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In vivo trafficking of endogenous opioid receptors

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Abbreviations: DOPR, δ opioid receptor; EGFP, enhanced green fluorescent protein; KOPR, κ opioid receptor; MOPR, μ opioid receptor

Abstract

Studies on trafficking of endogenous opioid receptors *in vivo* are subject of the present review. In many of the *in vivo* studies, the use of semi-quantitative immuno-electron microscopy is the

approach of choice. Endogenous opioid receptors display differential subcellular distributions with μ opioid receptor (MOPR) being mostly present on the plasma membrane and δ- and κ-opioid receptors (DOPR and KOPR, respectively) having a significant intracellular pool. Etorphine and DAMGO cause endocytosis of the MOPR, but morphine does not, except in some dendrites. Interestingly, chronic inflammatory pain and morphine treatment promote trafficking of intracellular DOPR to the cell surface which may account for the enhanced antinociceptive effects of DOPR agonists. KOPR has been reported to be associated with secretory vesicles in the posterior pituitary and translocated to the cell surface upon salt loading along with the release of vasopressin. The study of endogenous opioid receptors using *in vivo* models has produced some interesting results that could not have been anticipated *in vitro*. *In vivo* studies, therefore, are essential to provide insight into the mechanisms underlying opioid receptor regulation.

Introduction

Opioid receptors belong to the seven-transmembrane receptor superfamily and are coupled with $G_{i/o}$ proteins. Three types of opioid receptors have been cloned, μ- , δ- and κ-opioid receptor (MOPR, DOPR and KOPR, respectively).

Opioid receptors can be activated by a variety of naturally occurring or synthetic opiates and several endogenous neuropeptides. When the opioid receptors are activated upon binding of these ligands, a common regulatory event involves internalization of the receptor from the cell surface to intracellular sites. Agonist-induced endocytosis of opioid receptors has been studied extensively in cell models. Briefly, following binding of agonists to opioid receptors on plasma membranes, receptors undergo conformational changes leading to activation of G proteins and translocation of G protein-coupled receptor kinases to the cell surface resulting in phosphorylation of the receptors. β-arrestins are recruited to the phosphorylated receptors, which are subsequently endocytosed via a clathrin-dependent pathway. The decrease in the numbers of cell surface opioid receptors may be an adaptive process to avoid over-stimulation and may account in part for tolerance to opioids. Internalized opioid receptors are either recycled back to cell surface, resulting in re-sensitization of the receptors or sorted to degradation pathways, leading to down-regulation (Liu-Chen, 2004; von Zastrow et al., 2003).

Like endocytosis, trafficking of opioid receptors to the cell surface may also be regulated. In dissociated dorsal root ganglion neurons, DOPR is sorted into large dense-core vesicles through interaction with protachykinin (Guan et al., 2005). Activation of surface DOPR causes elevation of intracellular Ca^{2+} mostly via an inositol triphosphate-dependent mechanism that results in insertion of large dense-core vesicles-associated DOPR onto the cell surface (Bao et al., 2003). Another mechanism leading to an increase of opioid receptors on the cell surface is the

pharmacological chaperone effects of opioid ligands. In cells transfected with opioid receptors, cell-permeant opioid ligands promote endoplasmic reticulum-to-Golgi trafficking of opioid receptors to enhance cell surface expression by facilitating correct folding of the newly synthesized receptors at the endoplasmic reticulum (Chen et al., 2006; Petaja-Repo et al., 2002; Wannemacher et al., 2007; Chaipatikul et al., 2003).

Most studies on opioid receptor trafficking were carried out in various *in vitro* cell models. The limitations of these models are obvious, including differences in cellular milieu and receptor expression levels. In this review, observations regarding *in vivo* trafficking of opioid receptors will be presented. While some findings are consistent with *in vitro* results, others are unanticipated.

Consideration of methods and approaches for subcellular localization of opioid receptors *in vivo*

Each type of opioid receptor has a distinct distribution in the central nervous system as revealed by receptor autoradiography studies (Mansour et al., 1988) and immunohistochemical approaches (Arvidsson et al., 1995a, b). Some regions are abundantly enriched in opioid receptors and these include the striatum, the locus coeruleus, the ventral tegmental area and the dorsal horn of the spinal cords. Therefore, these regions are commonly used for studies on endogenous opioid receptors.

