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Up-regulation and trafficking of δ opioid receptor in a model of chronic inflammation: implications for pain control

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Abstract

Pharmacological and physiological evidence supports a role for delta (δ) opioid receptors in the nociceptive mechanisms of inflammation. However, few data exist regarding δ opioid receptor expression and localization in such conditions. In this study, we have assessed the distribution and function of δ opioid receptors in the rat spinal cord following induction of chronic inflammation by intraplantar injection of complete Freund's adjuvant (CFA). Intrathecal administration of the selective δ opioid receptor agonist, D-[Ala^2, Glu^4] deltorphin, dose-dependently reversed thermal hyperalgesia induced by CFA. In situ hybridization and Western blotting experiments revealed an increase in δ opioid receptor mRNA and protein levels, respectively, in the dorsal lumbar spinal cord ipsilateral to the CFA injection site compared to the contralateral side and sham-injected controls. By electron microscopy, immunopositive δ opioid receptors were evident in neuronal perikarya, dendrites, unmyelinated axons and axon terminals. Quantification of immunopositive signal in dendrites revealed a twofold increase in the number of immunogold particles in the ipsilateral dorsal spinal cord of CFA-injected rats compared to the contralateral side and to sham-injected rats. Moreover, the relative frequency of immunogold particles associated with or in close proximity to the plasma membrane was increased in the ipsilateral dorsal spinal cord, indicating a more efficient targeting of δ opioid receptors to neuronal plasma membranes. These data demonstrate that CFA induces an up-regulation and increased membrane targeting of δ opioid receptors in the dorsal spinal cord which may account for the enhanced antinociceptive effects of δ opioid receptor agonists in chronic inflammatory pain models.

Keywords: Inflammation; Delta opioid receptor; electron microscopy; Pain; Receptor trafficking

1. Introduction

Endogenous opioid peptides and their receptors are integral components of neural circuits that modulate the cognitive and affective aspects of the pain experience (Fields and Basbaum, 1994). Opioids exert their effects through interaction with receptors (mu, delta (δ), and kappa) belonging to the superfamily of seven transmembrane domain G-protein coupled receptors (reviewed by Kieffer, 1999). At present, most clinically used opioids are agonists at the mu opioid receptor (Mason, 1999). Nonetheless, their unpleasant or adverse life-threatening effects, as well as their tendency to elicit tolerance and physical dependence, typically restrict opioid therapy for pain management. In contrast, selective agonists that activate the δ opioid receptor are suggested to produce antinociception with minimal side effects (Porreca et al., 1984; May et al., 1989; Sheldon et al., 1990; Szeto et al., 1999) and are thought to elicit nominal physical dependence compared to morphine-like drugs that activate the mu opioid receptor (Cowan et al., 1988).

While the validation for δ opioid receptor involvement in analgesia arises from numerous pharmacological studies using acute pain tests (Mattia et al., 1991, 1992; Porreca et al., 1984; Malmberg and Yaksh, 1992; Stewart and Hammond, 1993), more recent studies have explored the role of these receptors in animal models mimicking the characteristics of chronic or persistent pain. For example, intrathecal administration of selective δ opioid receptor agonists dose-dependently reversed thermal hyperalgesia induced by intraplantar injection of carrageenan in the rat (Stewart and Hammond, 1994) or of complete Freund's adjuvant (CFA) in mu opioid receptor knockout mice (Qiu et al., 2000). Furthermore, δ opioid receptor agonists were also shown to be anti-hyperalgesic in the CFA model when

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administered supraspinally in the rat (Hurley and Hammond, 2000; Fraser et al., 2000).

Autoradiographic binding (Quirion et al., 1983; Mansour et al., 1987; Sharif and Hughes, 1989), in situ hybridization (Mansour et al., 1994), and immunocytochemical studies (Arvidsson et al., 1995; Cahill et al., 2001) have identified δ opioid receptors in the rat spinal cord. Within the dorsal spinal cord, δ opioid receptors were found to be associated with primary afferent terminals as well as with the soma and dendrites of intrinsic neurons (Besse et al., 1992; Mansour et al., 1994; Minami et al., 1995; Cheng et al., 1995; Cahill et al., 2001). However, there is little information available concerning the regulation and/or trafficking of these receptors during persistent inflammatory conditions. Furthermore, studies that have investigated the fate of opioid receptors following induction of peripheral inflammation have reported either no change or a down-regulation of δ opioid receptors in the rat dorsal spinal cord and dorsal root ganglia (Ji et al., 1995; Maekawa et al., 1995). These results are difficult to reconcile with the reported increase in the antinociceptive effects of selective δ opioid receptor agonists in this model system (but see Besse et al., 1992).

In this context, the aim of the present study was to reassess the effects of CFA-induced peripheral inflammation on the expression and distribution of δ opioid receptors in the dorsal spinal cord of the rat using biochemical and neuroanatomical approaches. We used a model of CFA injection into the hindpaw of the rat that was previously shown to produce inflammation and hyperalgesia that are limited to the injected paw (Iadarola et al., 1988; Hylden et al., 1989). This experimental paradigm was chosen for its persistent nature and the fact that it is thought to be highly predictive of thermal hyperalgesia in humans (Montagne-Clavel and Oliveras, 1996). Moreover, the allodynia and hyperalgesia precipitated in this model resemble the components of many pain states encountered clinically, such as post-operative pain and arthritis.

2. Methods

2.1. Animals

Experiments were performed on adult male Sprague Dawley rats (220–250 g; Charles River, Quebec, Canada) housed in groups of two or three per cage. Rats were maintained on a 12/12 h light/dark cycle and were allowed free access to food and water. Experiments were carried out according to a protocol approved by AstraZeneca R&D Montreal and in accordance with policies and guidelines of the Canadian Council on Animal Care.

2.2. Induction of chronic inflammation

Chronic inflammation was induced by a subcutaneous injection of 100 μl CFA (Sigma, St Louis, MO) in the plantar surface of the right hindpaw under brief isoflurane anaesthesia. Control (sham) rats received a similar injection consisting of vehicle (50% mineral oil in saline). Rats were sacrificed 72 h following injection of CFA, corresponding to the peak inflammatory response.

