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# Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain

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**Abstract**

The analgesic effects of local administration of opioid agonists into peripheral tissues in alleviating pain have been well documented in both clinical and preclinical studies, although few studies have examined their effects in neuropathic pain. In this study, we investigated the anti-allodynic effects of peripherally acting delta opioid receptor (DOR) agonists in a rat model of neuropathic pain. Peripheral nerve injury (PNI) produced a time-dependent decrease in mechanical withdrawal thresholds that was attenuated by local administration into the hind paw of either morphine or the DOR agonist deltorphin II. Using Western blotting techniques, no change in DOR protein expression was detected in DRG ipsilateral to the site of injury compared to contralateral. However, an up-regulation of DOR protein was found in neuropathic DRG compared to sham, suggesting that there may be a bilateral increase in the expression of DOR following PNI. Results obtained from immunohistochemical studies confirmed up-regulation in small and large DRG neurons in neuropathic compared to sham animals. Additionally, there was an increase in DOR protein within the ipsilateral sciatic nerve of neuropathic animals compared to sham and contralateral neuropathic conditions indicating the occurrence of receptor trafficking to the site of injury. Taken together, our findings suggest that functional peripheral DORs are present in sensory neurons following PNI and validate the development of selective DOR agonists for alleviating neuropathic pain.
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*Keywords:* Chronic constriction injury; Deltorphin II; DPDPE; Morphine; Dorsal root ganglia; Pain

## 1. Introduction

In the peripheral nervous system, opioid receptors (OR) are synthesized in the cell bodies of primary afferent neurons located in dorsal root ganglia (DRG) and targeted to terminals present in the superficial dorsal spinal cord and peripheral sensory nerve endings (Fields et al., 1980; Hassan et al., 1993; Coggeshall et al., 1997). While most studies have evaluated the effects of OR agonists following systemic administration, the analgesic effects of peripherally restricted opioid agonists has been more limited (Stein et al., 2003; DeHaven-Hudkins and Dolle, 2004; Riviere, 2004). For example, local peripheral administration of mu-, delta-, and kappa-OR agonists have been shown to elicit dose-dependent, stereo-selective anti-hyperalgesic effects in models of inflammatory pain (Stein et al., 1989). Furthermore, mu OR (MOR) agonists have also been shown to reverse bradykinin- and prostaglandin E2-induced hyperalgesia following their injection into the experimental paw (Levine and Taiwo, 1989; Taiwo and Levine, 1991). Clinical studies examining peripheral opioid analgesia have shown that intra-articular administration of morphine exerts potent and long-lasting postoperative analgesia following knee surgery (Stein et al., 1991; Stein et al., 1999). In addition, locally administered morphine has also been shown to elicit effective

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analgesic effects in various clinical pain syndromes including burn pain (Moiniche et al., 1993), visceral pain (Duckett et al., 1997), inflammatory pain (Dionne et al., 2001), osteoarthritis (Likar et al., 1997), rheumatoid arthritis (Stein et al., 1999), and tooth pain (Likar et al., 1998).

Relatively few studies have examined the effects of locally administered opioid agonists in models of neuropathic pain and most of those studies restricted their observations to the effects of MOR agonists on pain behaviour and neuropathy-induced changes in MOR expression. Accordingly, morphine, DAMGO, endomorphin-1 and endomorphin-2, and the peripherally selective kappa-OR (KOR) agonist, asimadoline, have been shown to exert anti-allodynic and anti-hyperalgesic effects in animal models of neuropathic pain (Walker et al., 1999; Pertovaara and Wei, 2001; Truong et al., 2003; Obara et al., 2004). To our knowledge, no studies have examined the effects of locally administered delta-OR (DOR) agonists on nerve injury-induced pain behaviour despite evidence that DOR agonists produce analgesia with minimal side effects as compared to their MOR agonist counterparts which produce undesirable side effects such as constipation, physical dependence, and respiratory depression (Porreca et al., 1984; May et al., 1989; Cheng et al., 1993). Although MOR-activating opioids such as morphine remain the mainstay of therapeutic treatment for this condition, their use is hampered by dose-limiting side effects. Investigating novel therapeutic agents and routes of drug delivery that would mitigate the side effects associated with the activation of MOR and of central ORs might lead to more effective therapies for the treatment of neuropathic pain.

In the present study, we demonstrate that local peripheral administration of a selective DOR agonist is effective in reversing mechanical allodynia in a model of neuropathic pain and that this effect is correlated with an increased expression of DOR protein in the primary afferent cell body in addition to providing evidence that DOR protein is targeted to the site of injury.

## 2. Methods

### 2.1. Animals

Adult male Sprague–Dawley rats weighing 200–350 g (Charles River, Québec, Canada) were housed in pairs in cages with beta chip bedding and free access to standard rodent chow and water. Animals were maintained under a standard 12/12-h light/dark cycle (lights on at 07:00 h), with ambient temperature set at 20–22 °C. Testing was performed during the light cycle. Animal protocols were approved by the Queen's University Animal Care Committee and were in accordance with the guidelines set by the Canadian Council on Animal Care and the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

### 2.2. Surgery

Peripheral nerve injury (PNI) was induced by chronic constriction of the sciatic nerve according to an adaptation of the Mosconi and Kruger (1996) model. Briefly, animals were anesthetized with isoflurane (2.5%, by inhalation). A skin incision was made at the left hind limb, followed by blunt dissection of the muscle to expose the sciatic nerve which was then isolated from the surrounding tissue. A 2 mm-long fixed-diameter Polyethylene (PE 90) tube was placed around the nerve at mid-thigh level proximal to the sciatic nerve's trifurcation point. Care was taken to ensure that the cuff enclosed the entire nerve and that the perineural blood flow was not disrupted. Sham animals underwent the same surgical treatment but the sciatic nerve was not manipulated. Animals were allowed to recover for a minimum of 4 days prior to behavioural testing. All animals received a single dose of acetaminophen pre-operatively and Q24 h post-operatively (60 mg/kg), lactated ringers (5 ml, subcutaneously (s.c.)) Tribrissen 24% (0.013 ml/100 g, s.c.), and topical Gentocin at the site of the incision.

