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ORIGINAL ARTICLE

Live molecular recognition: visualizing opioid receptors trafficking in vivo

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Abstract Receptors in biology are proteins which bind specific molecules, and trigger signaling events upon ligand binding. Opioid receptors are membrane receptors that belong to the large G protein-coupled receptor (GPCR) family. These receptors are expressed throughout the nervous system and bind endogenous opioid peptides to regulate pain and stress responses, as well as mood and well-being. These receptors also are also hijacked by exogenous opiates (morphine and heroin), and mediate their strong analgesic and addictive properties. Opioid receptor discovery and further breakthroughs have continually driven receptor research. Here we will summarize major findings in opioid receptor research. Recently, the direct visualization of delta opioid receptors in mice has opened a new area of investigation in GPCR research.

Keywords G protein coupled receptors · Opiates · Gene · Receptor imaging

This article is dedicated to Dr. Jacques Vicens and Pr. Jack Harrowfield on the celebration of their 65th birthday.

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Introduction

Receptors are key molecular actors in cell biology. These proteins respond to specific chemical stimuli and initiate cascades of signaling events within the cell. Among these are membrane receptors, which are located on the cell surface. Membrane receptors respond to extracellular stimuli, and convey environmental information within the cell to induce appropriate cell responses and ensure cell-cell communication in multicellular organisms. The largest family of membrane receptors is the G protein coupled receptor (GPCR) family. These receptors share a seven membrane-spanning topology and are functionally coupled to heterotrimeric G proteins, that relay information within the cell cytoplasm and nucleus [1]. Approximately 670 genes representing 2–3% of the human transcribed genome are dedicated to this family of receptors. GPCRs respond to an astonishing variety of stimuli, which include light, biogenic amines, lipids, neuropeptides, as well as small or large peptidic hormones, and also represent molecular targets for about 50% marketed drugs [2]. The structure of two representative mammalian GPCRs has recently been solved with atomic resolution [3, 4]. This review focuses on opioid receptors. These receptors are GPCRs, and their history has constantly brought pioneering concepts and findings in receptor research.

Opioid receptors: from opium to receptor genes

Discovery of opioid receptors stems from the use and abuse of opium in ancient history. Opium, extracted from poppy seeds (*Papaver somniferum)*, has powerful pain relieving properties and produces euphoria. This substance has been used both medicinally and recreationally for several

millenia. Morphine, named after the god Morpheus, is the most active ingredient of opium. Morphine was isolated in 1805 and rapidly became the treatment of choice to alleviate severe pain in the clinic. Heroin was synthesized chemically by morphine diacetylation in the late 1800s, and was commercialized as the first non-addictive opiate to treat cough and asthma. The strong addictive properties of heroin were soon acknowledged, and both heroin and opium were prohibited in 1910. Today morphine remains the best pain killer in contemporary medicine, despite a wide array of adverse side effects (respiratory depression, constipation, tolerance, dependence). Heroin is a main illicit drug of abuse, and heroin addiction represents a major public health issue. Because of their extraordinarily potent analgesic and addictive properties, opiates have prompted scientists to understand their mode of action in the brain.

In 1973, three independent teams showed that opiates bind to brain membrane preparations in a reversible and saturable manner [5–7]. The concept of "receptor" was born, and the binding sites were named opioid receptors. Opioid binding sites appeared heterogeneous and three receptors were defined (mu, delta and kappa), based on distinct binding and pharmacological properties of the many opioid drugs that were developed and tested throughout the years. After the receptor discovery, it became clear that morphine and heroin produce their potent activities by hijacking the receptors, and that both physiological function and natural activators of opioid receptors remained to be identified. The search for endogenous opioids was launched, and two pentapeptides (met- and leu-enkephalin) were isolated in 1975 [8] soon followed by other related peptides. Altogether, opioid peptides and their receptors form a complex neuromodulatory system, and two following decades of studies involving medicinal chemistry, biochemistry, pharmacology, anatomy and physiology provided a first view of the broad spectrum of opioid-mediated physiology and behaviors. In brief, the opioid system is expressed throughout the nervous system, and regulates responses to pain or stress, addictive and emotional behaviors, as well as autonomic, neuroendocrine and immune responses [9] (see Fig. 1).