The use of receptor autoradiography and electron microscopy was employed in the 1980s and 1990s (Moyse et al., 1997) where the opioid receptor ligands were labeled with ^{125}I. The localization of opioid receptors was detected using silver grains scattered by the radioactivity of

the bound radioligands. Although a useful approach at the time, this technique fell out of favor with the availability of specific antibodies that recognize each type of opioid receptors. Immunohistochemistry combined with confocal microscopy is another useful approach. Although confocal microscopes are more accessible than electron microscopes in most laboratory settings, the resolution of the former is much lower than that provided by the latter. Combining transmission electron microscopy with immunogold or immunoperoxidase labeling provides a high-resolution technique for the study of the subcellular distribution of endogenous opioid receptors in brain tissue. Although a more sensitive labeling approach, immunoperoxidase labeling is not as readily quantifiable for subcellular distribution as the labeling tends to be diffuse and has propensity to adsorb to membrane structures (Novikoff et al., 1972). In contrast, immunogold labeling is quantifiable generally by counting the sliver grains. Therefore, immunogold labeling is a major approach to quantify the subcellular localization of opioid receptors.

Importantly, it can not be over-emphasized that, with all immunohistochemical approaches, the validity of the results largely depends on the specificity and affinity of the antibodies. Specific antibodies recognizing each type of opioid receptors are available and have been characterized by different groups using complementary approaches.

Additional approaches have been used to investigate trafficking of epitope-tagged receptors artificially introduced into animals. A mouse line expressing DOPR tagged with enhanced green fluorescent protein (EGFP) at the C-terminus has been established using the gene targeting approach and allows examination of whether there is a correlation between receptor trafficking and *in vivo* pharmacology end points (Scherrer et al., 2006). Generation of such a knock-in mouse line is time-consuming and costly.

In another approach, exogenous opioid receptors, with epitope tags, have been introduced into and expressed in certain brain regions by use of viral vectors (Haberstock-Debic et al., 2003). Trafficking studies is carried out in a more physiological environment than in primary neurons. This review does not cover the findings from such an approach.

Differential subcellular localization of endogenous opioid receptors

MOPR: Several lines of evidence indicate that, irrespective of the brain region, the MOPR is mostly localized to plasma membranes (Fig. 1). For example, in the rat habenular nucleus, confocal microscopy has shown that MOPR immunoreactivity is associated primarily with plasma membranes of neurons (Keith et al., 1998). Using immunogold labeling combined with electron microscopy, Van Bockstaele and Commons (2001) showed that about 90% of MOPR immunoreactivity was located along the plasma membrane of somatodendritic processes in the rat locus coeruleus . MOPR has been shown to have a similar subcellular distribution in the striatal patches: 80% and 60% located on plasma membranes of dendritic spines and axon terminals, respectively (Wang and Pickel, 2001). In the rat ventral tegmental area, immunogold labeled MOPR was seen on plasma membranes of dendrites and axon terminals (Garzon and Pickel, 2001). In the dorsal horn of rat spinal cord, most of the peroxidase-labeled MOPR was associated with postsynaptic membranes of dendrites (Wang et al., 2003). Surprisingly, the majority of immunogold-labeled MOPR (> 70%) was found in cytoplasm of the dendrites of C1 adrenergic neurons in the rat rostral ventrolateral medulla (Drake et al., 2005). It may reflect the differential subcellular distribution of MOPR in brain regions.

DOPR: In contrast to the high percentage of MOPR associated with neuronal membranes, DOPR immunolabeling is typically located intracellularly (Fig. 1). Electron microscopic analysis

revealed that 80-90% of immunogold-labeled DOPR was found within the cytoplasm of rat spinal cord dorsal horn dendrites (Cahill et al., 2001a, b). Further, this pattern is similar in striatal patches (Wang and Pickel, 2001) where the prevalence of the intracellular distribution is even more apparent in perikarya (Cahill et al., 2001a). In the ventral division of the reticular oral pontine nucleus of the cat, the majority of DOPR immunoreactivity was located in the cytoplasm of dendrites (79%), axons (81%) and somata (Alvira-Botero and Garzon, 2006). In the rat and monkey dorsal root ganglia and dorsal horn, immunogold-labeled DOPR was frequently associated with the membranes of large dense-core vesicles (Zhang et al., 1998).

