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Oxycodone Plus Ultra-Low-Dose Naltrexone Attenuates Neuropathic Pain and Associated μ -Opioid Receptor– G_s Coupling

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Abstract

Both peripheral nerve injury and chronic opioid treatment can result in hyperalgesia associated with enhanced excitatory neurotransmission at the level of the spinal cord. Chronic opioid administration leads to a shift in μ -opioid receptor (MOR)–G protein coupling from $G_{i/o}$ to G_s that can be prevented by cotreatment with an ultra-low-dose opioid antagonist. In this study, using lumbar spinal cord tissue from rats with L_5/L_6 spinal nerve ligation (SNL), we demonstrated that SNL injury induces MOR linkage to G_s in the damaged (ipsilateral) spinal dorsal horn. This MOR– G_s coupling occurred without changing $G_{i/o}$ coupling levels and without changing the expression of MOR or $G\alpha$ proteins. Repeated administration of oxycodone alone or in combination with ultra-low-dose naltrexone (NTX) was assessed on the SNL-induced MOR– G_s coupling as well as on neuropathic pain behavior. Repeated spinal oxycodone exacerbated the SNL-induced MOR– G_s coupling, whereas ultra-low-dose NTX cotreatment slightly but significantly attenuated this G_s coupling. Either spinal or oral administration of oxycodone plus ultra-low-dose NTX markedly enhanced the reductions in allodynia and thermal hyperalgesia produced by oxycodone alone and minimized tolerance to these effects. The MOR– G_s coupling observed in response to SNL may in part contribute to the excitatory neurotransmission in spinal dorsal horn in neuropathic pain states. The antihyperalgesic and antiallodynic effects of oxycodone plus ultra-low-dose NTX (Oxytrex, Pain Therapeutics, Inc., San Mateo, CA) suggest a promising new treatment for neuropathic pain.

Keywords

Neuropathic pain; G protein coupling; tolerance; opioids; hyperalgesia; allodynia; μ -opioid receptor

Patients who have diseases such as cancer, arthritis, and diabetes often have development of painful neuropathies due to disease progression or medical treatment. Common first-line

approaches used in the clinic to treat these neuropathies include tricyclic antidepressants, dual-acting reuptake inhibitors, and anticonvulsants (eg, gabapentin) or combinations of these with nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids. However, these options often cause side effects such as dizziness and sedation and may not treat the various classifications of neuropathic pain states with comparable efficacy.^{30,32,55,77} Treatment of neuropathic pain that is not responsive to first-line therapies is often limited to opioids, which can be effective acutely but have decreased efficacy after chronic exposure, leading to the use of higher doses to achieve the same level of pain relief and often resulting in opioid-induced mechanical and thermal hypersensitivities.^{5,7,9,29,30,32,69–71,79,81} For patients taking higher doses of opioids to achieve pain relief, the presence of unwanted side effects including tolerance, dependence, respiratory depression, and constipation further limit the efficacy of the therapy.

Mechanistically, opioids act at G protein-coupled receptors (GPCRs) located in both peripheral and central sites. When opioid agonists bind to opioid receptors during acute pain states, G_i or G_o proteins are recruited to the receptor and activated. Subsequent signaling leads to decreased cyclic adenosine monophosphate (AMP) and hyperpolarization due to reduced calcium influx and increased potassium efflux, blocking pain transmission.^{24,41,42,45,47–49,58} Chronic opioid use has been shown paradoxically to cause excitatory rather than inhibitory effects on the cell, stimulating pain propagation.^{11,14,26,27,31,40,67,76} This enhanced pain and the underlying excitatory signaling have been proposed to be mediated by opioid receptors preferentially coupling to G_s proteins,^{19,60} or alternatively, via the $G\beta\gamma$ dimer of the $G_{i/o}$ proteins native to opioid receptors.^{36,76} More recent data have shown that chronic opioid administration leads to a shift in G protein coupling by μ -opioid receptors (MOR) from $G_{i/o}$ to G_s ,⁷⁴ with the $G\beta\gamma$ of this MOR-associated G_s protein also contributing to the excitatory signaling.⁷²

Remarkably, cotreatment with ultra-low-dose naloxone, an opioid antagonist, prevents the shift in G protein coupling by the MOR as well as the analgesic tolerance and dependence induced by chronic opioid treatment.⁷⁴ Recent data have revealed that a 4-picomolar interaction between the opioid antagonists naloxone or naltrexone (NTX) and the MOR-interacting scaffolding protein filamin A is responsible for the prevention of MOR's G protein coupling switch⁷³ rather than their direct interaction with opioid receptors that occurs at much higher doses. In both preclinical and clinical studies, ultra-low doses of NTX enhance opioid analgesia and prevent opioid dependence or withdrawal signs.^{22,25,61,62,78} Chronic pretreatment with ultra-low-dose NTX increases opioid sensitivity.⁸³ In addition, ultra-low-dose opioid antagonist cotreatment with opioids can attenuate tolerance-associated hyperalgesia.^{27,61–64} Oxytrex (Pain Therapeutics, Inc., San Mateo, CA) combines oxycodone (OXY) with ultra-low-dose NTX and is currently being developed as a novel painkiller anticipated to enhance analgesia while minimizing opioid tolerance, dependence, and certain side effects.