Control rats used in the behavioral experiments were naïve rats that did not receive any injection into the hindpaw. Control rats for all other experiments (in situ hybridization, Western blotting and immunohistochemistry) were injected with the vehicle for CFA (shams).

2.3. Behavioral testing

Withdrawal latencies from noxious heat using the plantar test were assessed in both CFA-injected and naïve rats ($n = 5$–7 per group), as previously described by Fraser et al. (2000). Rats were placed in Plexiglas® boxes positioned on a glass surface. Animals were allowed to habituate for 15 min before testing. Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Comerio, Italy). The heat source was positioned under the plantar surface of the affected hind paw and activated. The digital timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest 0.1 s. The intensity of the light beam was adjusted to give baseline latencies of 10–12 s in naïve rats in order to test for thermal hyperalgesia. A cut off time of 20 s was imposed to prevent tissue damage. The effects of the δ opioid receptor agonist, D-[Ala^2, Glu^4]deltorphin (deltorphin II, Tocris, Ellisville, MO), were tested 72 h after the injection of CFA. Deltorphin II was administered via lumbar puncture under brief isoflurane anaesthesia. Control animals were tested in parallel. Paw withdrawal latencies are expressed as raw data or converted to percentage maximum possible effect (M.P.E.) according to the formula:

$$\%\text{M.P.E.} = (\text{post drug latency} - \text{baseline}) \div (\text{Cutoff latency} - \text{baseline}) \times 100$$

Data are presented as means ± standard error of mean (s.e.m.). Differences between groups were analyzed by two-way analysis of variance with dose and time as between subject and within subject factors, respectively. Post hoc analyses were performed with Dunnett's or Tukey's multiple comparison test where appropriate. ED_{50} values were determined by linear regression analysis of the dose–response curves. All graphs were generated and statistical analysis performed using GraphPad Prism software 3.01 (San Diego, CA, USA).

2.4. Riboprobe synthesis

A 917 bp fragment of the rat δ opioid receptor, corresponding to amino acids 78–995, was obtained by polymerase chain reaction (PCR) from a plasmid, pcDNAI-rδOR (Gaudriault et al., 1997), containing the full-length rat δ opioid receptor (1366 bp). The 917 bp rat δ opioid receptor fragment was subcloned into the pGEM®-T Easy Vector

(Promega, Madison, Wisconsin) and the sequence confirmed. The plasmid constructs were linearized using Nco1 and Sal1 restriction enzymes. The rat δ opioid receptor sense and antisense probes were transcribed in vitro using either T7 or SP6 RNA polymerases (Amersham Pharmacia Biotech, Montreal, Quebec), respectively, in the presence of ^{35}S-UTP and ^{35}S-CTP (Amersham Pharmacia Biotech). After transcription, the DNA template was digested using DNAse I (Amersham Pharmacia Biotech). Riboprobes were subsequently purified using ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech). The quality of the labeled probe was verified by polyacrylamide–urea gel electrophoresis.

2.5. In situ hybridization

δ Opioid receptor mRNA expression was measured in the dorsal spinal cord of both CFA and sham-injected rats ($n = 8$ per group) using in situ hybridization as previously described (Cahill et al., 2001). In brief, rats were killed by decapitation, and the spinal cords were quickly removed, snap-frozen in isopentane at −40°C for 20 s and stored at −80°C. Frozen tissue (lumbar spinal cord level 3–6) was transversely sectioned at 16 μm in a Microm HM 500M cryostat (Germany) and thaw-mounted onto Superfrost Plus slides (VWR, Montreal, Quebec). Tissues were allowed to warm to room temperature (RT) prior to fixation with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) for 10 min at RT. After several washes with sodium citrate buffer, sections were equilibrated with 0.1 M triethanolamine, treated with 0.25% acetic anhydride in triethanolamine, followed by further washing with sodium citrate buffer and dehydration through an ethanol series. Hybridization was performed with a ^{35}S-labeled δ opioid receptor antisense probe at 55°C for 18 h in humidified chambers. As a control, adjacent sections were processed in parallel with ^{35}S-labeled δ opioid receptor sense probe. Sections were rinsed with citrate buffer, treated with 20 μg/ml RNase IA (Amersham Pharmacia Biotech) in RNase buffer. Slides were dipped in Kodak NTB2 emulsion diluted to 1:1 with distilled water and exposed for 6–8 weeks at 4°C before being developed with Kodak D-19 developer. Slides were then counterstained with cresyl violet acetate (Sigma) and viewed on Leitz Aristoplan photomicroscope (Leica, Canada). Densitometric quantification of mRNA expression was performed on ipsilateral and contralateral dorsal horns of both CFA- and sham-injected animals using a Biocom computer-assisted image analysis system (Historag; Les Ulis, France). Analysis was performed by counting grains in 8–12 selected regions throughout laminae I–II and laminae III–V for at least three spinal cord sections per animal. The total number of grains in each of the selected regions was averaged to give a total grain count per laminar region (i.e. I–II or III–V) for each side per animal. The grain counting and region selection were performed blind to the treatment. Data are presented as means ± s.e.m. Differences between groups were analyzed by one-way analysis of variance (ANOVA) and post hoc analysis was performed with Tukey's multiple comparison test.

2.6. Western blotting

For determination of δ opioid receptor protein levels, CFA- and sham-injected rats ($n = 3$ per group) were killed by decapitation and their spinal cords were quickly removed by spinal ejection and placed on ice for removal of the dura mater. Lumbar spinal cord regions were isolated and hemisected into ipsilateral and contralateral segments prior to homogenization with a Polytron in buffer A consisting of 50 mM Trisma base, pH 7.0 and 4 mM ethylenediaminetetraacetic acid (EDTA) with protease inhibitors (Complete™ Protease Inhibitor Tablets, Roche Molecular Biochemicals, Laval, Quebec). Samples were centrifuged at 4°C for 10 min at 1000 *g* (Sorval RC5C Plus). The supernatant was collected and the pellet resuspended in buffer A and centrifuged again at 4°C for 10 min. The supernatant from the second spin was combined with that of the first for each sample and centrifuged at 4°C for 10 min at 153,000 *g* (Sorval Discovery 90). The pellets were then resuspended in buffer B consisting of 50 mM Trisma base, pH 7.0 and 0.2 mM EDTA with protease inhibitors by vortexing and brief sonication (2 s).