The Mosconi and Kruger (1996) model, used in the present study, is an adaptation of, and induces behavioural characteristics such as mechanical allodynia, analogous to, the model initially described by Bennett and Xie (1988). Instead of constricting the sciatic nerve using four loose chromic gut ligatures as described by Bennett and Xie (1988), a loose polyethylene cuff of uniform length diameter is placed around the sciatic nerve. The latter intervention is easily performed, ensures lesser operator variability, since the tightness with which chromic gut ligatures are tied can vary with the experimenter, and produces a standardized injury. Hence the cuff model ensures decreased variation in the behavioural manifestations of pain, allowing for comparisons with findings from other published reports (Mosconi and Kruger, 1996; Fisher et al., 1998; Cahill et al., 2003; Coull et al., 2003).

### 2.3. Behavioural testing

#### 2.3.1. Behavioural habituation

Animals were handled by the experimenter and allowed to acclimate to the pain testing paradigms for 10 min for 5–7 days prior to testing. The same experimenter handled the animals on all occasions. Animals were also habituated to the testing apparatus for 10–20 min prior to experimentation on each test day.

#### 2.3.2. Mechanical threshold testing

To assess the effects of locally administered DOR and MOR agonists, mechanical withdrawal thresholds were assessed by examining response patterns to non-noxious tactile stimulation with von Frey filaments (Stoelting, Chicago, IL). The latter are monofilaments with differing force applied to the mid-plantar surface of the hind paws according to the up-down method described by Chaplan et al. (1994). Briefly, rats were placed in single compartments on a metal mesh platform. Testing was initiated when exploratory behaviour decreased and monofilaments of varying force were applied in ascending or descending intensity to determine the filament closest to the threshold of response. A lower-intensity stimulus was applied after a withdrawal

response and a higher-intensity stimulus was applied after a no withdrawal response. The minimum stimulus intensity was 0.25 g, and the maximum was 15 g. A value for 50% response threshold (g) was calculated based on the response pattern to the filaments and force of the final filament. The resulting pattern of positive and negative responses was tabulated using an “X” to denote a withdrawal and an “0” to represent no withdrawal. The 50% response threshold was interpolated using the formula:

$$50\%\ \text{g threshold} = (10^{[\chi_f + k\delta]})/10,000$$

where $\chi_f$ is the value (in log units) of the final von Frey filament used; $k$ is the tabular value (Chaplan et al., 1994) for pattern of positive/negative responses; and $\delta$ is the mean difference (in log units) between stimuli (here 0.224).

#### 2.3.3. Thermal nociceptive testing

Rats were placed in a Hargreaves Plantar Box (IITC Life Science, Woodland Hills, CA) in individual compartments and allowed to habituate for 15 min prior to baseline testing. A radiant heat source was applied to the plantar surface of the left and right hind paws, and the paw withdrawal latency was recorded using a digital timer connected to the heat source (accurate to the nearest 0.1 s). The light intensity was adjusted to yield a 6–8 s withdrawal latency in naïve rats. A cut-off time of three times baseline was imposed in order to minimize tissue damage.

#### 2.3.4. Formalin

The formalin test was employed as a model of pain involving tissue injury and inflammation whereby an intraplantar injection of formalin (2.5%, 50 μl injection volume) into one the rat’s hindpaws produces a characteristic biphasic nociceptive response. Formalin-induced nocifensive behaviours were assessed using a weighted score as previously described (Coderre et al., 1993). Briefly, nociceptive behaviour was assessed as: 1, no favouring; 2, favouring; 3, complete elevation of the hind paw from the floor; or 4, licking and/or vigorous flinching of the injected hind paw. Behaviour was evaluated in 5-min intervals and the severity of the response was determined by the following formula: $(\{0 \times \text{the time spent in category \#1}\} + \{1 \times \text{the time spent in category \#2}\} + \{2 \times \text{the time spent in category \#3}\} + \{3 \times \text{the time spent in category \#4}\})/300\ \text{s}$.

### 2.4. Drug testing

[D-Ala$^2$]deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-$NH_2$), DPDPE ([D-Pen$^2$, D-Pen$^5$]enkephalin), and naltrindole hydrochloride were purchased from Sigma (Saint Louis, MO). Morphine was obtained from Sabex (Boucherville, QC, Canada). Drugs were dissolved in saline and injected subcutaneously into the dorsum of the hind paw in a 50 μl volume using a 27-gauge needle. Drug injections were performed under brief halothane anesthesia, except in the formalin test, where no anesthetic was used to prevent masking of the initial pain response. Formalin was injected into the plantar surface of the hind paw and deltorphin II was injected 5 min prior to formalin. All behavioural testing was performed by the experimenter blind to drug treatment.