In knock-in mice expressing DOPR-EGFP, quantitative analysis of confocal images indicates that ~ 60% of DOPR-EGFP is present on the cell surface in the striatum (Scherrer et al., 2006). It is noteworthy that the B_{max} of [^{3}H]naltrindole binding to DOPR in DOPR-EGFP knock-in mice is twice as high as that in wild type mice. In addition, fusion of the DOPR at the C-terminus with EGFP may affect interactions of the DOPR with associated proteins. These two factors may affect expression, subcellular localization and trafficking of DOPR-EGFP.

KOPR: A number of neuroanatomical studies have shown that the KOPR is primarily distributed intracellularly (Fig. 1), similar to the DOPR. Harris et al. (2004) reported that ~55% of KOPR immuoreactivity was located intracellularly in the dendrites of rat spinal cords of both sexes. In axon terminals, ~55% and 70% of KOPR immunoreactivity was intracellular in male and female rats, respectively. We observed an even higher percentage (~70%) of KORP immunoreactivity located intracellularly in the dendrites of male rat spinal cord (Wang et al., submitted). Most of intracellular KOPR was not associated with any discernable organelles, but some immunoreactivity was associated with mitochondria and endosomes. In the rat posterior

pituitary, ~60% of immunogold-labeled KOPR was associated with large secretory vesicles in the axon terminals and only ~11% with plasma membranes (Shuster et al., 1999).

In contrast to its localization within dendrites, KOPR was frequently associated with small synaptic vesicles in axon terminals of the rat nucleus accumbens (Svingos et al., 1999, 2001; Meshul and McGinty, 2000). In addition, peroxidase-labeled KOPR immunoreactivity was detected along plasma membranes of presynaptic axon terminals, large dense-core vesicles and small vesicles of the hippocampus in guinea pigs (Drake et al., 1996). When interpreting these results, one must take into consideration the known diffusion of peroxidase reaction products and their possible absorption to membrane structures that may lead to an overestimation of the association of KOPR immunoreactivity with plasma membranes and synaptic vesicle membranes. Consistent with this notion is the finding that when KOPR was labeled with peroxidase it was predominantly associated with plasma membranes of glial cells in rat medial prefrontal cortex, but when labeled with immunogold, KOPR was mainly in the cytosol (Svingos and Colago, 2002).

In summary, *in vivo* experimental approaches have provided valuable insight into the differential subcellular distributions of opioid receptors. The predominance of MOPR on the cell surface and the greater prevalence of DOPR and KOPR intracellularly imply that the regulation of their trafficking is likely to be different.

Trafficking of opioid receptors *in vivo*

The studies on trafficking of opioid receptors *in vivo* are summarized in Table 1.

MOPR: It was first demonstrated in cell models that MOPR agonists had differential effects on internalization of the receptor. MOPR was internalized by acute treatment with enkephalins,

