuits driving motivated behavior.[116,117] Neuropeptides, such as EOPs, have long been understood to be central to modulating plasticity.[118] Currently, interest and focus on EOP fluxes has centered on putative roles in mediating affective reward though given their sluggish release and physically and pharmacologically wide-ranging actions perhaps the role of EOPs is primarily to modulate plasticity over longer time scales rather than to mediate acute events, such as brief and instantaneous changes in affect.

SUMMARY

Major progress has been made in demonstrating and uncovering roles for EOPs in numerous behaviors, especially those related to reward, addiction, pain, and feeding, using pharmacological and genetic approaches. However, our understanding of exactly how, where, when, and what induces changes in EOP transmission is rudimentary. Unlike monoamine signaling molecules, for example, there are currently no strong theories unifying the characteristics and functions of EOPs, such as their chemical nature, distribution, and nature of release. Addressing this gap in knowledge requires rising to the technical challenges of measuring a group of molecules that exists endogenously in very low quantities, are slow to release, and are structurally similar to hundreds if not thousands of other molecules.

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Author Contributions

N.P.M. conceived, drafted, revised, and completed all aspects of this review.

Notes

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