Following protein determination (Bradford, 1976), the tissue membranes were denatured using 6× Laemmli sample buffer (0.375 mM Trisma base, pH 6.8, 12% w/v sodium dodecyl sulfate (SDS), 30% v/v glycerol, 12% v/v 2-mercaptoethanol, 0.2% w/v bromophenol blue). Samples were subjected to SDS 10% polyacrylamide gel electrophoresis (PAGE) and the proteins were electroblotted onto nitrocellulose membranes. Uniform protein loading between wells was confirmed by Coomassie blue staining of the gels. Molecular mass markers (BioRad (Richmond, CA), New England Biolabs (Mississauga, Ontario)) were used to calibrate the gels. Nitrocellulose membranes were incubated with 1% bovine serum albumin (BSA) and 1% chicken albumin in 25 mM Trisma buffered saline (TBS) containing 0.1% Tween 20 at 4°C, overnight to block non-specific binding sites. Nitrocellulose membranes were then incubated for 48 h at 4°C with δ opioid receptor antibody (Chemicon, Temecula, CA) at a concentration of 0.2 μg/ml in TBS containing 0.1% Tween-20, 1% BSA and 1% chicken albumin. Bound antibodies and the biotinylated molecular weight marker were visualized using an HRP-conjugated goat anti-rabbit secondary antibody (Amersham Pharmacia Biotech) diluted 1:4000 and an HRP-conjugated anti-biotin diluted 1:10,000 (New England Biolabs), respectively, in TBS with 0.1% Tween-20 and 5% milk powder followed by chemiluminescent reagents (Perkin Elmer, Boston, MA). Specificity of the δ opioid receptor antibody was previously confirmed by pre-adsorption of the antibody with the appropriate peptide (Cahill et al., 2001). For quantification, blots were digitized with an Agfa Duoscan T1200

scanner and image processing was performed using Photoshop version 4.0/5.0 imaging software (Adobe Systems Inc., San Jose, CA) on an IBM-compatible computer. Density measurements of immunoreactive bands were performed using Scion Image software (NIH).

2.7. Immunohistochemistry for electron microscopy

For electron microscopic analysis of immunoreactive δ opioid receptor distribution, CFA-injected ($n = 3$) and sham-injected rats ($n = 3$) were anesthetized with sodium pentobarbitol (70 mg/kg, i.p.) and perfused through the aortic arch with 50 ml of heparin (75 U/ml heparin in 0.9% saline) followed by a 50 ml mixture of 3.75% acrolein and 2% PFA in 0.1 M PB, pH 7.4, and then by 400 ml of 2% PFA in the same buffer. Lumbar spinal cords were removed and post-fixed in 2% PFA in 0.1 M PB for 30 min at 4°C. Transverse sections (40 μm) were cut using a vibratome (Series 1000) and processed for δ opioid receptor immunogold labeling according to protocols previously described (Cahill et al., 2001). Briefly, sections were incubated for 30 min with 1% sodium borohydride to neutralize free aldehyde groups followed by copious rinses with PB. Sections were then incubated for 30 min in a cryoprotectant solution consisting of 25% sucrose and 3% glycerol in PB prior to snap freezing with isopentane (−70°C) followed by liquid nitrogen. They were then rapidly thawed in PB, rinsed with TBS, and pre-incubated for 1 h at room temperature in blocking solution consisting of 3% normal goat serum (NGS) in TBS. They were then incubated overnight at 4°C in δ opioid receptor antiserum diluted to 1 μg/ml in TBS containing 0.5% NGS. After rinsing, sections were incubated for 2 h at room temperature with colloidal gold (1 nm)-conjugated goat anti-rabbit IgG (1:50, Amersham Pharmacia Biotech, Montreal, Quebec) diluted in 0.01 M phosphate buffered saline (PBS) containing 2% gelatin and 8% BSA. Sections were then fixed for 10 min with 2% glutaraldehyde in PBS and rinsed with 0.2 M citrate buffer, pH 7.4. Immunogold particles were amplified by silver intensification for 7 min using an IntenSEM kit (Amersham Pharmacia Biotech). Sections were post-fixed by incubation for 40 min at room temperature with 2% osmium tetroxide in 0.1 M PB, rinsed and dehydrated with increasing concentrations of ethanol. Sections were embedded in Epon 812 plastic followed by placement between plastic coverslips at 60°C for 24 h. Ultrathin sections (80 nm) were collected and counter-stained with lead citrate and uranyl acetate for examination with a JEOL 100 CX electron microscope. Negatives were scanned using an AGFA Duoscan T1200 and images were processed using Photoshop version 5.5 (Adobe Systems, Inc.) on an IBM-compatible computer.

For quantification, 50 immunopositive dendritic profiles were counted from three independent experiments. The distribution of immunogold particles within each dendrite was then analyzed using computer-assisted morphometry (Biocom, Les Ulis, France), after verifying that the average size of dendrite sampled within each condition was not significantly different (sham: $3.39 \pm 0.18\ \mu m^2$, CFA ipsilateral: $3.91 \pm 0.44\ \mu m^2$, CFA contralateral: $3.11 \pm 0.31\ \mu m^2$). First, the subcellular distribution of immunoreactive δ opioid receptor gold particles associated with dendritic profiles was calculated per surface area. Second, gold particles were classified according to their association with either the plasma membrane or the cytoplasm. A gold particle was considered to be associated with the plasma membrane when it either contacted or overlaid it. Particles not in contact with the plasma membrane, even if in close proximity, were classified as intracellular. Third, the segmental distance of each gold particle from the plasma membrane was also calculated by measuring the shortest distance between the gold particle and the plasma membrane. Statistical analysis comparing the pattern of labeling between sham and CFA-injected animals was performed using a *t*-test on the total of gold particles per surface area as well as the segmental distance. All data are expressed as means ± s.e.m. and analysis was performed using Prism 3.01 (Graph Pad Software Inc.).