### 2.5. Western blotting

To investigate the effects of PNI on DOR protein levels in the DRG, animals were briefly anesthetized with halothane and decapitated on day 14 following neuropathic surgery. Dorsal root ganglia from lumbar segments L4–L6 and sciatic nerve at the site of injury were collected from neuropathic and sham-operated animals, both ipsilateral and contralateral to the site of nerve injury. Tissue was homogenized using a polytron homogenizer in buffer solution A (50 mM Trisma base, 4 mM EDTA, phenylmethyl sulfonyl fluoride (PMSF), protease inhibitors (Complete™ Protease inhibitor tablets, Roche Molecular Biochemicals, Laval, Quebec, Canada), pH 7.0). Samples were centrifuged at 1000 rpm (1000*g*), 4 °C for 10 min. The supernatant was collected and centrifuged at 40,000 rpm (25,000 g), 4 °C for 30 min. The pellets were resuspended in buffer solution B (50 mM Trisma base, 0.2 mM EDTA, PMSF, and protease inhibitor (Complete™ Protease inhibitor tablets, Roche Molecular Biochemicals, Laval, Quebec, Canada), pH 7.0) and sonicated for 2 s. Protein determination was conducted as described by Bradford (1976). Tissue membranes were denatured using 6× Laemmli sample buffer [0.375 mM Trisma base, 12% w/v sodium dodecyl sulfate (SDS), 30% v/v glycerol, 12% v/v β-mercaptoethanol, and 0.2% w/v bromophenol blue, pH 6.8]. Proteins (30 μg in 25 μl) were resolved on a 8% Tris–glycine precast gel (Novex, San Diego, CA) by SDS–PAGE. Proteins were electroblotted onto a nitrocellulose membrane. Gels were calibrated with Kaleidoscope molecular mass markers (Bio-Rad, Richmond, CA) and biotinylated protein ladder (Cell Signaling Technology, Beverly, MA). Nitrocellulose membranes were incubated in blocking solution consisting of 1% BSA and 1% chicken ovalbumin in TBS–T (25 mM Tris, 150 mM sodium chloride, and 0.075% Tween 20, pH 8.0) for 2 h at room temperature to minimize non-specific binding. Membranes were then incubated in DOR antisera (Cat # AB1560, Lot # 23040417; Chemicon, Temecula, CA) diluted 1:5000 in 1% BSA and 1% chicken ovalbumin in TBS-T overnight at 4 °C. This DOR antibody has been thoroughly characterized by Cahill et al. (2001). To allow visualization of bound antibody and biotinylated protein ladder, nitrocellulose membranes were incubated in HRP-conjugated goat-anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted 1:4000 and HRP-conjugated biotin antibody (Cell Signaling Technology, Beverly, MA) diluted 1:10,000 in 1% BSA and 1% chicken ovalbumin in TBS-T. Blots were then incubated in chemiluminescent reagents (Amersham Biosciences, Piscataway, NJ) and exposed to film. To confirm uniform protein loading, membranes were stripped and incubated in blocking solution, then β-actin antisera (Sigma, Saint Louis, MO) diluted 1:5000 overnight at 4 °C. Membranes were then incubated in HRP-conjugated goat-anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted 1:4000 and HRP-conjugated biotin antibody diluted 1:10,000 in 1% BSA and 1% chicken ovalbumin in TBS-T. Blots were digitized using HP Scanjet 4750c. The mean density of immunoreactive bands was quantified using ImageJ© (NIH) software. In order to normalize the data to account for any minor variations in protein loading, the DOR immunoreactive band density was divided by the corresponding β-actin band density. Calibration curves for each immunoblot were calculated using the distances

traveled by the molecular weight markers. Molecular weights of immunoreactive bands were estimated by interpolation from the calibration curve. Tissue was obtained from three groups of neuropathic and sham animals. Tissue from two to three animals per group was pooled in order to ensure sufficient amounts were present for protein analysis. Samples were run on gels in triplicate for each group.

### 2.6. Light and fluorescent microscopic immunohistochemistry

To assess the effects of PNI on the expression and cellular localization of DOR in the individual cell populations of the DRG, neuropathic (day 14 post-PNI) and sham rats were processed for immunohistochemistry, ($n = 5$ per group). Rats were anesthetized with sodium pentobarbital (70 mg/kg, intraperitoneal) and perfused though the aortic arch with 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4, at 4 °C. Dorsal root ganglia from lumbar segments L4–L6 (ipsilateral and contralateral to the operated hind limb) were isolated, removed, post-fixed in the same fixative solution for 30 min and cryoprotected in 30% sucrose in 0.1 M PB for 48 h at 4 °C. Dorsal root ganglia were sectioned (20 μm) using a cryostat and mounted on gelatin-coated glass slides. immunohistochemistry was performed according to the avidin-biotinylated-horseradish-peroxidase complex (ABC) using the ABC Elite™ kit (Vector Laboratories, Burlingame, CA). Slide-mounted sections were washed with TBS-T (100 mM Tris, 150 mM sodium chloride, and 0.2% Triton X-100, pH 7.4) and incubated in 0.3% $H_2O_2$ to reduce the endogenous peroxidase signal. Sections were incubated in blocking solution (5% NGS, 5% BSA in TBS-T) for 2 h at room temperature to minimize non-specific binding followed by incubation with rabbit polyclonal DOR antisera (Cat # AB1560, Lot # 23040417, Chemicon, Temecula, CA) diluted 1:5000 in TBS-T containing 1% BSA for 48 h at 4 °C. Sections were then incubated in goat-anti-rabbit biotinylated IgG (Vector Laboratories, Burlingame, CA) followed by ABC solution prepared according to manufacturer's protocols and Alexa Fluor 594 Streptavidin conjugate (1:200, Molecular Probes, Burlington, Ont.,, Canada) for 1 h at room temperature. Sections were mounted with the anti-fading agent, Aquamount (Polysciences, Warrington, PA).

### 2.7. Image acquisition and quantification

Images of DRG sections were acquired using a Leica DM 4000 microscope equipped with a (DFC 350 FX, Leica Microsystems, Cambridge, ON) digital camera. Images were captured using OpenLab© 4.01 image acquisition and analysis software (Improvision, Lexington, MA). Random images from four whole DRG per condition were acquired to obtain approximately 150 cells for neuropathic ipsilateral, neuropathic contralateral, and sham tissue for each experiment and a minimum of five experiments were quantified. Cell body diameter was measured using a calibrated micrometer within the software. Small and large DRG neurons were defined as having diameters less than 15 μm and greater than 30 μm, respectively, in accordance with previous DRG immunohistochemical studies (Mennicken et al., 2003). A threshold intensity was determined for each image above which most background noise was suppressed. The total number of DOR-positive cells was counted and divided by the total number of neurons in the DRG section to calculate the percentage of cells immunopositive for DOR. Only cells in which the nucleus could be clearly identified were counted. Intensity was measured by placing a rectangular box of varying size as dictated by cell size over the entire cell and the percentage of pixels within the box with an intensity above threshold was determined for all conditions. The results are expressed as raw values in arbitrary units for each condition. A calculated value for each class of DRG neurons was performed for each condition and a mean and standard error was then determined for each treatment group. Quantification of cell size, cell labeling and intensity of labeling were performed by an observer blind to the condition using Openlab© 4.01 software.