etorphine or DAMGO, but not morphine (Arden et al., 1995; Keith et al., 1996). Agonist-dependent internalization of MOPR has also been shown in tissues *in vivo*. Systemic injections of etorphine caused rapid internalization of MOPR in neurons in the myenteric plexus of the guinea pig as demonstrated by immunohistochemistry and confocal microscopy (Sternini et al., 1996). In contrast, acute morphine treatment (30 min) did not change localization of MOPR. Differential effects of etorphine and morphine on internalization of MOPR were also reported in neurons of the rat brain using the same approach (Keith et al., 1998). By counting the MOPR immunoreactive positive endosomes in confocal microscopy images, Trafton et al. (2000) reported similar findings for MOPR in the dorsal horn of rat spinal cord, which was internalized by DAMGO, remifentanil or endomorphin-1, but not morphine. Quantitative immunogold electron microscopy showed that acute etorphine treatment (15 min) significantly reduced the surface amount of MOPR in the dendrites in rat locus coeruleus (Fig. 1), whereas morphine, either acute (30 min) or chronic (5 days), had no effect (Van Bockstaele and Commons, 2001). In the dorsal horn of rat spinal cord, the endocytosed MOPR reappeared on cell surface within 60 min (Trafton et al., 2000). The magnitude of MOPR internalization in lamina II interneurons induced by intrathecal DAMGO correlated with the extent of antinociception. However, such a correlation did not exist in morphine-tolerant rats. Although the antinociceptive effect of DAMGO was greatly decreased in morphine-tolerant rats, it promoted internalization of MOPR to a similar extent as in control rats (Trafton and Basbaum, 2004), indicating the desensitized MOPR retains the capability to be internalized. Surprisingly, although endogenous opioids are expected to be released upon application of noxious stimuli, no MOPR internalization was detected in lamina II neurons in nociception models, which may be due to inadequate amount of

the released endogenous opioids (Trafton et al., 2000). The findings prompted the authors to suggest that released opioid peptides may act presynaptically.

Although morphine alone did not induce significant internalization of MOPR, morphine plus DAMGO, at a dose that did not cause endocytosis, internalized MOPR in the dorsal horns of rat spinal cord as demonstrated in confocal images. The combination also reduced the development of tolerance to chronic morphine treatment in rats (He et al., 2002). Recently, a knock-in mice expressing mutated MOPR with DOPR C-tail has been established (Kim et al., 2008). The mutant receptor in striatal neurons cultured from the knock-in mice were internalized by morphine *in vitro*; however, it was not examined *in vivo*. The knock-in mice showed significantly reduced tolerance and dependence to morphine (Kim et al., 2008). The authors concluded that these findings supported the notion that tolerance to opioid receptors is due to sustained activation of cell surface receptors.

Interestingly, trafficking of endogenous MOPR upon acute morphine treatment appears to be compartment-specific. Haberstock-Debic et al. (2003) reported that in the rat nucleus accumbens, morphine (30 min) translocated MOPR to intracellular sites in dendrites, but not in neuronal cell bodies or axons. Drake et al. (2005) also observed that, in the rostral ventrolateral medulla, morphine induced internalization of MOPR in dendrites that had diameters <1.4 μm, but not in larger dendrites. These findings imply that the abundance of molecules involved in internalization machinery may vary in different compartments of neurons. The impact from surrounding environment or neural circuitries may also play a role.

Confocal microscopy images showed that the endogenous MOPR1C, a splice variant of MOPR, in lateral septum was internalized by morphine administered intracerebroventricularly in mice

(Abbadie and Pasternak, 2001), but MOPR was not. The difference in the C-terminal domains is likely to account for their different abilities to be internalized.

Estrogen treatment also induced internalization of MOPR in medial preoptic nucleus and the posteriodorsal medial amygdala of ovariectomized rats when using the increase of the density of MOPR-immunoreactive fibers as an indicator for internalization (Eckersell et al., 1998). The internalization was rapid (within 30 min) and long lasting (>24 hr). The mechanisms underlying these observations are unknown.

DOPR: In DOPR-EGFP knock-in mice, acute treatment with SNC80 caused significant internalization of DOPR in caudate putamen neurons in a dose-dependent manner, concomitant with an increase in locomotor activity. In addition, DOPR internalization correlated with the occurrence of desensitization to the subsequent application of SNC80 in enhancing locomotor activity (Scherrer et al., 2006).

Since DOPR has a large intracellular pool, efforts were also devoted to investigating the stimuli that can promote cell surface expression of DOPR. Chronic inflammatory pain up-regulated mRNA and protein levels of DOPR in the dorsal horns of rat spinal cords, as demonstrated by in situ hybridization and immunoblotting (Cahill et al., 2003). Immunoelectron microscopy studies revealed that chronic inflammatory pain caused a significant increase of DOPR on the cell surface and in peripheral zones under plasma membranes, which may account for the increased antinociceptive efficacy of DOPR agonists in animals with chronic inflammatory pain (Cahill et al., 2003).

