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Mu-opioid receptor knockout prevents changes in delta-opioid receptor trafficking induced by chronic inflammatory pain

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Abstract

Previous studies from our laboratory have demonstrated that both chronic inflammatory pain, induced by intraplantar injection of complete Freund's adjuvant (CFA), and prolonged (48 h) stimulation of mu-opioid receptors (μOR) by systemic administration of a variety of selective agonists, resulted in enhanced plasma membrane targeting of delta-opioid receptors (δOR) in neurons of the dorsal spinal cord. To determine whether δOR trafficking induced by chronic inflammation was dependent on the activation of μOR, we investigated by immunogold cytochemistry the effects of intraplantar CFA injection on the plasma membrane density of δOR in μOR knockout (KO) mice. In untreated wild-type (WT) mice, only a small proportion of δOR was associated with neuronal plasma membranes in the dorsal horn of the spinal cord. The CFA-induced inflammation produced a significantly higher ratio of plasma membrane to intracellular receptors, as well as a 75% increase in the membrane density of immunoreactive δOR, in dendrites of the ipsilateral dorsal horn as compared to untreated mice. This increase in the membrane density of δOR was likely due to a recruitment of receptors from intracellular stores since no difference in the overall δOR immunolabeling density was evident between CFA-treated and untreated mice. Most importantly, the CFA-induced changes in δOR plasma membrane insertion seen in WT animals were not present in the spinal cord of μOR KO mice. These results demonstrate that the integrity of μOR is necessary for CFA-induced changes in δOR trafficking to occur and suggest that these changes could be elicited by stimulation of μOR by endogenous opioids released in response to chronic inflammatory pain.
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Keywords: Opioid receptors; Complete Freund's adjuvant; Receptor targeting; Spinal cord; Electron microscopy; Subcellular localization

1. Introduction

Administration of opioid analgesics directly into the central nervous system (CNS) produces a profound increase in nociceptive thresholds in various animal models of acute and chronic pain. For example, in models of chronic inflammatory pain, spinal administration of mu-(μ) or delta-(δ) opioid receptor (OR) agonists have been shown to reverse thermal hyperalgesia precipitated in these models (Cahill et al., 2003; Ossipov et al., 1995; Qiu et al., 2000; Stewart and Hammond, 1994). Moreover, δOR-induced anti-nociceptive effects were shifted to the left compared to controls, indicating an augmentation in analgesic potency, following induction of inflammation via carrageenan (Stewart and Hammond, 1994) or complete Freund's adjuvant (CFA) injection (Cahill et al., 2003; Fraser et al., 2000; Hylden et al., 1991; Qiu et al., 2000). Nonetheless, the mechanisms by which centrally administered opioids alleviate persistent inflammatory pain states are not well understood.

Plastic changes have been documented to occur in the spinal cord in response to persistent inflammatory pain (Julius and Basbaum, 2001). Some of these changes affect the expression and subcellular distribution of δOR in neurons of the dorsal horn of the spinal cord and dorsal root ganglia (Besse et al., 1992; Cahill et al., 2003; Ji et al., 1995). Under basal conditions, δOR are almost exclusively localized to intracellular compartments in neurons throughout the CNS,

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including the spinal cord (Arvidsson et al., 1995; Cahill et al., 2001a,b; Cheng et al., 1995; Morinville et al., 2003; Svingos et al., 1995; Wang and Pickel, 2001; Zhang et al., 1998). Hence, ultra-structural analysis revealed that in the rat spinal cord, plasma membrane-associated δOR accounted for less than 10% of the total amount of immunoreactive δOR detected within dendrites of dorsal horn neurons (Cahill et al., 2001a,b; Morinville et al., 2003). However, 72 h after a unilateral intraplantar injection of CFA into the hind paw, the proportion of δOR associated with plasma membranes increased almost twofold in dendrites from the dorsal spinal cord ipsilateral to the site of inflammation (Cahill et al., 2003). It was suggested that these changes in the cellular compartmentalization of δOR might contribute to the enhanced opioid anti-nociception observed in this pain state (Cahill et al., 2003).