### 2.8. Statistics

GraphPad Prism software 3.01 (San Diego, CA, USA) was used for statistical analyses and graph generation. The results were presented as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey's post hoc tests were utilized to analyze the parametric data generated from thermal antinociception tests, multiple dose comparison experiments, Western blotting experiments, and immunohistochemical quantification. Two-way ANOVA was used to analyze the effect of time and drug on formalin-induced nocifensive behaviour. The Mann–Whitney test was used to analyze non-parametric data generated from mechanical threshold assessment experiments. $P < 0.05$ was deemed significant. In general, the behavioural time course data are within subject, however some animals were removed from groups for molecular studies at various time points which accounts for the various numbers within the groups. Nevertheless, all data were analyzed by independent groups rather than within subject for more stringent statistical analyses.

## 3. Results

### 3.1. Behavioural studies

#### 3.1.1. Anti-allodynic effects of locally administered opioids in neuropathic animals

Peripheral nerve injury to the sciatic nerve resulted in a significant decrease in mechanical withdrawal thresholds on days 7, 14 and up to day 25 following surgery compared to baseline pre-surgical values (data not shown), which represents hypersensitivity to non-noxious tactile stimulation, and is interpreted as tactile allodynia. The mechanical withdrawal thresholds of sham-operated animals and of the un-operated contralateral (data not shown) hind paw of neuropathic animals were not altered and remained at the maximum value obtainable (15 g) throughout the during of all test days. Pre-surgery baseline values obtained were the maximum obtainable values imposed by the method of testing.

Local subcutaneous injection of morphine (10 μg) into the nerve-injured hind paw significantly reversed mechanical allodynia on days 7 (Fig. 1A) and 14 (Fig. 1B), but did not modify this behaviour on day 25 following surgery (Fig. 1C). The peak effect of morphine

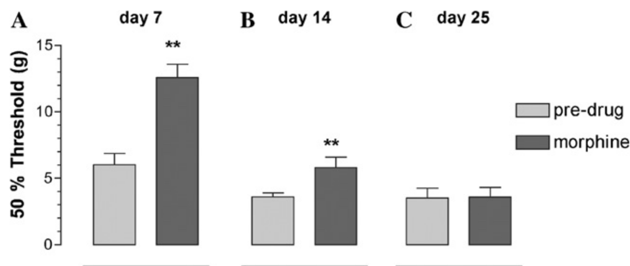


Fig. 1. The effects of local administration of morphine on mechanical allodynia in neuropathic animals. Morphine (10 μg) attenuated mechanical allodynia on days 7 (A) and 14 (B), but not on day 25 (C) following nerve injury. Data are presented at the 30 min time point following morphine injection. Data are presented as means ± SEM for at least $n = 8$ per group and represent within subject measurements. Statistical analyses performed using Mann–Whitney test [$P = 0.0011$ in (a); $P = 0.0058$ in (b); $P = 0.8785$ in (c)]. $^{**}P < 0.01$ denotes statistically significant differences compared to pre-drug baselines.

was observed 30 min following administration. Local administration of deltorphin II dose-dependently reversed allodynia on days 7 and 14 (Fig. 2) following PNI. The peak effect of deltorphin II was observed 20 min following administration. The 50 μg dose of deltorphin II significantly increased mechanical withdrawal thresholds on days 7, 14, and 21 (Fig. 3) following nerve injury. Unlike morphine, deltorphin II maintained its anti-allodynic effects at later time points following nerve injury. At the dose tested, morphine was without effect at 3 weeks following nerve injury. Interestingly,

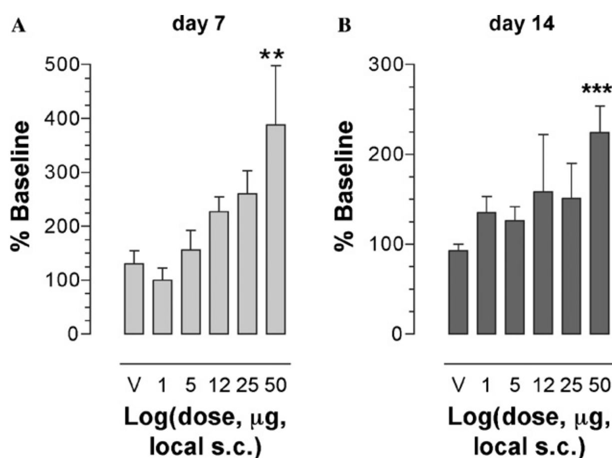


Fig. 2. Dose response curves of the anti-allodynic effects of deltorphin II on days 7 and 14 following PNI. Deltorphin II dose-dependently attenuated mechanical allodynia in neuropathic animals on days 7 (A) and 14 (B) following PNI. Mean ipsilateral mechanical withdrawal thresholds following drug injection are presented as a percentage of baseline thresholds (prior to drug administration). Data are presented at the 20 min time point following deltorphin II injection. Data are presented as means ± SEM for $n = 5$–15 in each group. Statistical analyses performed using One-Way ANOVA [$F(5, 39) = 3.704$ and $P = 0.0077$ in (A); $F(5, 46) = 4.155$ and $P = 0.0034$ in (B)] and Tukey's post hoc tests. $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistically significant differences compared to 1 μg dose. (s.c., subcutaneous, V, vehicle).