Interestingly, chronic treatment with morphine promoted movement of intracellular DOPR to the cell surface in the dorsal horn of rat spinal cord as shown by quantitative immunoelectron microscopy (Fig. 1) (Cahill et al., 2001b). The effect of morphine was mediated by MOPR which

was shown by using MOPR blockade and MOPR knock-out mice (Morinville et al., 2003). Different from chronic inflammatory pain, morphine treatment regulated subcellular localization of DOPR without affecting overall expression level of DOPR (Cahill et al., 2001b).

KOPR: Intrathecal injection of dynorphin A significantly decreased cell surface KOPR in the dorsal horns of rat spinal cord, but U50,488H did not, using quantitative immunoelectron microscopy (Wang et al., submitted). The differential effects of agonists may be due to the distinct receptor conformations they induce. However, the *in vivo* effect of dynorphin A is more complex. It has been reported that dynorphin A(2-17), the des-Tyr derivative of dynorphin A(1-17), can activate NMDA (Vanderah et al., 1996) or bradykinin (Lai et al., 2006) receptors at high concentrations. It may also affect the trafficking of KOPR via neuronal circuitry.

KOPR in the posterior pituitary is mostly associated with vesicles containing vasopressin (Shuster et al., 1999). When salt loading causes release of vasopressin, the KOPR is translocated to cell surface along with fusion of secretory vesicles with plasma membranes (Fig. 1) (Shuster et al., 1999).

Comparisons between *in vivo* and *in vitro* studies

MOPR: By and large, the results of the *in vivo* studies are similar to those of *in vitro* studies. Most of the MOPR is present on cell membranes in transfected cells and in neurons *in vivo*. DAMGO and etorphine cause significant internalization of MOPR, but morphine does not, both *in vitro* and *in vivo*. However, the *in vivo* study revealed that morphine promoted redistribution of endogenous MOPR in certain populations of dendrites. Its physiological significance is not clear at the present time.

DOPR and KOPR: While DOPR and KOPR expressed in cells are mostly localized on cell membranes, DOPR and KOPR in neuronal tissues *in vivo* are largely intracellularly located. There are several possibilities for the differences. It may be due to differences in cellular milieu between cell lines and neurons in the brain and spinal cord, including proteins involved in their trafficking and interacting proteins. In addition, immunohistochemistry for KOPR and DOPR *in vitro* was mostly performed with antibodies against an epitope tag added to the N-termini of the receptors, whereas *in vivo* studies were conducted with DOPR and KOPR antibodies against N- or C-terminal domain of the receptors. Antibodies against different epitopes may not recognize intracellular and cell surface receptors equally, thus producing different subcellular distribution patterns. Indeed, Cahill et al. (2001a) reported that antibodies directed against a C-terminal domain peptide of the DOPR recognized predominantly cell bodies and proximal dendrites, whereas those directed against an N-terminal domain peptide, labeled extensively dendritic and terminal arbors besides cell bodies. In addition, electron microscopy studies revealed that the two antibodies label differentially with antibodies against the C-terminal peptide staining twice as many DOPR-immunoreactivities on membranes compared to those against the N-terminal peptide. Moreover, when the receptor was epitope-tagged with FLAG, in most cases it contained a signal peptide to enhance endoplasmic reticulum membrane insertion and thus expression on plasma membranes (Guan et al., 1992), which may contribute to the differences.

Since DOPR has a dramatic difference in localization between *in vitro* and *in vivo*, the *in vivo* studies are focused on how intracellular DOPR is promoted to the cell surface, whereas the *in vitro* investigations have been on agonist-induced internalization and trafficking of internalized receptors.

For the KOPR, the *in vivo* studies are consistent with several *in vitro* findings that U50,488H did not internalize rat KOPR in cells (Li et al., 1999; Zhang et al., 2002).

Future studies

The *in vivo* studies have provided many descriptive observations. However, there is an obvious lack of mechanistic studies.