3. Results

3.1. Behavioral observations

There were no obvious signs of changes in weight, grooming or social behavior following CFA administration compared to either sham or naïve groups. Animals injected with CFA showed evident signs of inflammation at 72 h, whereas sham-injected rats exhibited no obvious indication of inflammation. The occurrence of inflammation was assessed by localized erythema and an increase in the swelling of the paw. As illustrated in Fig. 1A, there was a significant decrease in thermal threshold latencies in the ipsilateral hindpaw of CFA-injected rats compared to both the contralateral hindpaw and the right hindpaw of control rats ($F(2) = 4.563$, $P < 0.01$).

3.2. Antinociceptive effects of δOR agonist

Intrathecal administration of deltorphin II produced a dose-dependent increase in thermal withdrawal latencies in both control ($F(7) = 5.501$, $P < 0.01$) and CFA-injected rats ($F(14) = 8.818$, $P < 0.0001$). Peak antinociceptive effect consistently occurred between 10 and 20 min after injection for all doses and was essentially absent by 40 min with the exception of the highest dose tested (30 μg, 38.3 nmol). In addition, the dose–response curve for deltorphin II was significantly shifted to the left in response to stimulation of the ipsilateral hind paw of CFA-injected rats compared with either the contralateral hind paw or the hind paws of control rats ($F(21) = 5.411$, $P < 0.0001$, Fig. 1B). ED_{50} values obtained from dose–response curves generated by linear regression analysis were 0.4 μg for CFA-injected rats (ipsilateral) and 24 μg for control rats.

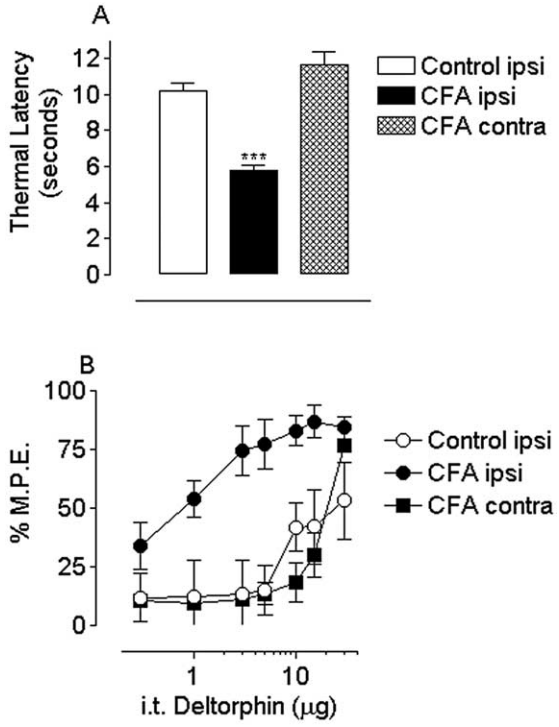

Fig. 1. (A) Thermal threshold withdrawal latencies using the plantar test were assessed in both ipsilateral (ipsi) and contralateral (contra) hindpaws of CFA-injected and naïve rats ($n = 5$–7 per group). Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus. (B) Dose-dependent antinociceptive effects of intrathecal administration of D-[Ala^2, Glu^4]deltorphin II were assessed at 20 min post-injection for both ipsilateral and contralateral hindpaws of CFA-injected as well as naïve rats ($n = 5$–7 per group). Data represent means ± s.e.m. Statistical analysis by one-way ANOVA indicate significant difference from ipsilateral control and equivalent contralateral hindpaw (***$P < 0.001$).

3.3. Effect of chronic inflammation on δOR mRNA and protein levels

As previously reported (Maekawa et al., 1995; Wang and Wessendorf, 2001; Cahill et al., 2001), δ opioid receptor expressing neurons were detected by in situ hybridization throughout the dorsal horn of the rat spinal cord (Fig. 2). These cells were few in number in the superficial dorsal horn (laminae I–II) and were more numerous and more intensely labeled in laminae III–V (Fig. 2A). Comparable patterns of δ opioid receptor mRNA expression were observed in CFA-injected (Fig. 2B) and sham-injected rats. However, densitometric quantification of silver grains in the dorsal spinal cord of CFA-injected rats revealed a significant increase in laminae III–V of δ opioid receptor mRNA in the ipsilateral compared to the equivalent contralateral side (Fig. 3B, also compare ipsilateral with contralateral side in Fig. 2B). In contrast, no significant difference in δ opioid receptor mRNA was observed between ipsilateral and contralateral regions for either superficial or deeper dorsal horn regions of the spinal cord of sham-injected rats (Fig. 3A). Additionally, comparison of δ opioid receptor mRNA levels in the dorsal horn of CFA versus sham-injected rats revealed a significant increase in δ opioid receptor mRNA expression within either the ipsilateral or contralateral dorsal horn regions of CFA-injected compared to sham-injected rats (Fig. 3C, D). Hence, there was a bilateral increase in δ opioid receptor mRNA in CFA-injected compared to sham-injected rats.

To investigate whether the increase in δ opioid receptor mRNA correlated with corresponding changes in protein levels, total δ opioid receptor proteins were measured by Western blotting in membranes prepared from the lumbar spinal cord of sham- and CFA-injected rats. As previously demonstrated in the spinal cord of adult naïve rats (Cahill et al., 2001), two specific immunoreactive bands at estimated molecular weights of 68 and 125 kDa were observed in CFA- and sham-injected rats (Fig. 4A). The lower molecular species most likely represents the monomeric form of the receptor, whereas the higher weight species presumably represents the receptor in different glycosylation states or dimerized receptors. A significant increase in the density of the higher molecular weight species was detected in