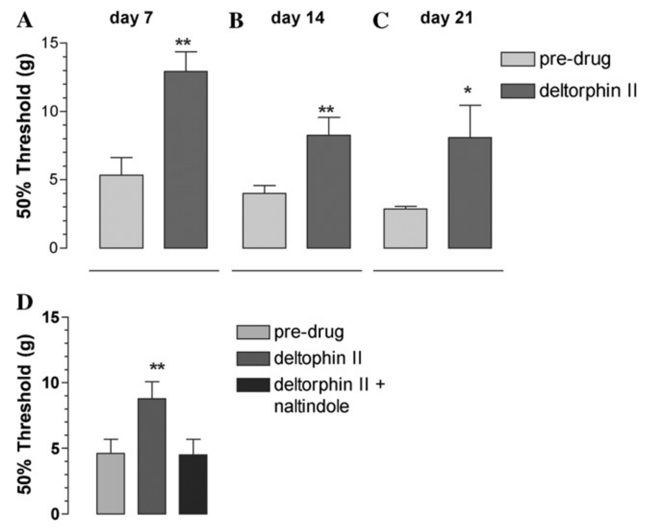


Fig. 3. The anti-allodynic effects of deltorphin II following local administration in neuropathic animals. Local subcutaneous injection of deltorphin II (50 μg) into the nerve-injured hind paw increased mechanical withdrawal thresholds. Deltorphin II attenuated mechanical allodynia on day 7 (A), day 14 (B) and day 21 (C) following PNI. Data are presented at the 20 min time point following deltorphin II injection. Co-administration of deltorphin II (50 μg) with naltrindole (58 μg) in a 1:2 molar ratio, into the ipsilateral hind paw suppressed deltorphin II's anti-allodynic effects on day 14 following PNI (D). Data are presented as means ± SEM [$n = 8$ in (A); $n = 11$ in (B); $n = 4$ in (C); $n = 7$ in (D)]. Statistical analyses performed using Mann–Whitney test [$P = 0.0047$ in (a); $P = 0.0095$ in (b); $P = 0.0286$ in (c); $P = 0.0001$ in (d)]. $^{*}P < 0.05$, $^{**}P < 0.01$ denote statistically significant differences compared pre-drug baselines.

[D-Pen$^{2,5}$]-Enkephalin (DPDPE) (40 μg) did not reverse mechanical allodynia when administered in an equimolar dose to deltorphin II (50 μg) on day 7 or 14 post-PNI (data not shown). Although this dose of DPDPE attenuated mechanical withdrawal thresholds following intrathecal administration by as much as 80%, demonstrating an almost complete return to pre-injury thresholds (data not shown). Increasing the dose of DPEPE twofold (80 μg) was also without effect following intraplantar injection on day 7 and 14 post-nerve injury (data not shown). Vehicle treatment did not affect tactile thresholds of neuropathic (data not shown) or sham-operated animals (data not shown).

In order to confirm that deltorphin II was producing its effects locally rather than having a systemic effect after gaining access to the systemic circulation, mechanical withdrawal thresholds were determined in the ipsilateral hind paw prior to and following local administration into the contralateral hind paw of neuropathic animals. Deltorphin II (50 μg) administered into the contralateral hind paw did not alter paw withdrawal thresholds at the ipsilateral hind paw on days 7 (data not shown) or 14 (data not shown) following nerve injury. To confirm that deltorphin II's effects were mediated via an opioid receptor-induced mechanism, naltrindole hydrochloride (58 and 100 μg) was co-injected with

deltorphin II in a 2:1 molar ratio. Naltrindole antagonized the deltorphin II-mediated increase in mechanical withdrawal thresholds on day 14 following nerve injury (Fig. 3D).

### 3.1.2. The effects of locally administered opioids on paw withdrawal latencies to noxious heat stimulation in naïve animals

To examine the ability of locally applied opioids to alter normal nociception, thermal antinociceptive thresholds were determined prior to and following local drug injection. Neither local deltorphin II (50 μg) nor morphine (10 μg), at doses that produced anti-allodynic effects in neuropathic animals, altered paw withdrawal latencies to noxious heat stimulation in naïve animals (data not shown).

### 3.1.3. The effects of locally administered opioids on formalin-induced pain behaviour

To investigate the effects of locally administered opioids in a tonic pain model, formalin-induced pain was assessed following local drug injection. Formalin produced bi-phasic nocifensive pain-like behaviour. Local subcutaneous administration of deltorphin II (50 μg) suppressed overall formalin-induced nocifensive behaviour in animals injected with 2.5% formalin solution (Fig. 4).

### 3.2. Western blot analysis of delta opioid receptor protein levels lumbar L4–L6 DRG neurons and sciatic nerve from neuropathic and sham animals

Probing tissue membranes with DOR antisera revealed one immunoreactive band at 65 kDa, which corresponds to the published molecular weight of the cloned DOR on both sham and injured L4–L6 DRG (Fig. 5A) (Kieffer et al., 1992). Quantification of DOR immunoreactive band density revealed a 1:1 ratio of DOR protein levels in ipsilateral versus contralateral neuropathic and sham DRG, indicating that there was no change in expression (Fig. 5B). Interestingly, the DOR protein levels in neuropathic compared to sham DRG were significantly higher, indicating that there was a bilateral increase in DOR protein levels in neuropathic animals compared to their sham counterparts (Fig. 5B and C).