The current study investigated whether OXY plus ultra-low-dose NTX could alleviate the hypersensitivities in the L_5/L_6 spinal nerve ligation (SNL) model of neuropathic pain over a period of 7 days when given locally or systemically. Finally, these studies addressed whether the neuropathic injury induces G_s coupling by MOR, as observed after chronic opioid

treatment, and whether OXY plus ultra-low-dose NTX similarly attenuates this signaling alteration.

Materials and Methods

Animals

Male Sprague-Dawley (Harlan, Indianapolis, IN) rats weighing 200 to 225 g were maintained on a 12-hour light/dark cycle with food and water available ad libitum. All preparations and testing were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, National Institutes of Health, and the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona.

Intrathecal Catheter Implantation

Rats were anesthetized with the use of intraperitoneal ketamine/xylazine 100 mg/kg and placed in a stereotaxic holder. The cisternum magnum was exposed, and an 8-cm catheter (PE-10) was implanted as described by Yaksh and Rudy,⁸² terminating in the lumbar region of the spinal cord. Catheters were sutured into place and externalized at the back of the neck. Animals were housed individually and allowed to recover for 3 to 5 days before baseline testing and spinal nerve ligation. Animals with signs of motor weakness or paralysis after surgery were not used.

Spinal Nerve Ligation

Nerve ligation injury produces signs of neuropathic dysesthesias, including tactile allodynia and thermal hypersensitivity. All nerve operations occurred 5 days after intrathecal catheter implantation. Rats were anesthetized with 2% isoflurane in O₂ anesthesia delivered at 2 L/min. The skin over the caudal lumbar region was incised and the muscles retracted. The L₅ and L₆ spinal nerves were exposed, carefully isolated, and tightly ligated with 4-0 silk distal to the dorsal root ganglion without limiting the use of the left hind paw of the animal.⁴⁴ Control animals underwent sham surgeries in which the nerve areas were exposed but not ligated. All animals were allowed 7 days to recover before any behavioral testing. Any animals exhibiting signs of motor deficiency were euthanized.

Intrathecal Compound Administration

To evaluate treatments and the development of tolerance, compounds were administered twice daily (separated by 6–7 hours) for 7 consecutive days, with some groups continuing through 11 days to more fully assess the time course of treatment. Testing was conducted daily, 20 minutes after the morning injection. Drugs or drug mixtures were administered in 5- μ L volumes, followed by a 1 μ L air/9 μ L saline push.

Oral Compound Administration

Each treatment was administered twice daily (separated by 3–5 hours), via oral gavage with an 18-gauge, 3-inch gavage needle. Behavioral testing occurred 20 minutes after the first administration and was repeated for 7 consecutive days. For OXY plus NTX groups, 10

mg/kg of OXY was given in conjunction with 1 of 3 ultra-low doses of NTX (0.3 µg/kg, 1.0 µg/kg, or 3.0 µg/kg). Control groups included 10.0 mg/kg OXY alone and 3.0 µg/kg NTX alone, with sham surgery animals receiving vehicle administrations (1:5, ethanol: water).

Tactile Hypersensitivity Testing (von Frey, Innocuous)

Rats were acclimated in suspended, wire mesh cages for 20 minutes before baseline von Frey testing (before and after nerve ligation/exposure). Test days consisted of treatment administration and measurement of responses to calibrated von Frey filaments probed perpendicularly on the plantar surface of the left hind paw (ipsilateral to the SNL) for 7 seconds, 20 to 30 minutes after the first compound administration, using the up-down method.²¹ Paw withdrawal thresholds were calculated in grams, using the Dixon nonparametric test and expressed as the mean withdrawal threshold $\pm$ mean standard error (SEM) in Prism by Graph Pad Software Inc. (San Diego, CA). The contralateral paw was not tested, as injured animals placed more body weight on the uninjured paw when compared with the injured side.

Thermal Hypersensitivity Testing (Radiant Heat, Noxious)

Rats were allowed to acclimate in plexiglas holders for baseline testing (before and after nerve ligation/exposure) for 20 minutes. A mobile radiant heat source was used to direct heat to the plantar surface of the left hind paw.³⁹ Paw withdrawal latencies were measured in seconds, with an automatic shutoff of the heat source at 33.0 seconds. Pre-nerve injury baselines were established between 20 and 25 seconds. Postinjury baselines were obtained after the 7-day recovery period. On test days, animals were dosed, tested using von Frey filaments 20 minutes after dosing followed by 5-minute habituation, and given radiant heat testing. Paw withdrawal latencies were calculated and expressed as the mean withdrawal latency $\pm$ SEM in Prism by Graph Pad Software Inc. The contralateral paw was not tested, as this would be a biased test asking animals to bear weight on an injured leg to lift an uninjured paw.