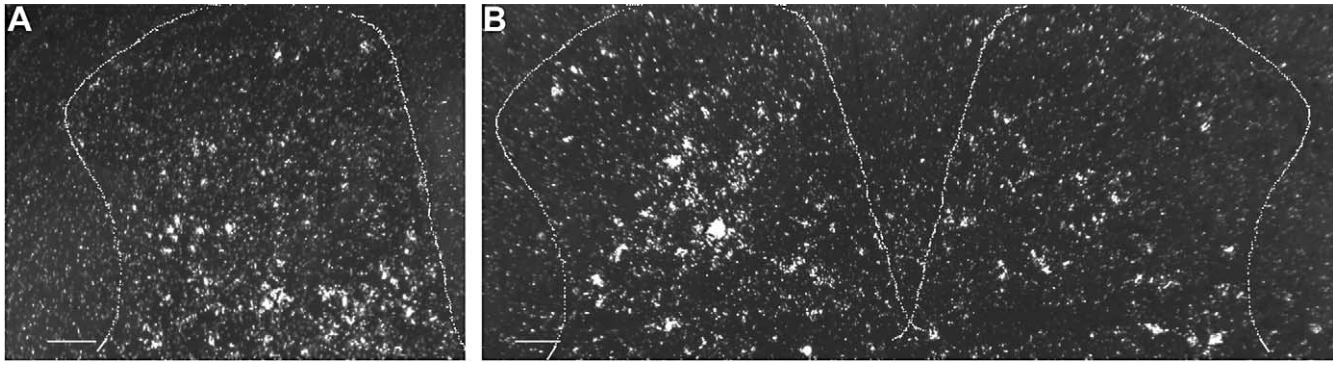

Fig. 2. Low magnification dark-field photomicrographs of the lumbar spinal cord (L5) illustrate the pattern of mRNA distribution for δ opioid receptor following CFA injection ((B), left side is ipsilateral to the site of injury) or sham ((A), ipsilateral to injection of vehicle) injection by in situ hybridization. Note the selective increase in δ opioid mRNA in the ipsilateral (left) compared to contralateral (right) dorsal spinal cord following CFA injection in panel (B). Transverse sections were hybridized with ^{35}S-labeled riboprobes directed to the δ opioid receptor. Dotted lines outline the dorsal horn grey matter of the spinal cord. Scale bar = 0.15 mm.

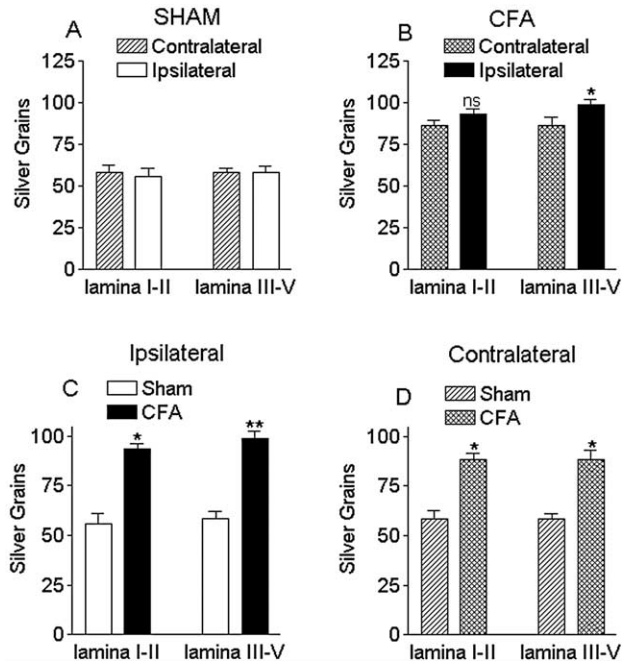

Fig. 3. Quantification of δ opioid receptor hybridization signal in laminae I–II and laminae III–V for ipsilateral and contralateral dorsal spinal cord of CFA- and sham-injected rats. (B) δ opioid receptor mRNA was significantly increased in laminae III–V of the ispilateral dorsal spinal cord of CFA-injected rats compared to the equivalent contralateral region. (A) No difference is evident between ipsilateral and contralateral dorsal horn regions of sham-injected rats. (C and D) The lower panels demonstrate that the density of silver grains representing δ opioid receptor hybridization signal was significantly greater throughout the dorsal horn of CFA-injected when compared to sham-injected rats. Densitometric quantification of silver grains was performed on at least 3–4 transverse spinal cord sections from $n = 8$ per condition. Data represent means ± s.e.m. Post hoc analysis for silver grain counts (Tukey's) indicates a significant difference between ipsilateral and equivalent contralateral regions of CFA-injected rats as well as comparing ipsilateral or contralateral regions of CFA-injected and sham-injected groups ($*P < 0.05$, $**P < 0.01$).

membranes prepared from the ipsilateral dorsal spinal cord of CFA-injected rats compared to membranes prepared from either the contralateral side or sham rats (Fig. 4B, $F(4) = 16.10$, $P < 0.001$). By contrast, no change was detected in the intensity of the lower immunoreactive band between any of the groups.

3.4. Electron microscopic localization of δ opioid receptor in CFA animals

The subcellular distribution of δ opioid receptor immunoreactivity was assessed by electron microscopic immunogold staining in laminae III–V of the dorsal horn of the spinal cord of sham- and CFA-injected rats. In both sham- and CFA-injected rats (ipsilateral as well as contralateral sides), the vast majority of immunolabeled δ opioid receptors was detected in association with perikarya and dendrites of small intrinsic neurons (Fig. 5). Most immunoreactive δ opioid receptors were intracellular rather than plasma membrane-bound as shown in the figure. Within the soma, gold particles were detected over rough and smooth endoplasmic reticulum, ribosomes, Golgi apparatus, and an array of small pleomorphic vesicles. Within dendrites, gold particles were primarily associated with microtubules and, to a lesser extent, with smooth endoplasmic reticulum and membranes of small and large clear vesicles as previously reported (Fig. 5, Cahill et al., 2001).