Recent studies from our laboratory have demonstrated that targeting of δOR to neuronal plasma membranes in the dorsal horn of rodent spinal cord could also be elicited via sustained morphine treatment and that the resulting enhanced number of pharmacologically available δOR increased δOR-induced anti-nociception (Cahill et al., 2001b; Morinville et al., 2003). Studies with transgenic animals revealed that this morphine-induced trafficking of δOR was prevented in μOR knockout (KO) mice, demonstrating that it was dependent on the activation of μOR (Morinville et al., 2003). Furthermore, the morphine-induced trafficking of δOR in dorsal horn neurons was no longer evident following dorsal rhizotomy, suggesting that this plastic event was predicated upon incoming somatosensory, including nociceptive, inputs (Morinville et al., 2004).

The aim of the present study was to assess whether the recruitment of δOR to the cell surface observed in a model of chronic inflammatory pain was, like the one observed after prolonged morphine treatment, dependent on the activation of μOR in the dorsal horn of the rodent spinal cord. To address this issue, chronic inflammation was induced by injection of CFA in the hind paw of wild-type (WT) and transgenic mice lacking the μOR and quantitative ultrastructural analysis using immunogold electron microscopy was used to investigate the subcellular distribution of δOR in neurons of the dorsal spinal cord. We report here that like in the rat, CFA-induced inflammation increases the cell surface density of δOR on dendrites of the dorsal horn ipsilateral to the site of inflammation and that this increase can no longer be elicited in the spinal cord of μOR-KO mice.

2. Methods

2.1. Animals

The studies used homozygous female and male C57BL/6 μOR KO mice as well as male and female WT C57BL/6 mice. The generation of μOR KO mice has been described in detail previously (Matthes et al., 1996). These mice were between 9 and 12 months of age at the time of the experiments. All animals were maintained on a 12/12 h light/dark cycle and were allowed free access to food and water. Experiments were approved by the animal care committee at McGill University and complied with the directives of the Canadian Council on Animal Care.

2.2. Induction of chronic inflammation

Chronic inflammation was induced by a subcutaneous injection of 130 μg/25 μl complete Freund's adjuvant (CFA; Sigma, St Louis, MO) in the plantar surface of the right hind paw under ketamine/xylazine (60/6 mg/kg, i.p.) anaesthesia. All mice were used 72 h following injection of CFA.

Two sets of controls were used for the study: WT and μOR KO C57BL/6 mice that did not receive an injection of CFA. The results from these two sets of control mice were previously published independently (Morinville et al., 2003; data reproduced with permission of the Society for Neuroscience). Experimental animals (i.e. WT and μOR KO mice injected with CFA to establish the effects of chronic inflammation on δOR trafficking) were obtained from the same batches of mice and were immunohistochemically processed in the same fashion, by the same experimenter, and within the same time frame as control animals.

2.3. Electron microscopic studies

Mice were anaesthetized with sodium pentobarbitol (7.5 mg/100 g) and perfused through the left aortic arch with 50 ml of heparin (6 U/ml) in 0.9% saline followed by 35 ml of a mixture of 3.75% acrolein in 2% paraformaldeyhde (PFA), pH 7.4, and then with 250 ml of 2% PFA in 0.1 M phosphate buffer (PB) (pH 7.4). Lumbar spinal cords were removed and post-fixed in 2% PFA in 0.1 M PB for 30 min at 4 °C. Transverse sections (50 μm) were cut using a vibrating microtome and collected in 0.1 M PB. Sections were processed for δOR immunogold labeling according to a previously described protocol (Morinville et al., 2003). Briefly, sections were incubated for 30 min with 1% sodium borohydride in 0.1 M PB to neutralize free aldehyde groups, followed by copious rinses with 0.1 M PB. Sections were then incubated at room temperature for 30 min in a cryoprotectant solution consisting of 25% sucrose and 3% glycerol in 0.1 M PB prior to snap-freezing with isopentane (−70 °C), then liquid nitrogen, and finally thawing in 0.1 M PB. Sections were rinsed with 0.1 M Tris-buffered saline (TBS) (pH 7.4) and pre-incubated for 30 min at room temperature in a blocking solution consisting of 3% NGS in TBS. They were then incubated for 36–48 h at 4 °C with a δOR antiserum (Chemicon, Temecula, CA; Cat No. AB1560, Lot No. 20100177) diluted 1:4000 in TBS