Mathematical Calculations

SNL injury percent activities (% antihyperalgesia and % antiallodynia):

$$\% \text{Activity} = 100 \times (\text{withdrawal latency after drug treatment} - \text{post SNL baseline latency}) / (\text{pre SNL baseline latency} - \text{post S.})$$

$$\% \text{Activity} = 100 \times (\text{withdrawal threshold after drug treatment} - \text{post SNL baseline threshold}) / (\text{pre SNL baseline threshold} - \text{post S.})$$

Sham-operated percent activities (% analgesia, cutoff values are listed in sections above)

$$\% \text{Activity} = 100 \times (\text{withdrawal latency after drug treatment} - \text{post Sham baseline latency}) / (\text{pre Sham baseline latency} - \text{post Sham baseline latency})$$

Tissue Collection

All animals underwent intrathecal catheterization, followed by a 3-day recovery period. On the fourth day, animals received a left-side, L₅/L₆ spinal nerve ligation (ipsilateral) except

for those indicated by “sham” surgery as the control. After a 7-day recovery period, animals received twice-daily injections (intrathecal) of vehicle, OXY, NTX, or OXY plus NTX, for 7 days. On the eighth day of injections, animals received 1 intrathecal bolus of the respective compound and were killed 30 minutes after administration by decapitation. The lumbar spinal cord was harvested and quartered into the ipsilateral/dorsal, ipsilateral/ventral, contralateral/dorsal, and contralateral/ventral sections. Tissue was fast-frozen in liquid nitrogen within 1 minute of removal. Western blotting was performed with the use of lumbar spinal cord tissue either ipsilateral to the side of injury or contralateral as a comparison to the injured side 15 days after nerve injury was induced and 8 days after compound or vehicle treatment was begun.

Direct Coupling Protocol

To investigate G protein coupling to MOR, synaptic membranes were prepared from spinal dorsal horn ipsilateral and contralateral to the SNL in rats from experiment 1 (intrathecal administration). These experiments were conducted with 5 to 7 tissue samples for each condition. We used a coimmunoprecipitation procedure described below, which is an established procedure for examining the association of GPCRs with G proteins.^{13,33,43,74,75,85,86} In addition, G proteins, adenylyl cyclase, and receptors interact via scaffolding proteins such as lipid rafts and caveolae, suggesting that such protein interactions survive a coimmunoprecipitation procedure.⁴¹ Further data show that GPCRs form stable signaling complexes with their effectors during receptor activation.⁴⁷ Because we used Gpp(NH)P with agonist to ensure persistent receptor activation, these signaling complexes should be stable under our experimental conditions. All data points are n = 5 to 7.

Synaptic membranes (200 µg) were incubated with 1 µM morphine for 5 minutes at 37°C in Krebs Ringer solution. Membranes were solubilized in immunoprecipitation buffer (25 mM HEPES, pH7.5; 200 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.2% 2-mercaptoethanol, 50 µg/mL leupeptin, 25 µg/mL pepstatin A, 0.01 U/mL soybean trypsin inhibitor, 0.04 mM phenylmethylsulfonyl fluoride) containing 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40. G protein-coupled MORs were immunopurified by using immobilized anti-Gα antibodies. MOR protein associated with specific Gα proteins was assessed by Western blotting, using an antibody directed against the amino-terminal region of MOR (Santa Cruz Biotechnology, Santa Cruz, CA). The specificities of antibodies directed against MOR or Gα proteins were rigorously demonstrated in an earlier publication.⁷⁴

Compounds

Oxycodone hydrochloride was provided by Noramco (Wilmington, DE), and naltrexone was purchased from Sigma Aldrich (St. Louis, MO). Both compounds were dissolved in a saline vehicle for intrathecal administration and in an ethanol:water vehicle for oral delivery.

Statistical Analysis

Thermal and tactile hypersensitivity data were analyzed by nonparametric 1-way ANOVA and 2-way ANOVA (post hoc, Newman-Keuls) in FlashCalc (University of Arizona, Tucson, AZ). Statistical significance for direct-coupling data was determined by ANOVA followed

by Newman-Keuls comparisons. Differences were considered to be significant at a value of $P < .05$.