Functional consequence of receptor trafficking in vivo: Scherrer et al. (2006) have demonstrated in DOPR-EGFP mice that SNC80 enhances DOPR internalization in caudate putamen neuron, which renders the animals less sensitive to the subsequent SNC80 administration (see above). In addition, Cahill et al. (2003) reported that inflammatory pain promoted trafficking of DOPR to cell surface in dorsal horn of the rat spinal cord, leading to enhanced response to DOPR agonists. More studies are needed to address the functional significance of MOPR and KOPR trafficking *in vivo*. McLaughlin et al. (2004) found that chronic U50,488H administration in mice enhanced KOPR phosphorylation and caused tolerance to KOPR-mediated antinociception. Whether the tolerance is related to KOPR internalization requires further study.

Mechanisms underlying the differential subcellular distribution of endogenous opioid receptors: Although the three opioid receptors are highly homologous in their amino acid sequences, in neuronal tissues MOPR is mostly on cell surface, whereas DOPR and KOPR are predominantly intracellular. Since their sequences in the C-terminal domains are highly divergent, it is tempting to speculate that the differences in this region result in their interactions with different proteins, which play an important role in their subcellular localization. However,

the majority of a mutated MOPR with the C-terminal domain replaced with that of the DOPR was still found on cell surface in primary neurons cultured from the knock-in mice (Kim et al., 2008). It will be interesting to directly examine the subcellular distribution of these mutant receptors *in vivo*. Another possibility that can not be ruled out is that the differential distribution may result from the differential recognition of the antibodies. Therefore, it is critical to further characterize the subcellular localization of endogenous opioid receptors using antibodies against different epitopes.

Drake et al.(2005) reported the majority of MOPR was located intracellularly in the dendrites of C1 adrenergic neurons in the rat rostral ventrolateral medulla, in contrast to other brain regions. Therefore, the differences in *in vivo* milieu, such as interacting proteins involved in trafficking, may lead to their differential subcellular distribution in brain regions. Identification of the interacting proteins that are involved in trafficking may help to elucidate the differences.

Constitutive internalization and recycling of endogenous opioid receptors may affect their subcellular distribution. It has been reported that opioid receptors were differentially regulated in the trafficking pathways *in vitro*. While internalized MOPR is mostly recycled, the majority of endocytosed DOPR is sorted to lysosomes for degradation (von Zastrow et al., 2003). Antagonists can be used to stop constitutive internalization and their effects on subcellular localization of the receptors can be examined.

Most receptors in transfected cells appear to be on cell surface; therefore, the *in vitro* systems do not always reflect the *in vivo* situations. One important task is to establish an *in vitro* system in which subcellular distributions of opioid receptors mimic those in tissues. Kim and von Zastrow (2003) found that treatment of PC12 cells with nerve growth factor caused cell differentiation and retained the transfected DOPR intracellularly; whereas transfected MOPR is mostly on cell

surface. This may be a good *in vitro* system that allows studies on mechanisms underlying differential subcellular distribution.

Mechanisms underlying the compartment-selective internalization of MOPR by morphine: MOPR is internalized by morphine treatment *in vitro* when G protein-coupled receptor kinase 2 is over-expressed (Zhang et al., 1998). It is possible that different compartments of neurons may have distinct compositions and/or abundance of internalization machinery components. In addition, we have reported previously that MOPR displayed differential glycosylation in different brain regions (Huang et al., 2008). Thus, it will be interesting to examine if the MOPR in different neuronal compartment may have distinct post-translational modifications.

Mechanisms underlying the promotion of intracellular DOPR to the cell surface: Morphine treatment enhances cell surface level of endogenous DOPR and the MOPR is required for this action. Mechanisms for this process are not clear. MOPR and DOPR have been demonstrated to form dimers *in vitro* (George et al., 2000; Gomes et al., 2000); however, there is no definitive evidence showing their *in vivo* dimerization. It will be interesting to study if MOPR-DOPR dimerization is involved. Unfortunately, there are no reagents that can promote or block dimerization of MOPR-DOPR. Alternatively, morphine may act on the MOPR via neuronal circuitry and ultimately leads to enhancement in cell surface expression of the DOPR. If this is the case, the neuronal circuitry needs to be identified.