containing 0.5% NGS, followed by 2 h at room temperature with a 1:50 dilution of colloidal gold (1 nm) conjugated goat anti-rabbit IgG (BBInternational, Cardiff, UK) diluted in 0.1 M phosphate buffered saline (PBS) (pH 7.4) containing 2% gelatin and 8% BSA. After thorough washing, sections were fixed with 2% glutaraldehyde and immunogold deposits were enhanced by incubation with ionic silver (IntenSE M Silver Enhancement Kit, Amersham Biosciences). Sections were post-fixed with 2% OsO_4, dehydrated in graded alcohols, and flat-embedded in Epon.

Ultrathin silver-gray sections (80 nm) were cut from the surface of each Epon block using a Reichert (Heidelberg) ultramicrotome. Sections were serially collected on copper grids as soon as tissue appeared, counter-stained with uranyl acetate and lead citrate, and examined with a JEOL 100CX transmission electron microscope (JEOL USA, Inc., Peabody, MA). Sections sampled for analysis were typically from grids #2 and 3 (first whole sections). Negatives were scanned using an AGFA Duoscan T1200 and images were processed using Photoshop version 6.0.1 (Adobe Systems, Inc.) on an IBM-compatible computer.

2.4. Data analysis

For quantification of the distribution of immunolabeled δOR, a minimum of 50 immunopositive dendrites were sampled in the dorsal horn (lamina III–V) of CFA-treated WT ($n = 3$) and μOR KO mice ($n = 3$), both ipsi- and contralaterally to the side of CFA injection. Dendrites were considered immunopositive if overlaid with 3 gold particles or more. Labeled dendrites of all sizes were included in the sampling, irrespective of their angle of sectioning (i.e. longitudinal versus oblique or transversal). The data were compared with those obtained from similarly sampled dendrites in untreated WT ($n = 3$) and μOR KO ($n = 3$) mice (Morinville et al., 2003). Raw data for each experimental condition (sum of $n = 3$) are provided in Table 1. The following quantitative analyses were carried out in each animal for each experimental condition:

Table 1
Number of immunopositive dendrites and gold particles counted in each experimental group

Condition	Total number of dendritic profiles sampled	Total number of gold particles counted	Total number of plasma membrane associated gold particles counted
WT naive	206	1835	124
WT CFA contra	202	2068	185
WT CFA ipsi	232	2620	294
μOR KO naive	176	2092	137
μOR KO contra	218	2137	168
μOR KO ipsi	291	2480	230

Values correspond to the sum of $n = 3$ for each condition.

(1) The number of δOR per unit area (μm^2) was calculated by dividing the sum of the total number of gold particles detected over the entire cross-sectional profile of all sampled dendrites by the sum of these surface areas (measured by computer-assisted morphometry; Biocom, Les Ulis, France).

(2) The percentage of membrane-associated to total gold particles was calculated by dividing the sum of the number of gold particles found at the cell surface by the sum of immunogold particles detected in all sampled dendrites. Gold particles were considered to be membrane-associated only if in actual contact with the plasma membrane; gold particles in close proximity to but not touching the plasma membrane were considered to be intracellular. To correct for possible differences in the perimeter/area ratios of sampled dendrites between the different conditions, the calculated percentages for each condition were normalized as follows:

$$\text{Corrected percentage} : [(\text{percentage of condition 1}) \times (\mu m/\mu m^2 \text{ of the control})/(\mu m/\mu m^2 \text{ of condition 1})]$$

(3) The density of immunoreactive δOR per unit length of membrane was calculated by dividing the total number of gold particles detected at the surface of all sampled dendrites by the sum of the respective perimeters. This average δOR plasma membrane density was then divided by the average plasma membrane density of the respective control (untreated WT or untreated μOR KO) and the data expressed as percentage of control.

(4) The segmental distance of each gold particle from the plasma membrane was calculated in a subpopulation of dendrites of comparable sizes (mean diameters ranging between 2.4 and 2.6 μm) randomly selected from the original sampling (30 dendrites per condition), by measuring the shortest distance between the gold particle and the plasma membrane; these segmental distances were then averaged for each condition.

Calculations and statistical analyses were performed using Excel 97 (Microsoft Corp.) and Prism 3.02 (Graph Pad Software Inc.). Details of the statistical analysis are provided in the figure legends.