Results

SNL Induces MOR Coupling to G_s in Ipsilateral Spinal Cord Dorsal Horn

Both injury and treatment effects on G protein coupling by MOR were demonstrated by co-immunoprecipitation of $G\alpha$ proteins with MOR from spinal dorsal horn tissue of sham control animals (Fig 1A, top blots) or nerve-injured animals (all other blots in Fig 1A) receiving either vehicle, oxycodone alone, NTX alone, or oxycodone plus NTX intrathecally. First, the L_5/L_6 SNL injury itself induced the recruitment of G_s proteins to MOR, as indicated by the presence MORs in the $G\alpha_s$ immunoprecipitate from ipsilateral dorsal horn in SNL animals receiving vehicle but not in sham control animals (Fig 1). This SNL-induced, novel coupling was found only in the spinal dorsal horn ipsilateral to the lesion and was not present on the contralateral side of vehicle-treated or NTX-treated SNL rats. Second, oxycodone treatment augmented this MOR- G_s coupling in SNL animals, because G_s coupling was observed in both the ipsilateral and contralateral dorsal horn in oxycodone groups (but only in ipsilateral dorsal horn in SNL rats treated with vehicle or NTX alone) and because ipsilateral levels of MOR- G_s coupling were higher in oxycodone-treated than in vehicle-treated SNL rats. Third, NTX plus oxycodone cotreatment significantly attenuated the level of MOR- G_s coupling in SNL animals as compared to oxycodone-treated SNL animals without NTX. This attenuation occurred in both ipsilateral and contralateral dorsal horn, suggesting that this attenuation of the MOR- G_s coupling included the oxycodone-induced component. Interestingly, the fact that the ipsilateral level of G_s coupling of oxycodone plus NTX animals was similar to that of vehicle-treated SNL animals may suggest that the injury-induced contribution to G_s coupling was not itself alleviated by the NTX cotreatment. In all cases, in vitro morphine augmented coupling by MOR with similar magnitude but not altering the patterns of G protein coupling seen for each treatment and spinal cord side. Fig 1B shows only the densitometric quantification of MOR coupling stimulated by in vitro morphine. Basal (Krebs Ringer) coupling levels from Fig 1A are omitted for simplicity. The top panels of Figs 1A and 1B show sham-operated, vehicle-treated animals; all other panels represent L_5/L_6 SNL-operated animals. Changes in G protein coupling were not due to changes in protein expression levels of MOR or $G\alpha$ proteins (Data not shown).

Ultra-Low-Dose NTX Enhances the Effects of Intrathecal Oxycodone

Before injury, all animals had an average paw withdrawal latency of 21.3 ± 0.4 seconds in response to a noxious, thermal stimulus, and SNL reduced this value to 11.9 ± 0.3 seconds, indicating thermal hypersensitivity. The peak effect of oxycodone ($10.0 \mu\text{g}$) over an 11-day administration period occurred on day 2, with a mean withdrawal latency of 19.0 ± 1.2 seconds (Fig 2A). Animals given a combination injection of oxycodone ($10.0 \mu\text{g}$) and NTX (0.33 ng) exhibited prolonged paw withdrawal latencies of 22.6 ± 1.2 seconds, 22.6 ± 1.6 seconds, and 23.2 ± 1.8 seconds on days 3, 4, and 5, respectively. Although the peak antihyperalgesic effect occurred on these 3 days, the reversal of thermal hypersensitivity continued through day 11 of testing (Fig 2A). Animals treated with NTX (0.33 ng) withdrew

the injured paw with an average baseline of 11.5 ± 0.8 seconds after SNL and 12.5 ± 0.5 seconds across the 7-day testing period. Vehicle control animals showed no significant antihyperalgesia on any testing day, with a mean withdrawal of 13.8 ± 0.9 seconds. Additional testing on days 9 through 11 was not conducted for vehicle or on days 8 through 11 for NTX animals, as withdrawal latencies of these groups were not significantly different from post-SNL paw withdrawal baseline latencies. A >1000-fold higher dose of NTX (1.0 μ g) was used in combination with 10 μ g oxycodone, following the same testing paradigm as above. This higher dose of NTX acting to antagonize the opioid $G\alpha_i$ significantly reversed opioid antinociceptive behavior in the SNL animals on all testing days compared with oxycodone alone ($P < .01$, data not shown).

To quantify the antihypersensitivity observed, percent responses were calculated for each group (Fig 2B). The vehicle control group and the NTX alone group did not result in a significant response throughout the 11 days of testing. The greatest reversal of thermal hypersensitivity for animals receiving oxycodone alone occurred on day 2 ($60.1\% \pm 7.0\%$), with the rapid development of analgesic tolerance thereafter. In contrast, animals cotreated with oxycodone plus NTX showed a peak, $75.2\% \pm 9.0\%$ reversal of thermal hypersensitivity on day 4, with more than 50% antihyperalgesia on all other days through 11 days of treatment and testing (Fig 2B). Preliminary data collected for optimization of the amount of NTX to be used in conjunction with oxycodone were used to analyze a possible dose-dependent effect. A dose-response curve for intrathecally administered NTX combined with 10 μ g oxycodone (intrathecal) was plotted from the percent activity on day 4 for antihyperalgesia (Fig 2C). The A_{50} was calculated to be 0.020 ng NTX. The 95% confidence interval was determined to be 0.001 ng to 0.160 ng NTX. The R^2 value was 0.995, indicating that ultra-low-dose naltrexone acts in a dose-dependent manner to attenuate SNL-induced thermal hyperalgesia.