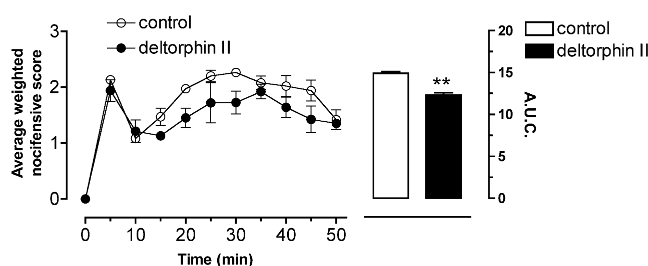


Fig. 4. The effect of deltorphin II on formalin-induced pain behaviour. Average weighted nocifensive behavioural scores of animals injected with 2.5% formalin (no drug; hollow circle), or deltorphin II (50 μg) and 2.5% formalin (deltorphin II; solid circle) into the hind paw. Local administration of deltorphin II suppressed overall pain behaviour induced by formalin as indicated by the area under the curve (AUC). Scoring of nocifensive behaviour was conducted for a 50-min period following formalin injection. Deltorphin II was injected 5 min prior to formalin. Nociceptive behaviour was assessed as: 1, no favouring; 2, favouring; 3, complete elevation of the hind paw from the floor; and 4, licking and/or vigorous flinching of the injected hind paw. Data are presented as means ± SEM [$n = 5$ (no drug); $n = 4$ (deltorphin II)]. Statistical analyses performed using two-way ANOVA [$F(1, 70) = 18.62$ and $P < 0.0001$ for drug; $F(9, 70) = 8.195$ and $P < 0.0001$ for time].

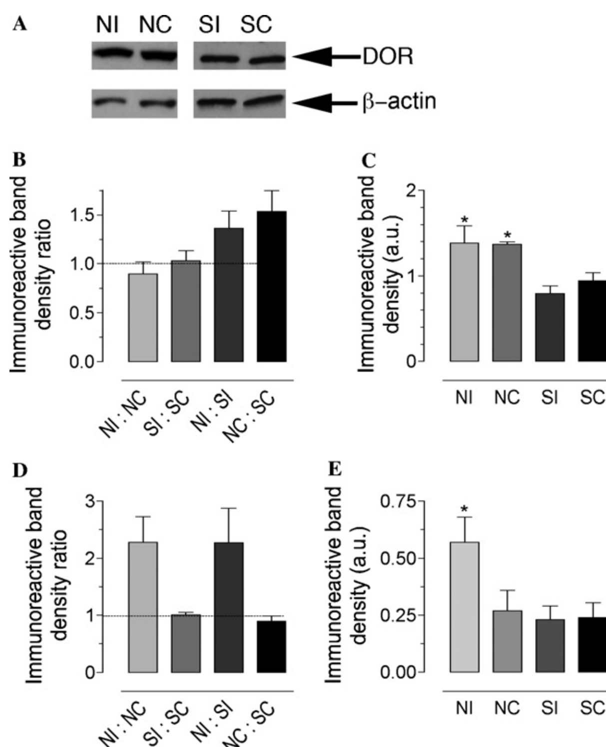


Fig. 5. Identification and quantification of delta opioid receptor protein expression in lumbar L4–L6 dorsal root ganglia and sciatic nerve from neuropathic and sham animals. Identification of DOR protein in DRG by Western blotting (A). Membranes from L4 to L6 DRG and nerve were isolated and proteins were resolved by 8% SDS–PAGE followed by electroblotting onto nitrocellulose membranes. Immunoblot analyses revealed an immunoreactive band at the 65 kDa molecular weight range, which corresponds to the published molecular weight of DOR. Membranes were reprobed with β-actin to normalize for protein loading. Quantification was performed by digitizing images and measuring DOR immunoreactive band density using ImageJ software (NIH). The immunoreactive bands presented are from one blot, but are representative of the immunoreactive labeling in all samples. Immunoreactive band densities from DRG neurons (B and C) and sciatic nerve (D and E) were normalized to β-actin. Ratios are presented to illustrate the change in band density within a set of samples whereas raw immunoreactive band density quantification is depicted in (C and E). Statistical analyses were performed using a one-way ANOVA followed by a Tukey's post hoc comparison on raw data. *$P < 0.05$ denotes statistically significant differences compared to sham ipsilateral ratio. NI, neuropathic ipsilateral; NC, neuropathic contralateral; SI, sham ipsilateral; and SC, sham contralateral.

Quantification of immunoreactive bands resolved from sciatic nerve tissue revealed a 1.5:1 ratio of DOR protein levels in the ipsilateral versus contralateral neuropathic and sham treatments (Fig. 5D). There was no evidence of an increase in contralateral DOR immunoreactive bands compared to sham (Fig. 5E).

### *3.3. Immunohistochemical localization of delta opioid receptors in lumbar L4–L6 dorsal root ganglia neurons*

Fig. 6 represents a photomicrograph of DOR immunoreactivity in DRG neurons. Moderate to intense DOR immunoreactivity was observed over small, medium and large cells of neuropathic ipsi- and contralateral, and naïve DRG. Quantitative analysis of labeling intensity revealed no significant difference in the percentage of large, medium, or small cells immunopositive for DOR (Fig. 7A and C). However, a significant increase was identified in DOR labeling intensity in small and large DRG neurons in neuropathic ipsilateral and contralateral DRG compared to sham tissue (Fig. 7B and D). There was no change in DOR immunoreactive labeling intensity in medium size cells (data not shown).