3. Results

3.1. Effect of CFA injection on the subcellular distribution of δOR in mouse spinal cord

By electron microscopy, silver-intensified immunogold particles, corresponding to immunoreactive δOR, were detected in association with neuronal perikarya

and dendrites, as well as with axons and axon terminals, throughout laminae III–V of the dorsal horn in both untreated and CFA-treated WT mice (Fig. 1A and B). Within these immunolabeled neurons, most immunoreactive δOR were intracellular rather than on the plasma membrane (Fig. 1A and B; Table 1).

Unilateral injection of CFA in the hind paw of WT mice did not produce any significant change in the density of immunogold particles detected per unit area of dorsal horn dendrites when compared to untreated WT animals, either ipsi- or contralateral to the side of the CFA injection (Fig. 2A). However, the percentage of gold particles associated with plasma membranes (as opposed to intracellular) was significantly increased (from 6.6 to 13% of total) in dendritic profiles within the ipsilateral dorsal horn of CFA-treated mice as compared to untreated animals ($P < 0.01$, Fig. 2B; also compare A and B in Fig. 1 and see Table 1). In addition, we noted a small increase in the percentage of gold particles associated with plasma membranes on the side contralateral to the injected hind paw as compared to untreated mice, but this increase did not reach statistical significance (Fig. 2B). These distributional changes translated into a 75% increase in the density of gold particles per unit length of plasma membrane in dendrites of the ipsilateral dorsal horn in CFA-treated as compared to untreated WT mice (Fig. 2C). This increase was significantly different ($P < 0.05$) from that (23%) observed on the contralateral side (Fig. 2C).

To confirm that the increase in the proportion of gold particles associated with the plasma membrane was due to a mobilization of intracellular receptors towards the cell surface, we compared, in dendrites of comparable sizes randomly selected from CFA-treated and untreated WT mice, the average segmental distance separating each gold particle from the closest plasma membrane. As can be seen in Fig. 3, this distance was significantly shorter ($P < 0.001$) in dendrites from the dorsal horn ipsilateral to the side of CFA injection than from the dorsal horn of untreated animals.

3.2. *Effect of μOR gene knockout on CFA-induced up-regulation of cell surface δOR*

As previously reported (Morinville et al., 2003), the cellular and subcellular distribution of immunoreactive δOR in the dorsal horn of the spinal cord of untreated μOR KO mice was similar to that observed in untreated WT mice (Table 1). Specifically, no significant difference was noted in the density of immunogold particles over dendrites from the dorsal horn of WT as compared to μOR KO mice (Fig. 2A). Likewise, the proportion of dendrite-associated immunogold particles found over plasma membranes was identical to that observed in untreated WT mice (Fig. 2B; Table 1).

As in WT animals, we observed no difference in the density (per surface area) of gold particles over dendrites of the dorsal horn between untreated and CFA-treated μOR KO mice (Fig. 2A). However, in contrast to WT animals, CFA-treated μOR KO mice did not show an increase in the percentage of membrane-associated gold particles in dendrites of the ipsilateral dorsal horn (Figs. 1C and 2B). In addition, the density of gold particles per unit length of dendritic membrane (expressed as percentage control), was not significantly different between sides ipsi- and contralateral to CFA injection in μOR KO mice (Fig. 2C). Accordingly, the average segmental distance separating each gold particle from the closest plasma membrane was the same in dendrites from the ipsilateral dorsal horn of

