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Dissociation of the Opioid Receptor Mechanisms that Control Mechanical and Heat Pain

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SUMMARY

Delta and mu opioid receptors (DORs and MORs) are inhibitory G protein-coupled receptors that reportedly cooperatively regulate the transmission of pain messages by substance P and TRPV1-expressing pain fibers. Using a DOReGFP reporter mouse we now show that the DOR and MOR are, in fact, expressed by different subsets of primary afferents. The MOR is expressed in peptidergic pain fibers, the DOR in myelinated and nonpeptidergic afferents. Contrary to the prevailing view, we demonstrate that the DOR is trafficked to the cell surface under resting conditions, independently of substance P, and internalized following activation by DOR agonists. Finally, we show that the segregated DOR and MOR distribution is paralleled by a remarkably selective functional contribution of the two receptors to the control of mechanical and heat pain, respectively. These results demonstrate that behaviorally relevant pain modalities can be selectively regulated through the targeting of distinct subsets of primary afferent pain fibers.

INTRODUCTION

The delta and mu opioid receptors (DOR and MOR) are inhibitory G protein-coupled receptors (GPCRs) through which endogenous opioids (endorphins and enkephalins) regulate a variety of physiological functions, including pain control, emotional tone, and reward (Kieffer and Gaveriaux-Ruff, 2002). The MOR also mediates the pain-relieving effects of some of the most clinically efficacious drugs. For example, the analgesia produced by morphine is lost in mice in which the gene that encodes the MOR is inactivated (Matthes et al., 1996; Sora et al., 1997b). The contribution of the DOR to pain processing is much less clear. Although some studies report that DOR-selective agonists exert potent analgesic effects (Narita and Suzuki, 2003; Onofrio and Yaksh, 1983; Porreca et al., 1987), others found that DOR agonists are relatively weak, particularly compared to morphine (Gallantine and Meert, 2005; Scherrer et al., 2004).

Yet another perspective is that a functional interaction between the two receptors contributes to opioid agonist-mediated pain control at the level of the spinal cord. For example, it has been reported that genetic inactivation or pharmacological blockade of the DOR can potentiate the pain-relieving effect of MOR agonists (Gomes et al., 2004; Gomes et al., 2001) and can counteract development of the tolerance that occurs with chronic morphine treatment (Zhu et al., 1999). This apparent negative cooperativity between the MOR and DOR may involve a direct interaction of the two receptors, via the formation of MOR-DOR heterodimers (Gomes et al., 2004; for review, see Rozenfeld et al., 2007). In fact, immunohistochemical studies demonstrated that the MOR and DOR are coexpressed in the same subpopulation of primary afferent "pain" fibers (nociceptors), namely in the small-diameter, peptidergic substance P- (SP) and calcitonin gene-related peptide (CGRP)-containing unmyelinated afferents (Arvidsson et al., 1995a; Ji et al., 1995). As these peptidergic afferents express the heat-sensitive TRPV1 channel (Caterina et al., 2000) it follows that both MOR and DOR agonists would regulate heat pain sensitivity, which is indeed what many studies have reported (Matthes et al., 1996; Narita and Suzuki, 2003; Sora et al., 1997b).

Processing of the two GPCRs in these neurons, however, is thought to be very different (Cahill et al., 2007; Zhang et al., 2006). In contrast to prototypical GPCRs, such as the MOR, the DOR is reportedly absent from the plasma membrane of the synaptic terminal of nociceptors, under resting conditions (Cahill et al., 2001; Gendron et al., 2006; Morinville et al., 2003; Patwardhan et al., 2005; Walwyn et al., 2005; Zhang et al., 1998). Rather, the DOR is transported to central terminals via the regulated secretory pathway, which results in the DOR being stored in the membrane of large, peptide-containing dense core vesicles (LDCVs; Bao et al., 2003; Zhang et al., 1998). Functionality of the DOR only occurs when stimuli trigger exocytosis of LDCVs, resulting in their integration into the plasma membrane (Bao et al., 2003). This, in turn, renders the DOR accessible to

opioid ligands. Recently, Guan et al. (2005) provided insights into the mechanism through which the DOR is sorted to LDCVs. These authors discovered an interaction of SP with an extracellular loop of the DOR that is essential for proper DOR trafficking. When the SP-DOR interaction was disrupted, in mice in which the gene encoding SP was inactivated (*ppt-A* gene), the DOR was no longer transported to the terminals of nociceptors in the spinal cord.

Here we show that many of the existing conclusions concerning the DOR are not tenable. Using a DOReGFP reporter knockin mouse, we provide a substantially different view of the DOR and MOR distribution, function and relationship to the processing of pain messages.