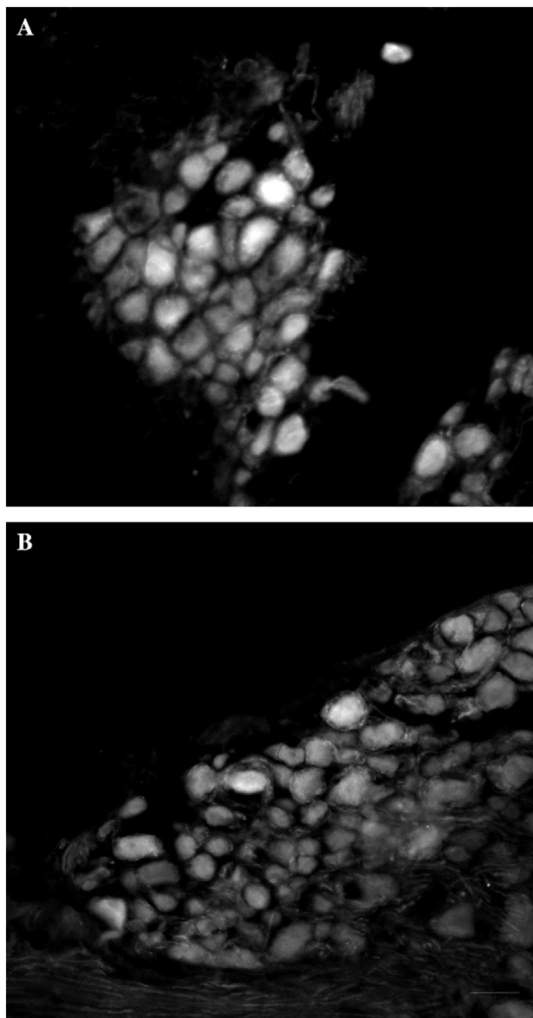


Fig. 6. Photomicrograph of delta opioid receptor immunoreactive neurons in lumbar L4–L6 dorsal root ganglia neurons from neuropathic animals. Immunhistochemical localization of DOR in neuropathic ipsilateral (A) and contralateral (B) DRG. Moderate to intense DOR immunoreactivity was observed over small, medium and large neurons. Large arrows point to large-diameter cells, medium arrows to medium cells, and small arrows to small cells. Tissue was harvested 14 days following PNI. Scale bar = 50 μm.

## 4. Discussion

Summary of findings: this study provides the first evidence that local administration of the DOR selective agonist deltorphin II exerts local, dose-dependent and opioid receptor-mediated anti-allodynic effects in a model of neuropathic pain. Furthermore, the anti-allodynic dose of deltorphin II did not alter normal nociceptive thresholds to noxious thermal stimuli in naïve animals, but suppressed overall formalin-induced nocifensive behaviour. Western blotting experiments revealed no change in DOR protein expression in DRG ipsilateral to the site of nerve injury as compared to the contralateral site. However, an up-regulation of DOR protein was found in neuropathic DRG compared to sham, that appeared to be due to enhanced expression in both small and large DRG neurons. These results suggest that there may be a bilateral increase in the expression of DOR following nerve injury. Finally, DOR trafficking to the site of injury was suggested by the increase in DOR protein at the site of PNI.

Deltorphin II-induced anti-allodynic effects were completely blocked by co-administration with the DOR antagonist, naltrindole, providing evidence that deltorphin II is mediating its effects via activation of DOR. Further support for deltorphin II producing its effect via activation of DOR is provided by the selectivity of deltorphin II for this receptor. Hence, deltorphins are considered to be the most selective natural ligands for the DOR, exhibiting 1000-fold greater selectivity for the DOR than for its MOR counterpart (Erspamer et al., 1989). Similar to the effect produced by deltorphin II, local subcutaneous administration of morphine also produced anti-allodynic effects in the PNI model of neuropathic pain. Nevertheless, morphine at the dose assessed in this study lost its anti-allodynic effects at later time points. It is perhaps not surprising that morphine was ineffective in attenuating tactile allodynia at later time points. Hence, previous studies examining the anti-allodynic effects produced by intraplantar injection of morphine have demonstrated that doses greater than 100 μg were required to attenuate tactile allodynia in the spinal nerve ligation model of neuropathic pain (Pertovaara and Wei, 2001) or doses in excess of 500 nmol were required to produce significant anti-allodynic effects in a chronic constriction injury model (Obara et al., 2004). Although dose-dependent effects

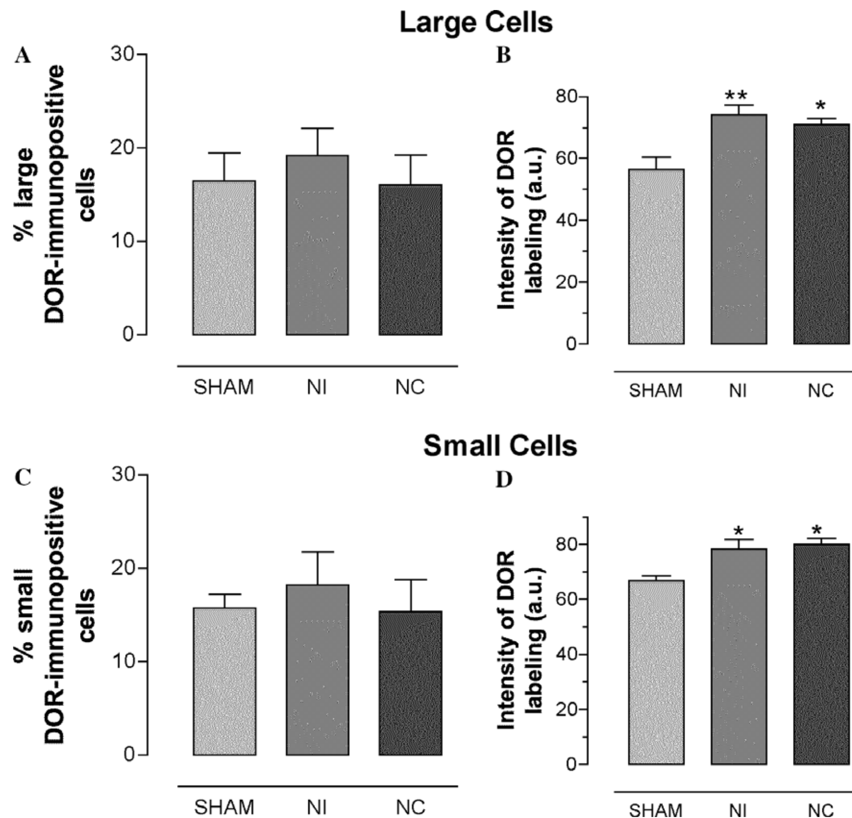


Fig. 7. Quantification of the percentage of delta opioid receptor immunoreactive neurons and delta opioid receptor immunoreactive density in lumbar L4–L6 dorsal root ganglia neurons from neuropathic and naïve animals. The intensity of DOR immunoreactivity was significantly increased in small and large DRG neurons in neuropathic compared to sham rats (B and D). No difference in the proportion of large- (A), and small-diameter (C) neurons immunopositive for DOR in neuropathic ipsi- and contralateral, and sham DRG was observed. Tissue was harvested 14 days following PNI. Statistical analyses performed using one-way ANOVA [$F(2, 9) = 8.88$ and $P = 0.0074$ in (B); $F(2, 9) = 7.761$ and $P = 0.011$ in (D)], $*P < 0.05$, $**P < 0.01$ denotes statistically significant differences compared to sham. NI, neuropathic ipsilateral and NC, neuropathic contralateral.