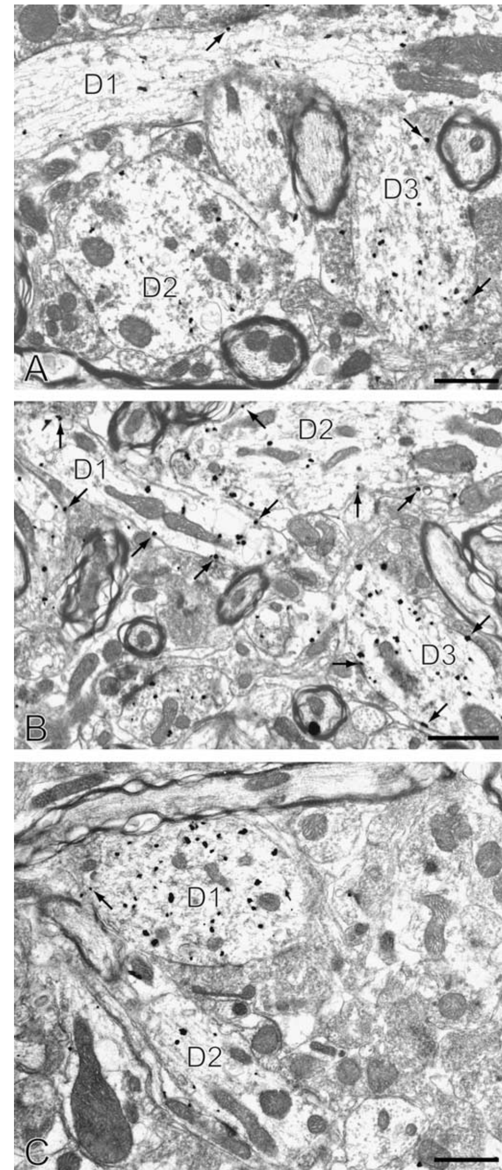

Fig. 1. Subcellular localization of δOR in dendrites from the lumbar (L5) spinal cord of an untreated wild-type (WT) mouse (A), and of CFA-treated (ipsilateral to the injected paw) WT (B) and μOR KO mice (C). In all cases, the majority of immunogold particles is intracellular. However, gold particles are more numerous over the plasma membrane (arrows) of CFA-treated WT than of either untreated WT or CFA-treated μOR KO mice. D, dendrite. Scale bar A, B, C: 1 μm.