Tactile allodynia was assessed using calibrated von Frey filaments. Preinjury baseline thresholds averaged 14.5 ± 0.2 g. After the nerve ligation, tactile thresholds fell to 3.0 ± 0.3 g. Paw withdrawal thresholds measured after SNL and over the 7-day testing period did not vary significantly. The peak response of oxycodone-treated animals was a threshold of 4.2 ± 0.5 g on day 1 of testing (Fig 3A). Animals receiving oxycodone plus NTX showed a slight return toward the preinjury threshold on days 1, 2, and 4 (7.4 ± 1.4 g, 6.5 ± 1.0 g, and 6.3 ± 1.1 g, respectively; Fig 3A); these were statistically significant when compared with the oxycodone-alone-treated group ($P < .05$). The percent reversal of allodynia seen throughout the testing period was below 20% for all SNL control groups (oxycodone, NTX, and vehicle; Fig 3B). On day 1, animals given oxycodone plus NTX showed a $41.2\% \pm 9.3\%$ return to the preinjury threshold; on days 2 and 4, the combination therapy produced $30.8\% \pm 6.9\%$ and $33.6\% \pm 7.6\%$ anti-allodynia, respectively (Fig 3B). Compared with the oxycodone alone, these percent activities of the combination therapy were significantly different ($P < .05$). In animals receiving >1000-fold higher dose of NTX (1.0 μ g) with 10 μ g oxycodone, nerve injury-induced allodynia was significantly blocked in comparison to those receiving 10 μ g oxycodone alone ($P < .05$, data not shown).

Ultra-Low-Dose NTX Enhances the Effects of Oral Oxycodone

As in the intrathecal experiment, thermal sensitivity after oral oxycodone with or without NTX was assessed by latency to withdraw the paw from infrared heat (Fig 4A). The mean preinjury paw withdrawal latency was 21.7 ± 0.6 seconds, whereas the mean postsurgery baseline was 11.5 ± 0.7 seconds in SNL animals. Oxycodone alone (10 mg/kg) produced a mean withdrawal latency of 20.7 ± 2.3 seconds on day 1, representing analgesia; however, by day 4 this latency had shortened to 15.5 ± 2.9 seconds, suggesting the development of antinociceptive tolerance. On days 5 through 11 of treatment, the mean withdrawal latency was not significantly different from the postsurgery baseline. In contrast, animals receiving the combination gavage of oxycodone (10 mg/kg) + NTX (3 μ g/kg) showed prolonged paw withdrawal latency, peaking on days 2 and 3 (27.4 ± 2.3 seconds and 28.9 ± 1.9 seconds, respectively). Only on the last day of the 7-day testing period did the latency of this group approach the postsurgery baseline, with a paw withdrawal latency of 12.6 ± 2.5 seconds. Paw withdrawal latencies of animals treated with 3.0 μ g/kg NTX alone, as well as those of vehicle-treated animals, did not significantly vary from the postinjury baseline across the 7-day period.

To quantify the antihypersensitivity, percent responses were calculated for each group (Fig 4B). Oxycodone alone reversed the SNL-induced thermal hypersensitivity by $72.3\% \pm 12.4\%$, $52.6\% \pm 14.1\%$, and $64.5\% \pm 13.2\%$ on days 1, 2, and 3, respectively. Animals cotreated with oxycodone plus NTX showed a 100% reversal of injury-induced hyperalgesia on day 2 of treatment and significant reversal of the ligation-induced hyperalgesia through day 6, with mean percentages ranging from $98.9\% \pm 1.4\%$ to $71.7\% \pm 14.7\%$ (Fig 4B). Neither NTX-treated animals nor vehicle-treated animals showed any significant reversal of thermal hypersensitivity throughout the 7-day time course (<20% attenuation).

A dose-response curve for NTX combined with 10 mg/kg oxycodone was calculated from percent activity of the oral combinations for antihyperalgesia on day 4. The A_{50} was determined to be 0.050 μ g/kg NTX. The 95% confidence interval was calculated to be 0.009 to 0.288 μ g/kg NTX. The R^2 value was 0.998, indicating that oral ultra-low-dose NTX with 10 mg/kg oxycodone reverses SNL-induced thermal hyperalgesia in a dose-dependent manner (Fig 4C).

Using calibrated von Frey filaments, tactile allodynia was again assessed before and after SNL. All animals had preinjury tactile threshold averaging 13.2 ± 0.7 g, and these dropped to 1.8 ± 0.2 g after nerve ligation. Oxycodone-treated control animals showed partial return toward preinjury thresholds on days 1 through 3 (10.7 ± 1.5 g, 8.7 ± 2.0 g, and 6.8 ± 1.8 g, respectively). From day 4 through the experiment, the paw withdrawal thresholds of oxycodone-alone animals were not significantly different from postinjury levels (Fig 5A). Animals receiving oxycodone plus NTX responded to the von Frey probing at thresholds near preinjury values on days 1, 2, and 3, with the peak effect of 15.0 ± 0.0 g (day 3, Fig 5A). These values were significantly different from post-SNL baseline paw withdrawal thresholds and the same-day paw withdrawal thresholds of animals treated with oxycodone alone, indicating the dual therapy evoked an antiallodynic response. By day 6, the withdrawal thresholds of this group had nearly returned to postinjury levels (2.7 ± 0.8 g). No

significant effect was observed in either vehicle-treated or NTX-alone-treated groups over the 7-day time course in the nerve-injured animals.