Until 1992, receptors had remained a concept in pharmacology, and opioid binding the sole- and indirect- way to detect, localize and manipulate the receptors. Biochemical analysis had revealed the proteic nature of opioid receptors, but all attempts to isolate these low abundant and highly unstable proteins had failed. Gene cloning had

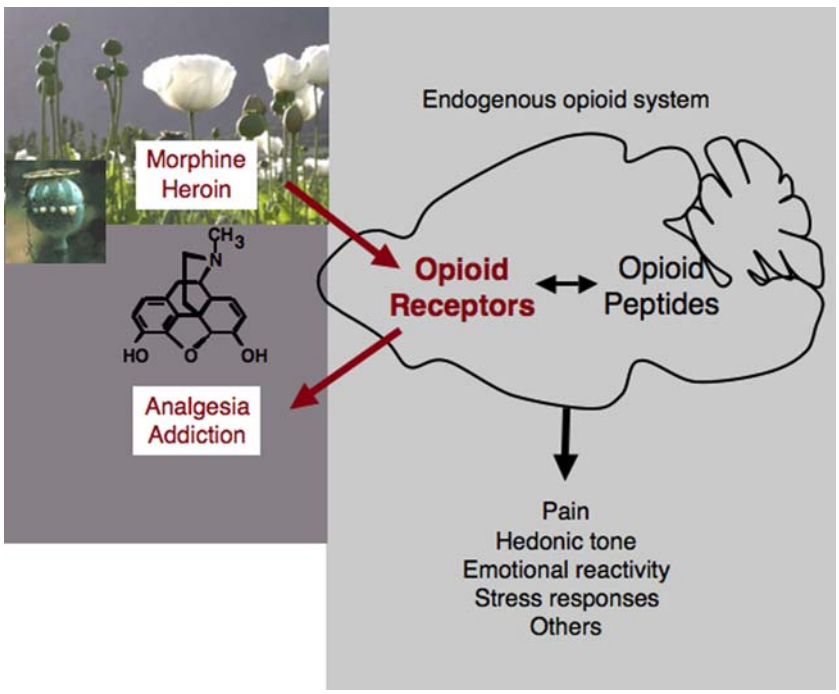

Fig. 1 The opioid system. *Dark grey box*: exogenous opiates activate opioid receptors. Morphine is the main active ingredient of opium and has strong analgesic and addictive properties, which result from a direct interaction of the drug with opioid receptors. Heroin is a di-acetylated form of morphine, and is a major drug of abuse worldwide. *Light grey box*: the endogenous opioid system is a complex neuromodulatory system. Three opioid receptors (mu, delta and kappa) naturally interact with a family of opioid peptides (enkephalins, dynorphin, Bendorphin), produced by proteolytic cleavage of three large precursor proteins. Receptors and peptides are broadly expressed throughout the nervous system and control nociceptive pathways (pain), mood and well-being (hedonic status), anxiety and depressive-like behaviors (emotional reactivity), as well as responses to stress. The system also controls respiration, gastro-intestinal motility, endocrine and immune functions (others). This figure was adapted from [29]

become a necessary step to evolve our understanding of opioid receptors. The first opioid receptor gene was isolated by expression cloning in 1992 [10, 11]. Because of strong sequence homology across receptors, the entire opioid receptor gene family was readily cloned in several species [12]. Gene sequence analysis confirmed that opioid receptors belong to the G protein coupled receptor superfamily, with a seven-transmembrane topology, as predicted by the pharmacology. Altogether three genes, encoding mu, delta and kappa receptor sites, were characterized at the molecular level.