Quantitative analysis of the ultrastructural distribution of immunoreactive δ opioid receptors in dendrites revealed that the number of immunogold particles was significantly higher in the ipsilateral dorsal spinal cord of CFA-injected rats than in the equivalent contralateral region or in sham-

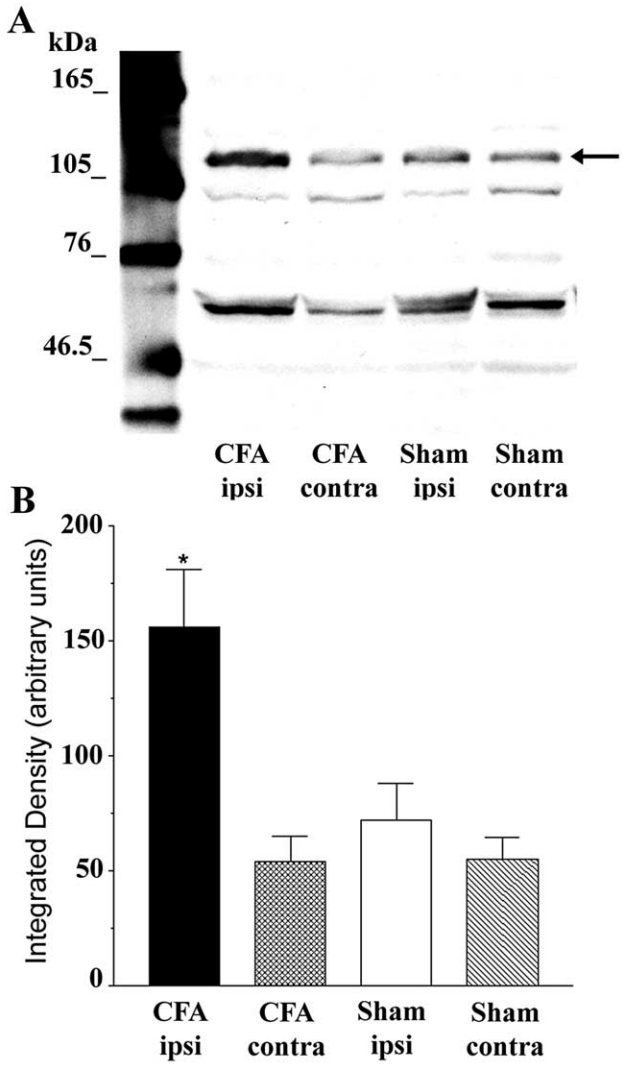

Fig. 4. (A) Identification of δ opioid receptor proteins by Western Blotting. Membranes from spinal cord tissue were isolated and proteins resolved by 10% SDS-PAGE followed by electroblotting onto nitrocellulose membranes. Immunoblot analysis reveals two immunoreactive bands and a significant increase in the higher molecular species ipsilateral to the site of injection of CFA-injected compared to sham-injected rats. (B) Quantification was performed by digitizing the images and measuring the integrated density of the immunoreactive bands using Scion Image (NIH). Uniform protein loading of lanes within the gel was confirmed by Coomassie blue staining of the gel after protein transfer onto nitrocellulose membranes.

injected rats (Fig. 6A, $P < 0.05$). Within all labeled dendrites, there was a significantly higher ratio of plasma membrane-associated over total number of immunogold particles in ipsilateral CFA-injected as compared to the contralateral side of injury as well as the ipsilateral spinal cord of sham-injected rats (Fig. 6B first column set, distance