Percent activity was also used to quantify antiallodynia (Fig 5B). Oxycodone alone produced a $68.1\% \pm 10.9\%$ antiallodynic effect on day 1 that declined to $52.8\% \pm 14.4\%$ and $40.1\% \pm 12.8\%$ on days 2 and 3. Animals exhibited less than 20% antiallodynia by day 4. Oxycodone plus NTX completely reversed injury-induced allodynia on day 3 ($100.0 \pm 0.0\%$), with percent activities of $89.52\% \pm 11.52\%$ and $86.1\% \pm 15.5\%$ on days 1 and 2, respectively. The antiallodynia of the combination treatment declined to $66.8\% \pm 10.3\%$ on day 4 and was near 20% the final 3 days of the experiment. NTX and vehicle-treated animals did not exhibit any significant antiallodynic activity over the testing period.

Using additional oral doses of NTX, a dose-response curve for NTX was calculated from the percent activity of NTX combined with 10 mg/kg p.o. oxycodone on day 1 for antiallodynia. The A_{50} value was $0.999 \mu\text{g/kg}$, the 95% confidence interval was 0.669 to $1.49 \mu\text{g/kg}$ NTX, and the R^2 value was 0.994 (Fig 5C). Behavioral data for additional oxycodone/NTX combinations used in creating dose-response curves are not shown.

Discussion

The present work demonstrates that L_5/L_6 SNL induces MOR coupling to G_s proteins in the spinal dorsal horn, indicating emergence of excitatory signaling by these receptors instead of the typical inhibition via $G_{i/o}$ coupling. This excitatory signaling by MOR may contribute to the hyperalgesia associated with neuropathic injury and the lack of efficacy of chronic morphine administration in some patients. Animal models of neuropathic pain have shown that nerve injury induces a host of pronociceptive, neuroplastic changes, including an up-regulation of dynorphin in the spinal cord, enhanced levels of cholecystokinin, increased activation of phosphorylated p38 MAP kinase, and increased content and evoked release of substance P and CGRP.^{1-4,10,34,52,66,80} Upregulation of cytokine receptor mRNA in the sciatic nerve and dorsal root ganglion after partial spinal nerve ligation has been noted, and SNL has also been reported to decrease MOR density in injured peripheral axons in the ipsilateral Lamina I of dorsal horn, leading to changes in presynaptic- and postsynaptic-evoked responses of MOR-containing fiber.^{2,46,59} These synaptic changes include a decrease in presynaptic MOR-mediated inhibition, reduced inhibition of the miniature EPSP in substantia gelatinosa neurons, a shortened afterhyperpolarization duration in C-fibers in the L_5 dorsal root ganglion, and increased repetitive firing of A δ -fibers during sustained depolarization.^{46,59} In this study, we demonstrate a nerve injury-induced neuroplastic alteration in MOR-G protein coupling profile in ipsilateral lumbar spinal cord tissue of SNL animals.

Although we previously showed that chronic opioid administration causes a shift in MOR-G protein coupling from $G_{i/o}$ to G_s ,⁷⁴ we now show that SNL injury induces this novel MOR- G_s coupling ipsilateral to the injury without changing the extent of coupling to $G_{i/o}$ proteins. Chronic oxycodone administration to nerve-injured rats contributed to this G_s coupling, as chronic oxycodone treatment significantly enhanced the level of G_s coupling compared with that seen in the ipsilateral dorsal horn of vehicle-treated SNL animals. Additionally, MOR-

G_s coupling was detected in both ipsilateral and contralateral dorsal horn of oxycodone-treated SNL rats, whereas the SNL injury itself induced G_s coupling only in the ipsilateral dorsal horn. This alternative coupling by MOR and its augmentation by chronic oxycodone treatment may be an indication of opioid-induced hyperalgesia and decreased efficacy of chronic opioids in neuropathic pain states observed in animals and in some humans.^{6,23,28,30,32,51,54,65,71} Prolonged opioid treatment has also been shown to alter the function of $G\beta\gamma$ -subunits of G proteins coupling to MORs, resulting in multiplicative excitatory effects such as phosphorylation of $G\beta$ and enhanced $G\beta\gamma$ stimulation of adenylyl cyclase, phosphorylation of G protein receptor kinase_{2/3} and $G\beta$ via protein kinase C, and increased activity of PKA, which can also be activated by G_s stimulation of adenylyl cyclase.^{15–20,37,57} The $G\beta\gamma$ that associates with adenylyl cyclases during opioid tolerance has now been shown to originate from the G_s protein coupling to MOR.⁷² In conjunction with increased excitatory signaling via MOR-associated $G\alpha_s$ in an injury state, the $G\beta\gamma$ released by these G_s heterotrimeric proteins may further enhance the analgesic tolerance and hyperalgesia observed clinically.