From the gene to a visible receptor in vivo

The isolation of opioid receptor genes opened the way to molecular manipulations of the receptors, both in artificial systems and in vivo. First, the isolation of receptor DNA sequences allowed the generation of cell lines expressing mutant opioid receptors and characterize requirements for ligand binding and selectivity for the different opioid receptors, as well as molecular determinants for receptor activation and signaling [13, 14] (see Fig. 2). Second, the identification of receptor DNA sequences in humans launched novel drug discovery programs involving 3D-computer receptor modeling and high throughput drugs screening. Access to human sequences also enabled extensive gene expression pattern analysis in human brain tissues, as well as large-scale studies in human populations to address genetic bases of opioid sensitivity and vulnerability to drug abuse [15, 16]. Third, gene cloning paved the way to the creation of mice lacking opioid receptors that could dissect out the role of each receptor in exogenous and endogenous opioid-mediated behaviors [17]. The latter mutant mice unambiguously demonstrated the primary role of mu receptor in mediating both artificial (drugs of abuse) and natural rewarding stimuli [18–20], the unique role of delta receptors in controlling emotional reactivity [21], the implication of kappa receptors in dysphoria and stress [22, 23], and finally the specific role of each receptor in reducing distinct pain modalities [24, 25]. All these studies provided a comprehensive view on receptor structure and function at both cellular and integrated levels.

Recently, another genetic manipulation in mice allowed a major breakthrough towards understanding receptor function in vivo. A mouse line was engineered to produce fluorescent delta opioid receptors, instead of native receptors [26]. To do so, the Green Fluorescent Protein (GFP) gene was appropriately inserted into the receptor gene, in order to encode a C-terminally GFP-tagged receptor.

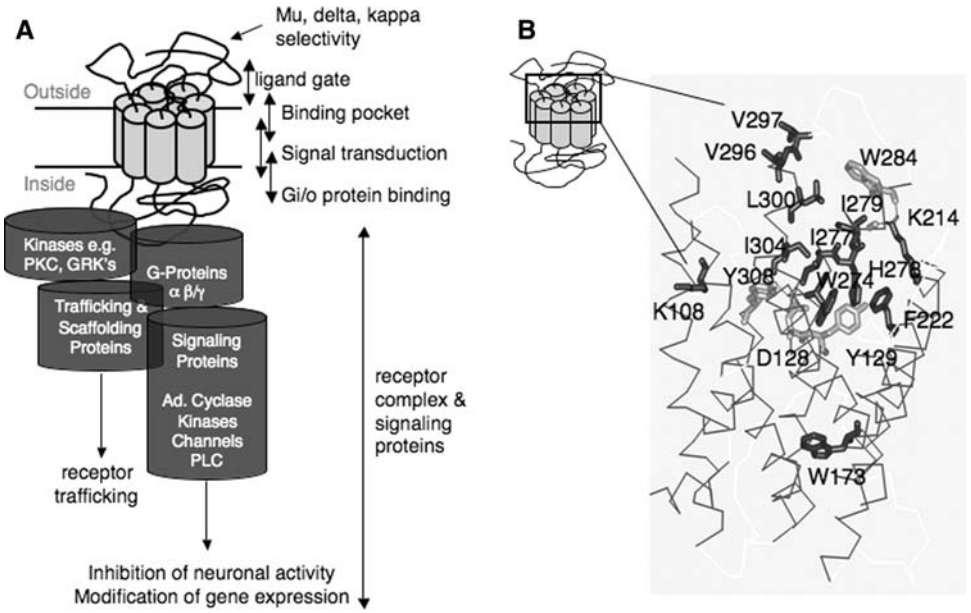

Fig. 2 Opioid receptor structure and signaling. **a** Opioid receptors are coupled to inhibitory G proteins and form signaling complexes with many protein partners. Opioid receptor activation modifies ion channel activities (decreased neuronal excitability or neurotransmitter release), decreases cAMP levels via inhibition of adenylate (Ad.) cyclase and activates phosphorylation pathways that lead to transcriptional regulations. As for all GPCRs, signaling is highly regulated by receptor phosphorylation and trafficking via scaffolding proteins. A current hypothesis is that different agonist ligands confer different patterns of receptor signaling in vivo. **b** Lateral view of a 3D model of the human delta opioid receptor. Helices are indicated as ribbons, side chains of aminoacids implicated in binding (*dark gray*) or both binding and activation (*light grey*) are shown as sticks. The opioid binding site forms a pocket penetrating half-way into the helical bundle, and is similar across mu, delta and kappa receptors. **b** This figure was adapted from [14]