Chronic inflammation also enhances cell surface DOPR. Biochemical processes leading to the enhancement remains to be determined. It is likely that chemical mediators of inflammation and subsequent activation of their receptors and down-stream effectors may be involved.

Functional significance of intracellular pool of KOPR: KOPR has a large intracellular pool in dorsal horns of the rat spinal cord, which was mostly dispersed in the cytosol without association

with any organelles. It will be interesting to investigate whether the intracellular KOPR can be translocated to cell surface under certain physiological or pathophysiological conditions. Infusion of U69,593, a selective KOPR agonist, to rostral ventromedial medulla produced antinociceptive effects against chemical or mechanical stimuli (Schepers et al., 2007). The efficacy of U69,593 was significantly enhanced in animals that had chronic inflammatory pain induced by hind paw injection of complete Freund's adjuvant. The presence of KOPR in rostral ventromedial medulla has been reported (Drake et al., 2007). Whether the enhanced efficacy of U69,593 is due to the increase in the number of cell surface KOPR and/or down-stream signaling needs further investigation.

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Figure Legend

Fig. 1 Illustration of electron microscopy findings on subcellular distribution of endogenous opioid receptors and their trafficking upon stimulation *in vivo*.

Upper panel, MOPR is predominantly present on cell surface. MOPR is internalized following treatment with etorphine or DAMGO, but not morphine, in spinal cord, myenteric plexus and several brain regions. Morphine causes internalization of MOPR in the dendrites, but not in the cell body, in the nucleus accumbens.

Middle panel, DOPR is mostly intracellular. Pretreatment with morphine or chronic inflammatory pain enhances trafficking of intracellular DOPR to cell surface in the spinal cord.

Lower panel, KOPR has a significant intracellular pool. In the posterior pituitary, salt loading promotes the insertion of KOPR on vasopressin-containing vesicles into cell surface of axon terminals.

Table 1. Summarization of *in vivo* trafficking of endogenous opioid receptors

Receptor	Method	Animal	Region	Stimulation	Findings	Reference
MOPR	immunohistochemistry + confocal microscopy	guinea pig	myenteric plexus	acute etorphine or morphine	internalized by etorphine, but not by morphine	(Sternini et al., 1996)
		rat	dorsal horn of spinal cord	DAMGO, remifentanil, endomorphin-1 or morphine; noxious stimuli	internalized by DAMGO, remifentanil, endomorphin-1, but not by morphine or noxious stimuli	(Trafton et al., 2000)
		morphine tolerant rat	dorsal horn of spinal cord	DAMGO	DAMGO caused similar magnitudes of internalization in control and tolerant rats	(Trafton and Basbaum, 2004)
		rat	dorsal horn of spinal cord	DAMGO (low dose that did not promote internalization) + Morphine	Promoted internalization	(He et al., 2002)
		rat	Lateral septum	acute morphine	MOPR1C internalized	(Abbadie and Pasternak, 2001)
		OVX rat	medial preoptic nucleus; posterodorsal medial amygdale	estrogen	internalization	(Eckersell et al., 1998)
	immunogold-labeling + electronmicroscopy	rat	locus coeruleus	acute etorphine or morphine	internalized by etorphine, but not by morphine	(Van Bockstaele and Commons, 2001)
			nucleus accumbens	acute morphine	internalized in dendrites, but not in cell bodies	(Haberstock-Debic et al., 2003)
			rostral ventrolateral medulla	acute morphine	internalized only in the dendrites with diameter <1.4 μm	(Drake et al., 2005)
DOPR	confocal microscopy	knock-in mouse	striatum	SNC80	internalization correlated with tolerance	(Scherrer et al., 2006)
	immunogold-labeling + electron microscopy	rat	dorsal horn of spinal cord	Chronic inflammatory pain	up-regulation and translocation to surface	(Cahill et al., 2003)
				Chronic morphine treatment	translocation to surface	(Cahill et al.,2001b)
KOPR	immunogold-labeling + electron microscopy	rat	dorsal horn of spinal cord	U50,488H or dynorphin A	internalized by dynorphin A	(Wang et al. submitted)
			hypothalamus	salt loading	inserted into cell surface	(Shuster et al., 1999)

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