Chronic cotreatment with ultra-low-dose naloxone diminishes the MOR- G_s protein coupling induced by chronic opioid administration in intact uninjured rats.⁷⁴ In this study, the MOR- G_s coupling after SNL injury was significantly attenuated by ultra-low-dose NTX cotreatment. Although ultra-low-dose NTX might only have reduced the chronic opioid-induced MOR- G_s coupling and may not have specifically reduced the injury-induced MOR- G_s coupling, the combination treatment resulted in a significant enhancement in antihyperalgesic and antiallodynic behavioral responses when compared with oxycodone treatment alone. Furthermore, rapid analgesic tolerance occurred to the effects of oxycodone alone, whereas tolerance to the antihyperalgesic and antiallodynic effects of oxycodone plus NTX was delayed. Both intrathecal and oral cotreatment with ultra-low-dose NTX enhanced the antihypersensitivity effects and diminished the tolerance seen with oxycodone alone. These doses of NTX alone were without effect, illustrating an interaction with the effects of the opioid agonist and underscoring NTX's mechanism via high-affinity binding to the scaffolding protein filamin A.⁷³ A dose >1000-fold higher, delivered intrathecally, blocked the opioid effect completely for nerve injury-induced hypersensitivities by directly antagonizing opioid receptors.

The dose-response curve generated from noxious, thermal stimulation had a gradual slope, indicating slight dose-dependency for ultra-low-dose NTX in the reversal of thermal hyperalgesia. Oral delivery of the combination therapy also produced a dose-dependent antiallodynic effect. These data are encouraging because opioid therapy has little efficacy in ameliorating the allodynia of neuropathic pain.^{8,68} Previous studies using ultra-low-dose NTX for analgesia in intact animals have not noted dose-response effects within a fairly wide but effective ultra-low-dose range and have noted a decreased response only with increasing doses of NTX when classic receptor antagonism begins to interfere with the opioid agonist effect.^{12,53} Again, the relatively flat dose-response curves can be explained by saturation of the high-affinity, filamin A binding sites well before antagonism at opioid receptors can occur.⁷³ Interestingly, the doses of NTX used in this study are 10- to 100-fold higher than the NTX doses shown to enhance analgesia and prevent tolerance in intact animals yet 100-fold to 1000-fold lower than doses used to block opioid analgesia via

MORs.^{25,35,50,56,74,84} Although we believe this to be the first study to assess oral delivery of an ultra-low-dose opioid antagonist in animals, a direct comparison with intrathecal NTX was possible with the study by Powell et al.⁵⁶ The multitude of neuroplastic changes underlying neuropathic pain, including the extent of MOR-G_s coupling now thought to be controlled by filamin A,⁷³ may contribute to the need for somewhat higher doses of NTX in alleviating hypersensitivities in nerve-injured animals compared with NTX doses reported to enhance opioid analgesia in intact rodents.

In conclusion, the present work demonstrates that SNL injury, like chronic opioid treatment, induces G_s coupling by MOR. This aberrant signaling may contribute to the excitatory neurotransmission in spinal dorsal horn after neuropathic injury as well as to the decreased efficacy of endogenous and exogenous MOR agonists in alleviating neuropathic pain. The MOR-G_s coupling may also contribute to the development of opioid analgesic tolerance, opioid-induced hyperalgesia, and differences in μ -opioid efficacy.^{19,24,38,67,74,76} Although the ultra-low-dose NTX attenuation of injury-induced MOR-G_s coupling was mild compared with the suppression of chronic opioid-induced MOR-G_s coupling in intact animals, oxycodone plus ultra-low-dose NTX produced profound and significant antihypersensitivities in the L₅/L₆ SNL model. These data suggest a common mechanism of action for certain ultra-low-dose opioid antagonist/opioid combinations in preventing analgesic tolerance and in alleviating hypersensitivities in SNL rats. These data also suggest that oxycodone plus ultra-low-dose NTX (Oxytrex; Pain Therapeutics, Inc.) may be a promising treatment for neuropathic pain.

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Perspective

The current study investigates whether Oxytrex (oxycodone with an ultra-low dose of naltrexone) alleviates mechanical and thermal hypersensitivities in an animal model of neuropathic pain over a period of 7 days, given locally or systemically. In this report, we first describe an injury-induced shift in μ -opioid receptor coupling from $G_{i/o}$ to G_s , suggesting why a μ -opioid agonist may have reduced efficacy in the nerve-injured state. These data present a novel approach to neuropathic pain therapy.

twice-daily injections (intrathecal) of vehicle, OXY, NTX, or OXY+NTX, for 7 days. On the eighth day of injections, animals received 1 intrathecal bolus of the respective compound and were killed 30 minutes after administration. The lumbar spinal cord was harvested and quartered into the ipsilateral/dorsal, ipsilateral/ventral, contralateral/dorsal, and contralateral/ventral sections. Tissue was fast-frozen in liquid nitrogen within 1 minute of removal. Membranes prepared from these tissues were incubated with either vehicle or 1 μ M morphine, solubilized, and immunoprecipitated with immobilized anti-G α antibodies. The MOR content in the anti-G α immunoprecipitates were then analyzed by Western blotting. Experiments were performed by using lumbar spinal cord tissue either ipsilateral to the side of injury or contralateral as a comparison to the injured side after 8 days of compound or vehicle treatment and 15 days after the nerve injury was induced. **(B)** MOR immunoreactivity from rats with lumbar spinal nerve injury or sham operation: Densitometric quantification of Western blots showing in vitro morphine-stimulated coupling in **(A)**. $\ddagger P < .01$ when compared with vehicle-treated, sham-operated animals $\#P < .01$ compared with the contralateral side; $*P < .05$, $**P < .01$ when compared with respective SNL-operated, vehicle-treated group; $+P < .05$ compared with respective oxycodone-treated group.