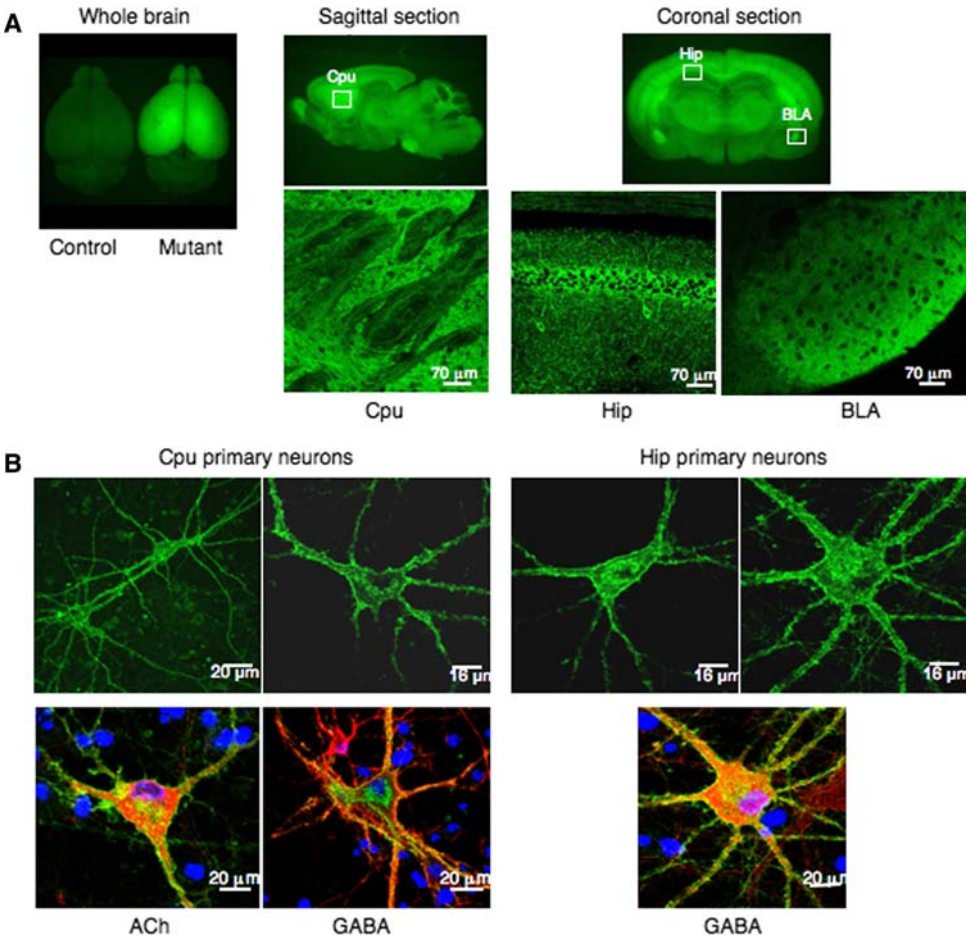

Fig. 3 Receptors at rest are located at the cell surface. Receptor imaging in mutant mice expressing fluorescent delta receptors. **a** Delta-GFP receptors in the brain. *Left panel*, epifluorescence microscopy shows strong receptor expression in intact brains from mutant but not control mice. *Right panels*, confocal microcopy of sagittal and coronal sections of the brain show distinct fluorescence architectures at the level of the Caudate putamen (Cpu), hippocampus (Hip) and basolateral amygdala (BLA), reflecting the natural distribution of the receptor. Higher magnification images (*bottom*) are from areas indicated by insets (*top*). **b** Delta opioid receptors in primary neurons from mutant mice. Confocal imaging of primary neurons reveals receptor localization within distinct neuronal compartments. Top panels, neurons prepared from the Cpu (*left*) and Hip (*right*) show receptors mainly targeted at the cell surface and uniformly distributed along cell bodies and processes (*green*). Immunostaining with specific antibodies reveals that delta receptors are expressed in GABAergic (GABA) and cholinergic (ACh) neurons of the Cpu, as well as in GABAergic neurons from the Hip (*red*)