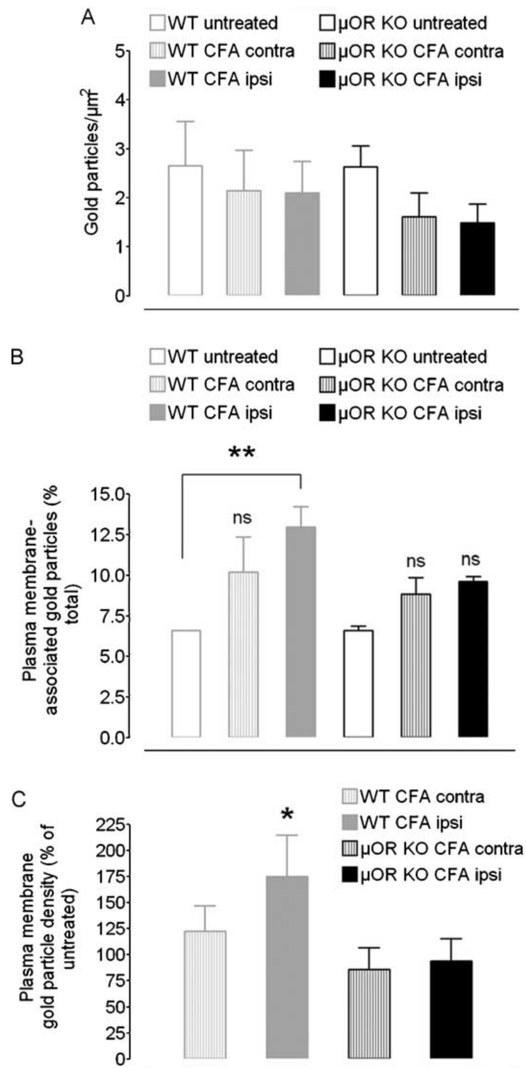

Fig. 2. Quantitative analysis of the subcellular distribution of immunoreactive δOR in dendrites from the lumbar (L5) spinal cord of untreated and CFA-treated wild-type (WT) and μOR KO mice. (A) Density of immunoreactive δOR over dendritic profiles. Each bar represents the mean (± SEM) number of gold particles per unit area (in μm^2; $n = 3$ for each group). No statistically significant differences is observed between any of the groups (ANOVA, $P > 0.05$). (B) Percentage of dendrite-associated gold particles associated with plasma membranes. Note that this percentage is significantly higher on the side ipsilateral (one way ANOVA, Bonferroni's MCT, $P < 0.01$; denoted by an asterisk) but not contralateral ($P > 0.05$, denoted by ns) to the side of CFA injection in WT mice when compared to untreated WT mice. No difference in the percentage of membrane-associated gold particles is observed between untreated and either contra- or ipsilateral sides of CFA-treated μOR KO mice (ANOVA, $P > 0.05$; denoted by ns). (C) Plasma membrane density of immunoreactive δOR (number of gold particles per unit length of dendritic membrane) in the spinal cord of CFA-treated WT and μOR KO mice expressed as percent of corresponding untreated animals. Note that the percent increase over untreated mice is higher on the side ipsilateral to the CFA injection as compared to the contralateral side ($P < 0.05$, denoted by an asterisk) in WT animals but not in CFA-treated μOR KO mice. Statistical significance was determined by means of a repeated measures one-way ANOVA followed by a Bonferroni's MCT; ns, not significant.

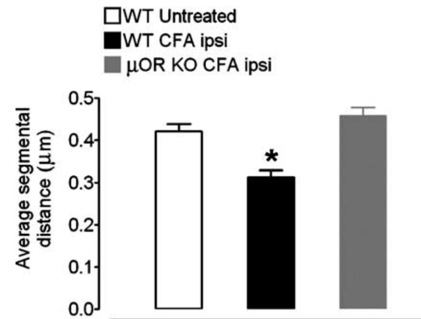

Fig. 3. Mean distance separating individual gold particles from the closest plasma membrane in δOR-immunoreactive dendrites of matched sizes. Note the statistically significant difference between CFA-treated (ipsilateral side; $n = 373$) WT mice and both untreated WT ($n = 352$) and CFA-treated μOR KO mice (ipsilateral side; $n = 339$) (one-way ANOVA followed by a Tukey's MCT). No significant difference is observed between untreated WT mice and CFA-treated μOR KO mice.

CFA-treated μOR KO mice as in the dorsal horn of untreated μOR KO animals (Fig. 3).

4. Discussion

The present study demonstrates that in the mouse, as in the rat, chronic inflammatory pain induced by intraplantar injection of CFA results in an up-regulation of cell surface δOR in neurons of the dorsal horn of the spinal cord, ipsilateral to the side of inflammation. This up-regulation is no longer observed in μOR KO mice, indicating that it requires the presence of μOR.

4.1. Effect of CFA injection on the subcellular distribution of δOR in mouse spinal cord

In a previous report, we showed that a unilateral injection of CFA in the hind paw of the rat resulted in an increase in the cell surface density of δOR in neurons from the deeper laminae of the ipsilateral dorsal horn 72 h after injection of the bacterium (Cahill et al., 2003). Using a similar experimental protocol and sampling of the dorsal horn (laminae III–V), we found here that chronic inflammatory pain similarly affected δOR trafficking in the dorsal spinal cord of the mouse. Hence, CFA-induced inflammation resulted in a significantly higher ratio of plasma membrane to intracellular receptors, as well as in a 75% increase in the membrane density of immunoreactive δOR, in dendrites of the dorsal horn ipsilateral to the side of CFA injection as compared to untreated mice. Since there was no difference in the overall density of δOR (per surface area) over labeled dendrites, these findings were taken to reflect a re-distribution of receptors from intracellular stores towards the plasma membrane. Concordant with this interpretation, the mean segmental distance separating intracellular δOR from the plasma membrane was significantly shorter in CFA-treated than in untreated animals.

The lack of change in the overall density of intraneuronal δOR was surprising in light of our earlier observations that in the rat, the CFA-induced redistribution of δOR was accompanied by an increase in δOR expression and content (Cahill et al., 2003). This discrepancy could be due to interspecies differences in the time course of δOR regulatory mechanisms induced by CFA injection. It could also be due to an experimental bias whereby ultrathin sections would have been sampled at different depths in immunoreacted spinal cord slices from CFA-treated and untreated mice. Such a bias would not have affected cell surface/intracellular ratios, but could have influenced the absolute number of gold particles detected and hence density appraisals. Further experiments (i.e. measurements of δOR mRNA and δOR protein levels) will be needed to definitively settle this issue. In any event, the net effect of CFA-induced inflammation in both species was a robust increase in the cell surface density of δOR, which likely accounts for the greater analgesic potency of δOR agonists documented in conditions of chronic inflammatory pain in both mouse (Hylden et al., 1991; Qiu et al., 2000) and rat (Cahill et al., 2003; Stewart and Hammond, 1993).