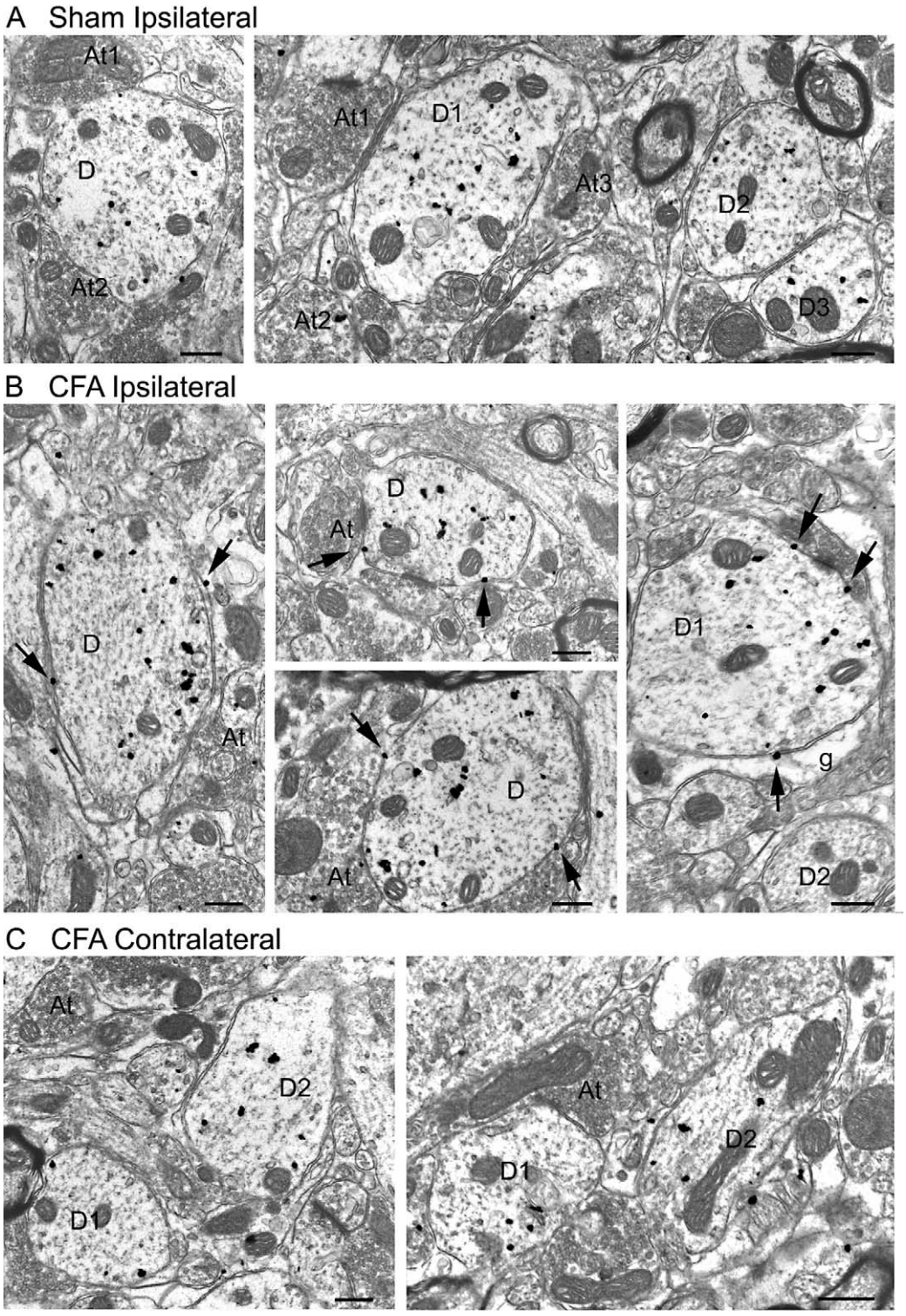

Fig. 5. Electron micrographs showing the distribution of immunoreactivity of immunogold-labeled δ opioid receptors in dendritic profiles of (A) sham-injected ipsilateral dorsal spinal cords, as well as (B) ipsilateral and (C) contralateral dorsal spinal cords of CFA-injected rats. Silver-intensified immunogold labeling of δ opioid receptor demonstrates predominantly intracellular localization. Sampling was obtained from lamina III–V for each condition. Arrows indicate gold particles associated with the plasma membrane. D, dendrite; At, axon terminal; g, glia. Scale bar = 0.2 μm.

'0'). Furthermore, the mean distance separating intracellular immunogold particles from the plasma membrane was significantly shorter in the ipsilateral (269 ± 12 nm) than in the contralateral side (354 ± 10 nm) or the ipsilateral side of sham-injected rats (425 ± 9.4 nm) indicating that CFA treatment resulted in a mobilization of intracellular δ opioid receptor towards the plasmalemmal region (Fig. 6B).

4. Discussion

This study demonstrates that a selective δ opioid receptor agonist is a more effective antinociceptive agent following tissue injury associated with chronic inflammation and hyperalgesia than in control animals. This enhanced antinociceptive effect of the δ opioid receptor agonist is correlated with an up-regulation of δ opioid receptor protein and recruitment of spinal δ opioid receptors from intracellular stores to the plasma membrane.

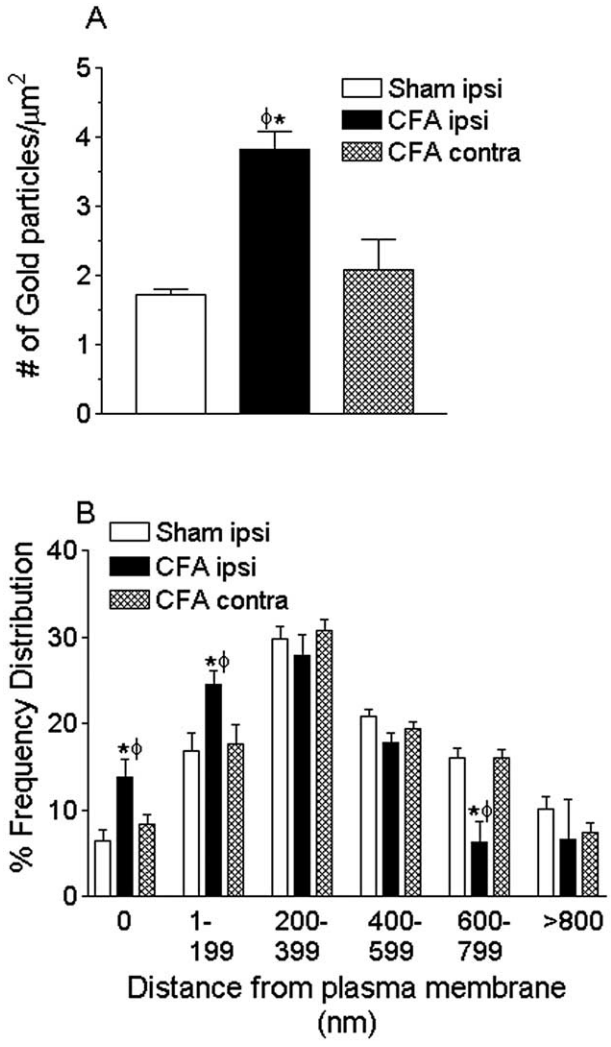

Fig. 6. Quantitative analysis of the ultrastructural distribution of immunoreactive δ opioid receptor. (A) Distribution of immunoreactive labeling over dendritic profiles ipsilateral and contralateral to the site of CFA injection as well as sham-injected rats. (B) Percentage of grains located relative to the distance (nm) from the plasma membrane where '0' indicates association with the membrane. Percentages are based on counting 50 immunopositive dendritic profiles per animal for three different CFA- and sham-injected rats. Data represent means ± s.e.m. Statistical analysis on intracellular segmental localization was performed using a two-way ANOVA ($P < 0.05$). Post hoc analysis (Tukey's) indicates significant difference between ipsilateral and equivalent contralateral regions ($*P < 0.05$) or sham values (ϕ $P < 0.05$).

The present behavioral findings complement those of previous reports suggesting that δ opioid receptor agonists reverse peripheral hyperalgesia and exhibit enhanced potency following intrathecal administration (Hylden et al., 1991; Qiu et al., 2000) or administration into supraspinal structures (Fraser et al., 2000; Hurley and Hammond, 2000) in rodents with unilateral hindpaw inflammation. Electrophysiological studies have also demonstrated that δ opioid receptor agonists suppress the hypersensitivity of the flexor motor reflex in CFA-injected rats, but have no effect in control animals (Cao et al., 2001). In the current study, we demonstrate that the antinociceptive potency of deltorphin II is shifted to the left by a factor greater than 50 in CFA-injected compared to naïve rats. While no previous study has evaluated changes in spinal δ opioid receptor potency following chronic inflammation, an increase in deltorphin II potency (sixfold) was demonstrated in the CFA model following microinjection of the drug into the rostral ventromedial medulla of the rat (Hurley and Hammond, 2000).