Importantly the fluorescent receptor was fully functional, and responded to agonists normally at cellular and behavioral levels, thereby reflecting endogenous receptor physiology. The mutant (delta-GFP) mice proved to be an extraordinary tool to study receptor biology. For the first time, the precise receptor localization of a GPCR was visible throughout the nervous system, with a cellular resolution (see Fig. 3). Detailed receptor anatomy is currently being examined in these animals, and these data will help understanding mechanisms whereby delta receptors control emotional responses and alleviate chronic pain within neural networks. In addition, these mutant mice will enable functional analysis of delta receptor-expressing neurons at electrophysiological and molecular levels.

Imaging receptors at work

Stimulation of a GPCR by an extracellular stimulus triggers receptor signaling via G proteins. This process requires tight regulation, in order to maintain cellular homeostasis. Hence, receptor activation is typically followed by desensitization of receptor responses. A wide range of events contributes to the desensitization process, which are not fully understood. Many studies have demonstrated that stimulated receptors are internalized within the cell, and that receptor endocytosis is followed by either receptor degradation within lysosomal compartments or receptor recycling back to the cell surface [27]. From there, receptor trafficking from and to the cell surface has been

postulated as a key process in the regulation of receptor signaling. At present, however, most studies have been conducted in artificial cell systems, which cannot adequately model physiological environments and the complexity of integrated responses observed in the whole animal. Hence the in vivo significance of GPCR internalization remains an open question.

Delta-GFP mice represent a unique tool to readily examine the subcellular localization of endogenously expressed receptors in neurons and correlate receptor trafficking with the behavioral effects of agonists in vivo. In addition, the GFP fusion strategy allows studying real-time receptor trafficking in live neurons.

Primary neurons from mutant mice were exposed to opioid ligands and rapid receptor internalization was observed upon exposure to several delta agonists (see [26] and Fig. 4). Delta-GFP receptors were targeted to lysosomes, definitely classifying delta receptors among slow-recycling/fast degrading GPCRs. In a next set of experiments, mutant mice were treated with the delta agonist SNC80 and massive receptor endocytosis throughout the nervous system (Fig. 4), together with locomotor activation. Interestingly, mice with internalized receptors did not respond behaviorally to a second drug administration [26]. This was a first indication that internalization may impact delta receptor signaling in vivo. A further study analyzed the pain-relieving effects of two delta agonists with similar signaling potencies and efficacies, but distinct internalizing properties. An initial treatment with the high (SNC80) or low (ARM390) internalizing agonist equally reduced pain. However, subsequent drug treatment produced highly distinct responses. Animals initially treated with SNC80 showed receptor internalization throughout the nervous system and were unable to respond to a second dose of either delta agonist. This behavioral desensitization was temporary, since full analgesic responses were restored