RESULTS

DOR Is Expressed in Myelinated and Nonpeptidergic Unmyelinated Pain Fibers

We recently described a reporter knockin mouse in which a functional DOReGFP fusion receptor replaces the endogenous receptor (Scherrer et al., 2006). Here, we took advantage of this mouse to address the contribution of the DOR to pain processing. We first examined the DOReGFP distribution in sensory neurons of dorsal root ganglia (DRG) immunostained with an antibody against GFP (Figure 1A) and found that 17% of sensory neurons expressed DOReGFP. Positive cells show intense labeling of the plasma membrane and the perinuclear region (Figure 1B) under resting conditions. Intrathecal delivery of the DOR agonist SNC80 (i.e., directly into the CSF by lumbar puncture) triggered a profound internalization of DOReGFPs (Figure 1B). Based on these observations we conclude that the subcellular distribution and trafficking of DORs are characteristic of those of a prototypical GPCR, and that DORs expressed in sensory neurons can be targeted via the intrathecal route.

Based on previous studies using antibodies against the DOR, we expected that the DOReGFP+ DRG cell bodies would overlap with the peptide-containing subpopulation of unmyelinated nociceptors. This was not the case (Figures 1C and 1D and Table S1). Instead, more than 61% of DOReGFP cells were of medium to large size and expressed NF200 (Figures 1D and 1E), a neurofilament marker of neurons with myelinated axons. In fact, 65% of the DOReGFP myelinated neurons coexpressed TRPV2, a channel that is restricted to myelinated afferents (Figure 1F and Table S1).

Strikingly, when we directly assessed the extent of colocalization of DOReGFP with markers of peptidergic unmyelinated nociceptors, SP, CGRP and the capsaicin and heat-sensitive channel, TRPV1, we found no overlap (Figures 1G and 1H). Rather, all small-diameter DOReGFP neurons, which represent 39% of all DOReGFP DRG cells, bound the lectin IB4 (Figure 1I) and coexpressed the purinergic receptor, $P2X_3$ (Figure S1), two features of the nonpeptidergic population of unmyelinated nociceptors. We found some colocalization of CGRP and DOReGFP, predominantly in the NF200+ neurons (Figure 1J). Together, our results indicate that DOReGFP is expressed in myelinated and nonpeptidergic unmyelinated DRG neurons, not in peptidergic unmyelinated nociceptors.

Dissociation of DOReGFP and Substance P

Given the large literature reporting coexpression and functional interactions of the DOR and SP, our finding that DOReGFP almost never colocalizes with SP+ DRG neurons was completely unexpected. Because rapid transport of the DOReGFP from the cell body to the terminals of SP+ cells could have made detection of the DOReGFP in DRG cell bodies difficult, we next costained for SP and DOReGFP in both the central and peripheral terminals of primary sensory neurons. As expected, we found that SP+ terminals in the spinal cord are concentrated in the most superficial laminae (I and outer II) of the dorsal horn (Hökfelt et al., 1977) (Figure 2A). By contrast, the DOReGFP predominates in terminals in the most inner part of lamina II, a region defined by its large number of PKCγ interneurons (Figures 2C and 2D). That result is of particular interest as we recently reported that the PKCγ layer of interneurons, which has been implicated in the development of injury-induced persistent pain (Malmberg et al., 1997), receives a myelinated primary afferent input (Neumann et al., 2008).

We also observed a less dense band of DOReGFP staining in lamina I, but even here confocal analysis showed that the SP+ and DOReGFP+ terminals in lamina I do not overlap (Figure 2B). Figures 2A–2C also show that there is light, relatively uniform DOReGFP staining throughout the gray matter (dorsal and ventral horns), which agrees with the distribution pattern of the DOR revealed in radioligand binding studies (Mennicken et al., 2003, Figure S2).

In the skin, we observed a dense plexus of DOReGFP axons that course through the dermis and epidermis, but no colocalization with SP axon terminals (Figures 2E and 2F). We conclude that there is no overlap of the DOR and SP, in either the central or peripheral terminals of nociceptors. Finally, we show that the segregated expression of DOR and SP is not restricted to somatic afferent nociceptors, but is particularly apparent for afferents that innervate viscera (Supplemental Results, Figures 2G–2I, Figure S3). Together these results not only indicate that the DOR is not expressed in SP+ nociceptors, but also that the DOR is largely excluded from the innervation of visceral organs.

The DOR Is a Prototypical GPCR that Is Trafficked via the Nonregulated Pathway, Independently of SP

Our finding that the DOR is expressed in myelinated and nonpeptidergic unmyelinated primary afferents and localized at the cell surface under resting conditions differs greatly from the prevailing view that is based on studies using anti-DOR antibodies. We therefore reexamined the specificity of the immunoreactivity generated with DOR antisera. The staining pattern that we obtained was identical to that reported in the literature, however, it did not change in tissues from two different mouse strains with a deletion of the *dor* gene (Filliol et al., 2000; Zhu et al., 1999) (see Supplemental Results, Figures S4 and S5). We conclude that this anti-DOR antibody, which is the most widely used, does not recognize the DOR in immunohistochemical preparations, but rather must cross-react with an as yet unidentified molecule. Additionally, we have tested several other commonly used anti-DOR antibodies, all of which equally immunostain tissue from wild-type and *dor* null mice (Figure S6).