4.2. Effect of μOR gene knockout on CFA-induced up-regulation of cell surface δOR

The most noteworthy finding of the present study was that the changes in δOR trafficking observed in dendrites from the ipsilateral dorsal horn following unilateral intraplantar injection of CFA were no longer apparent in the spinal cord of μOR KO mice. In fact, all indices of δOR plasma membrane insertion (percentage of receptors associated with the membrane, plasma membrane receptor density, and mean segmental distance separating labeled receptors from the closest plasma membrane) were the same in CFA-treated μOR KO mice as in untreated WT animals. This lack of CFA-induced effect cannot be attributed to abnormal expression and/or function of δOR inherent to the μOR KO phenotype since earlier biochemical and histochemical studies failed to reveal any substantial difference in δOR agonist binding, G-protein activation, δOR subcellular distribution or δOR mRNA expression levels between WT and μOR KO mice (Chen et al., 2000; Hosohata et al., 2000; Kitchen et al., 1997; Loh et al., 1998; Matthes et al., 1996; Morinville et al., 2003; Narita et al., 1999; Sora et al., 1997; for review, see Kieffer and Gaveriaux-Ruff, 2002). Therefore, the lack of changes in δOR trafficking observed in μOR KO mice had to be attributed to the suppression of a CFA-induced activation of μOR in these animals.

This interpretation is supported by previous evidence for a μOR-induced regulation of δOR (Cahill et al., 2001b; Morinville et al., 2003) and implies that the changes in δOR trafficking produced by CFA inflammatory pain may be due to chronic stimulation of μOR by endogenously released opioids. It has been well established that chronic inflammation produces a dramatic up-regulation of endogenous opioid peptides in rodent spinal cord and primary afferent neurons. For instance, peripheral inflammation produced an increase in the number of enkephalin mRNA-positive dorsal root ganglion neurons (Ji et al., 1994) and an up-regulation of pre-proenkephalin gene expression in the spinal cord (Iadarola et al., 1988; Nogushi et al., 1992). In addition, stimulation of nociceptive inputs has been linked to the release of endomorphin-2 from C-type nociceptive primary afferents (Przewlocka et al., 1999) and of met-enkephalin from intrinsic circuit neurons in the dorsal horn of the spinal cord (Bourgoin et al., 1990; Cesselin et al., 1989; Le Bars et al., 1987). Endogenous opioids, including enkephalins, have long been known to inhibit nociceptive transmission through their action on either descending or other sensory inputs that activate local circuit neurons (reviewed in Stanfa and Dickenson, 1995). Indeed, transgenic mice deficient in pre-proenkephalin have altered responses to noxious stimuli (Konig et al., 1996). Some of these inhibitory effects are exerted through activation of μOR, to which both met-enkephalin and endomorphin-2 bind with high affinity (Corbett et al., 1993; Zadina et al., 1997). We propose here that sustained stimulation of μOR by endogenous opioids may also result in increased trafficking of δOR to neuronal plasma membranes. In the case of CFA-induced inflammatory pain, this effect could even be compounded by the up-regulation of μOR reported to occur within the superficial layers of the dorsal horn in this model system (Mousa et al., 2002). The mechanisms by which stimulation of μOR induces targeting of δOR to the plasma membrane are yet to be elucidated, but, given the time frame of the phenomenon, likely involve protein neosynthesis, such as the synthesis of a chaperone molecule as previously proposed (Morinville et al., 2003).

5. Conclusion

We demonstrated that chronic inflammatory pain induced by intraplantar administration of CFA increases δOR targeting to neuronal plasma membranes in the ipsilateral dorsal horn of the mouse spinal cord. This membrane recruitment was no longer evident in μOR KO mice, suggesting that it is predicated on stimulation of μOR by endogenous opioids released in response to chronic pain. These results could imply that μOR agonists may be useful in the early phases of inflammatory pain, not only for their anti-nociceptive properties, but also as a primer for optimizing the analgesic potency of δOR agonists in the chronic phase of the disease.

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