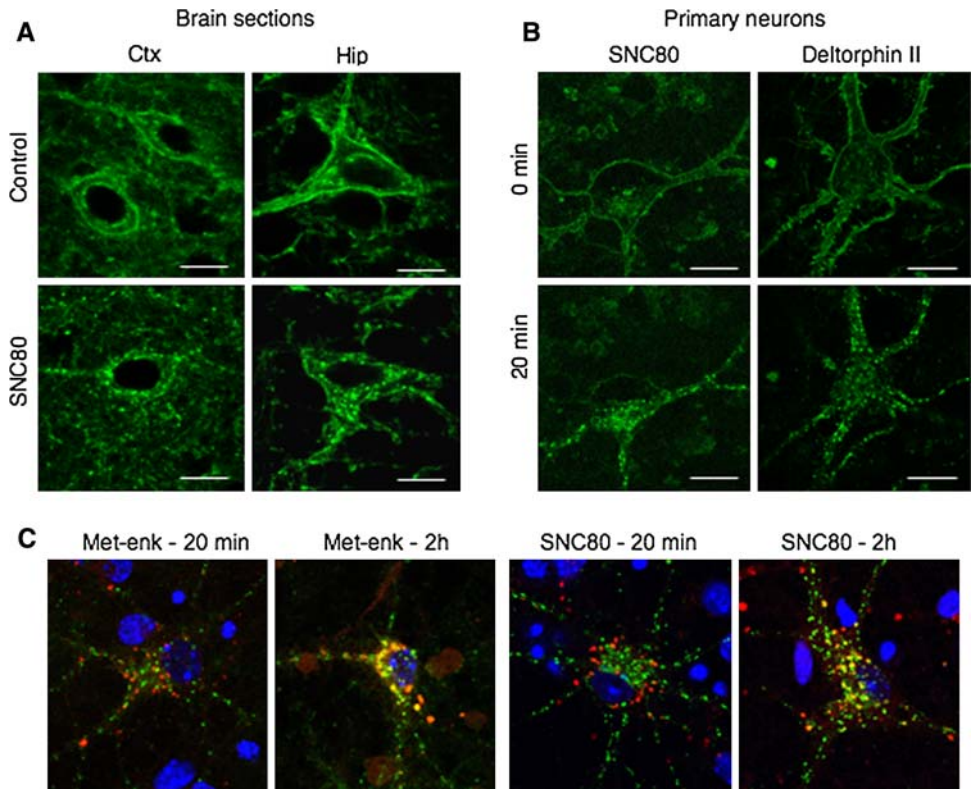

Fig. 4 Receptors activated by opioids internalize within neurons. Exposure of mice (**a**) or cultured primary neurons from mice (**b** and **c**) to a delta opioid agonist triggers receptor endocytosis. **a** Confocal imaging of brain sections from mutant animals treated with the agonist SNC80 shows that receptors disappear from the cell surface (*top*, control) and cluster within the cell cytoplasm (*bottom*, SNC80) forming bright intracellular vesicles. This occurs at all levels of the central nervous system and images are shown at the level of the cortex (Ctx, *left*) and hippocampus (Hip, *right*). **b** In cultured primary neurons from mutant mice, receptor internalization is observed after exposure to several agonists (SNC80, *left*; deltorphin II, *right*) and is complete after 20 minutes. The internalization phenomenon can be monitored in real-time using time-lapse confocal microscopy (see movie in [26]). **c** Two hours following agonist stimulation by either Met-enkephalin (Met-enk, *left*) or SNC80 (*right*), receptors (*green*) colocalize with a lysosomal marker (*red*). This reveals that after internalization, receptors (20 min) are committed to the degradation pathway (2 h). Altogether, these events prevent further responses to opioids and contribute to receptor desensitization at both cellular and animal levels. Receptor desensitization is transient and restoration of surface receptors occurs after 24 h (see text)

24 h later. In contrast, animals treated with ARM390 were fully responsive to a subsequent agonist injection, and receptor imaging showed that receptors had remained on the cell surface. These results establish that receptor internalization in vivo fully controls behavioral effects of opioid drugs, and is a main acute desensitization mechanism [28]. It will be important to investigate whether the non-internalizing drug remains efficient following repeated agonist treatment. The development of in vivo tolerance, even in the absence of receptor internalization, would indicate that neurons develop other strategies to counteract the drug effect. These studies have implications for drug design and the development of effective therapies for the treatment of chronic diseases.

Perspectives: receptor imaging in the living organism

Positron emission tomography and magnetic resonance imaging offer unique access to receptor anatomy and occupancy in the living brain, and are particularly suited for human studies. The spatial and temporal resolution of these approaches, however, remains limited. Because receptor subcellular localization and trafficking in neurons is critical to understand receptor function, there is tremendous interest in developing non-invasive receptor imaging approaches in animal models, that achieve subcellular resolution and allow direct visualization of receptor movements in live neurons. In this context, it is expected that strategies similar to the delta-GFP in vivo labeling approach described here will be developed for other receptors. The combination of mouse engineering with high-resolution optical imaging should definitely clarify how single receptor molecules operate in highly organized cellular networks, and orchestrate physiological or pathological processes in vivo.

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