of morphine were not assessed in this study, the ability of deltorphin II, but not morphine, to attenuate neuropathic pain-induced allodynia at thee weeks post injury provides further support that deltorphin II actions are mediated via DOR activation. In addition, the sustained effect of deltorphin II and the enhancement of DOR protein levels in the DRG suggest that DOR do not undergo down-regulation – a mechanism thought to be responsible for the meager analgesic effects of morphine in treating neuropathic pain (Ossipov et al., 1995).

To establish that the effects of deltorphin II were local and not due to the drug's access to the systemic circulation, the drug was injected into the contralateral hind paw and mechanical thresholds were assessed in the ipsilateral hind paw of neuropathic animals. Indeed, deltorphin II did not alter mechanical thresholds of the nerve-injured hind paw when injected into the contralateral hind paw, indicating that its anti-allodynic effects are local, presumably mediated by activating DOR on primary afferent neurons innervating the zdermatomes of the rat hind paw glabrous skin.

In our study, DPDPE, unlike deltorphin II, did not reverse neuropathic allodynia when administered subcutaneously into the hind paw. It is unknown why DPDPE had no effect, however, its ineffectiveness may be due to the rapid degradation of this peptide by endogenous enkephalinases whereas deltorphin II has been shown to be relatively resistant to enzymatic breakdown (Erspamer et al., 1989). In agreement with this hypothesis is the positive outcome of demonstrating that DPDPE attenuated mechanical allodynia following spinal administration. An alternative explanation for the lack of a peripheral effect is that DPDPE may be activating another molecular form of DOR, which in turn may be selectively down-regulated following nerve injury or perhaps not present in primary afferent neurons. In keeping with this hypothesis, Abdulla and Smith (1998) reported that DPDPE was without effect in altering evoked currently in acutely isolated DRG neurons from either control or nerve injured rats.

A role for DORs in providing an endogenous tone in suppressing neuropathic pain has recently been demonstrated in that mice with null mutation of the DOR protein exhibited exacerbated mechanical and thermal alloydnia compared to their wild type littermates (Nadal et al., 2006). It would be of interest to determine if

part of this endogenous tone was peripherally mediated. Very few studies have examined the change in DOR expression within the peripheral nervous system following nerve injury with the exception of a study that demonstrated a decrease in DOR immunoreactive labeling following axotomy (Zhang et al., 1998). Many studies have inferred changes in DOR expression within primary afferent neurons from spinal cord studies with the assumption that much of DOR expression within the dorsal spinal cord is pre-synaptic. For example, radioligand binding studies have demonstrated a decrease in DSLET binding in the ipsilateral to contralateral ratio following nerve injury (Besse et al., 1992) whereas DPDPE binding was reduced bilaterally following chronic constriction injury of the sciatic nerve (Stevens et al., 1991). These studies are not inconsistent with our data as we failed to show efficacy of DPDPE. Nevertheless, Pol et al. (2006) reported no significant change in DOR mRNA levels in mice following partial nerve ligation. Whereas, spinal cord DOR immunoreactivity was shown to decrease following CCI (Stone et al., 2004), spinal nerve ligation (Stone et al., 2004) or nerve crush injury (Robertson et al., 1999). It remains unknown why our data do not support the previous studies in that we demonstrate a bilateral increase in DOR expression following nerve injury, however, one could surmise various explanations. Considering that studies that show a decrease in DOR protein have been on spinal cord tissue rather than cell bodies of primary afferents, one possible explanation is the shunting of DOR protein from the cell body to the site of injury with less protein being transported to the terminal arborization within the spinal cord. Indeed, we see an increase in DOR protein expression at the site of nerve injury. Previous reports have suggested that PNI produces a shunting or targeting of protein from the cell body to peripheral terminals at the site of injury causing a decrease in the central terminals. Indeed, Truong and colleagues (2003) showed that the expression of MOR in DRG and sciatic nerve and the trafficking and targeting of MOR to the periphery were all elevated ipsilateral to nerve injury, a phenomenon that contributed to the antinociceptive efficacy of peripherally administered opioid agonists. Studies that examined OR targeting in the peripheral nervous system in persistent inflammatory states have provided indirect evidence that the trafficking of DOR receptor could be enhanced in nerve injury states (Hassan et al., 1993). Hassan and colleagues (1993) ligated the sciatic nerve in order to assess changes in bi-directional transport of β-endorphin binding sites (β-endorphin is a non-selective MOR and DOR agonist) due to persistent inflammation, however, ligation of the sciatic nerve, in itself a model of neuropathic pain, and was found to enhance opioid receptor targeting to the periphery. Alternative explanations for the difference between our study and previous reports include the differences in models to induce neuropathic pain as well as a different antibody for assessing DOR protein. Indeed, we have previously reported that multiple molecular forms of DOR protein can be identified with different antibodies (Cahill et al., 2001) and therefore we may be recognizing a different molecular species than those reported the studies cited above.

## 5. Conclusion

This study demonstrated DOR-mediated anti-allodynic effects following local administration of deltorphin II in a model of neuropathic pain that persisted. These findings suggest that peripheral DORs may be an attractive therapeutic target in the treatment of neuropathic pain and validate further investigation into the development of effective, non-invasive, peripherally selective DOR agonists as a novel therapeutic approach for treating neuropathic pain conditions.

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