To better understand the mechanism of the enhanced antinociceptive effects of spinal δ opioid receptor agonists in chronic inflammation, we examined the changes in δ opioid receptor expression and localization within the rat spinal cord following peripheral inflammation. The distribution of δ opioid receptor mRNA in the dorsal horn of the rat spinal cord as revealed by in situ hybridization was consistent with that previously reported (Mansour et al., 1994; Maekawa et al., 1995; Wang and Wessendorf, 2001; Cahill et al., 2001). Following CFA-induced inflammation, δ opioid receptor mRNA expression was increased throughout the dorsal horn of the spinal cord. Furthermore, this increase, although more pronounced on the side of injury, was clearly bilateral as revealed by comparisons of CFA-injected to sham-injected rats. As a result, differences between ipsilateral and contralateral sides in the CFA-injected animals were only modest which might explain why an earlier study reported no change in δ opioid receptor mRNA expression in CFA-induced arthritis by comparing ipsilateral to contralateral levels (Maekawa et al., 1995). Comparison between ipsilateral and contralateral sides has previously been found to obscure the presence and/or magnitude of any change because neuroanatomical alterations can also occur contralateral to an unilateral injury (Donaldson, 1999; Koltzenburg et al., 1999). Although an up-regulation of δ opioid receptor mRNA was evident throughout the dorsal horn of CFA-injected rats, this increase in mRNA levels was presumably not translated into protein on the contralateral side as neither Western blotting experiments nor electron microscopy revealed an

increase in receptor protein in the CFA-injected contralateral dorsal spinal cord compared to sham-injected rats. Moreover, the functional consequences of this contralateral up-regulation of mRNA are unclear as no difference in the antinociceptive effects of intrathecal deltorphin II in the contralateral hindpaw of CFA-injected rats compared to naïve rats was evident.

It is of interest that the greatest increase in δ opioid receptor mRNA expression occurred within the deeper laminae of the ipsilateral dorsal horn in CFA-injected rats. Cells within laminae III–V include post-synaptic dorsal column and spinothalamic projection neurons as well as interneurons (Willis and Coggeshall, 1991). The large cells in the deeper dorsal horn laminae have large numbers of dendrites that project dorsally to penetrate the substantia gelatinosa where nociceptive small diameter primary afferents terminate and can thereby influence nociceptive synaptic activity. The deeper dorsal horn neurons also receive inputs from myelinated primary afferent fibres. Although most of these afferents transmit non-nociceptive information, a significant contingent comprises thinly myelinated A-delta nociceptive fibres that terminate in laminae IV and V. Therefore, the observed changes in the δ opioid receptor expression are ideally poised to affect spinal processing of primary nociceptive inputs.

In agreement with a previous study, Western blotting experiments on membranes prepared from rat spinal cord tissue demonstrated the presence of two specific δ opioid receptor immunoreactive bands at approximate molecular weights of 68 and 125 kDa (Cahill et al., 2001). These bands were postulated to correspond to the monomeric receptor (lower molecular weight band) and to either a heavily glycosylated or dimerized form of the δ opioid receptor, as the higher molecular weight band was approximately twice the molecular weight of the predicted molecular mass of the cloned receptor (Cahill et al., 2001). Quantification of the levels of protein in CFA and sham-injected rats demonstrated that there was a significant increase in the higher molecular weight species ipsilateral to the site of inflammation compared to the equivalent contralateral region or to sham-injected rats. Furthermore, an increase in δ opioid receptor immunoreactivity was observed in neurons of the ipsilateral dorsal horn of CFA-injected rats as compared to both the contralateral side and to the dorsal horn of sham-injected rats as demonstrated by quantitative electron microscopic immunohistochemistry. These results conform to the results of δ opioid receptor binding studies that demonstrated an increase in δ binding sites in the superficial dorsal horn of CFA-injected rats as compared to controls (Besse et al., 1992). By contrast, chronic inflammation was reported to decrease δ opioid receptor expression in neurons within the dorsal root ganglia (Ji et al., 1995), suggesting that chronic inflammation may differentially regulate pre- and post-synaptic spinal δ opioid receptors.

As previously reported by us (Cahill et al., 2001) and others (Cheng et al., 1995) for the naïve adult rat spinal cord, the bulk of δ opioid receptor immunoreactivity in the dorsal horn of the spinal cord of both CFA- and sham-injected rats was associated with intrinsic laminae III–V neurons. In control animals, only a small proportion of immunolabeled δ opioid receptors was observed over neuronal plasma membranes suggesting that, under steady-state conditions, the majority of δ opioid receptors are reserve receptors awaiting targeting to the plasma membrane in response to physiological changes. By contrast, following CFA-induced inflammation, there was a significantly higher ratio of plasma membrane receptors to intracellular receptors. Additionally, receptor proteins were redistributed towards the plasma membrane as evidenced by a decrease in the mean segmental distance separating intracellular δ opioid receptors from the plasma membrane. Thus, CFA-induced inflammation produced an outward movement of intracellular receptors from the core of the cell to the plasma membrane. Factored in together with the increase in the expression of immunoreactive δ opioid receptor protein, this outward movement translates into an eightfold augmentation in plasma membrane δ opioid receptor number. It is tempting to speculate that the δ opioid receptors newly recruited to the plasma membrane are the functional receptors that account for the enhanced antinociceptive effects of δ opioid receptor agonists in chronic inflammatory pain models.

In conclusion, we have demonstrated that chronic inflammatory pain induced by intraplantar administration of CFA increases δ opioid receptor mRNA expression and protein levels. Ultrastructural immunohistochemical analysis confirmed that chronic inflammation induced an up-regulation of δ opioid receptors and demonstrated that this up-regulation was accompanied by an increased recruitment of intracellular receptors to the plasma membrane. We suggest that the enhanced expression and targeting of δ opioid receptor to plasma membranes, evident in this chronic inflammatory pain model, accounts for the enhanced antinociceptive potency of selective δ opioid receptor agonists and justifies the exploration of novel d-acting compounds in treating clinical pain arising from an inflammatory insult.

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