Figure 2.

(A) Paw withdrawal latencies of nerve-injured animals injected intrathecally with oxycodone in the absence or presence of naltrexone (NTX): Twice-daily intrathecal injections of oxycodone (OXY, 10 μg/5 μL) plus naltrexone (NTX, 0.33 ng/5 μL) ($n = 16$) produced a partial and transient antihyperalgesic effect in the paw withdrawal latency compared with a much more subtle effect of OXY (10 μg/5 μL, $n = 14$) alone in L₅/L₆ SNL rats. Animals treated with vehicle ($n = 6$) or NTX alone ($n = 12$) did not vary significantly from postinjury baseline values. Days 1 through 11 correspond to the days of drug treatment and are equivalent to days 7 through 18 after the SNL. (B) Antihyperalgesic effect of intrathecally injected oxycodone in the absence or presence of NTX: Twice-daily intrathecal injections of OXY (10 μg/5 μL) plus NTX (0.33 ng/5 μL) produced a partial antihyperalgesic effect in the calculated percent response when compared with a much more subtle effect of OXY (10 μg/5 μL) alone in L₅/L₆ SNL rats. This effect was statistically significant on days 3 through 5 and 8 through 11 ($*P < .05$, ANOVA) compared with animals treated with OXY alone. Both OXY alone and combination therapy groups were significantly different from vehicle treated animals ($P < .05$). Animals treated with vehicle or NTX did not show any significant variance in calculated percent activity over the testing period or between each other. (C) Dose-dependent reversal of thermal hypersensitivity using oxycodone and varying doses of ultra-low-dose NTX: The calculated percent activity on day 4 of intrathecally injected OXY

+ultra-low-dose NTX showed a dose-dependent reversal of thermal hypersensitivity in nerve-injured animals. The A_{50} value for the dose of NTX to be used with 10 $\mu\text{g}/5\ \mu\text{L}$ oxycodone was calculated to be 0.024 $\text{ng}/5\ \mu\text{L}$ (95% confidence interval, 0.007–0.086 $\text{ng}/5\ \mu\text{L}$; $P = .05$, 1-way ANOVA, FlashCalc [Momentum Engineering Software, Inc., Freeport, TX]).

Figure 3.

(A) Paw withdrawal thresholds of nerve-injured animals injected intrathecally with oxycodone in the absence or presence of naltrexone (NTX): Twice-daily intrathecal injections of oxycodone (OXY, 10 μg/5 μL) plus NTX (0.33 ng/5 μL) produced a partial reversal of mechanical allodynia using von Frey filaments compared with a much more subtle effect of OXY (10 μg/5 μL) alone in L₅/L₆ SNL rats. The former was determined to be significantly different than animals treated with OXY alone. Animals treated with vehicle or NTX did not vary from postinjury baseline thresholds over the testing period. (B) Antiallodynic effect of intrathecally injected OXY in the absence or presence of NTX: Twice-daily intrathecal injections of OXY (10 μg/5 μL) plus NTX (0.33 ng/5 μL) produced a partial antiallodynic effect in the calculated percent response when compared with a much more subtle effect of OXY (10 μg/5 μL) alone in L₅/L₆ SNL rats. This effect was statistically significant on days 1, 2, 4, and 5 (**P* < .05, ANOVA) when compared with animals that had received OXY treatment alone. Treatment with OXY or the combination of OXY + ultra-

low-dose NTX showed significant attenuation of SNL-induced allodynia when compared with vehicle-treated animals ($P < .05$). Vehicle-treated and NTX-alone-treated animals did not show significant antiallodynia across the testing period.

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Figure 4.

(A) Paw withdrawal latencies of nerve-injured animals given oral oxycodone in the absence or presence of naltrexone (NTX): Antihyperalgesic effects of oral oxycodone (OXY) with or without NTX at various doses. The OXY-alone group ($n = 12$) received 30 mg/kg on day 1 and 10 mg/kg on days 2 through 7; all OXY+NTX, Oxytrex (Pan Therapeutics, Inc., San Mateo, CA), groups ($n = 6$) received OXY at 10 mg/kg. Statistically significant differences between OXY-treated animals and Oxytrex-treated animals were observed on days 2 through 4 ($*P < .05$, ANOVA, FlashCalc [Momentum Engineering Software, Inc., Freeport, TX]) in the thermal hypersensitivity behavioral test. Vehicle ($n = 6$) and NTX ($n = 6$) control animals showed no variance from post-spinal nerve ligation baseline paw withdrawal latencies. (B) Antihyperalgesic effect of oral OXY in the absence or presence of NTX: Antihyperalgesic effects of oral OXY with or without NTX at various doses. The OXY-alone group received 30 mg/kg on day 1 and 10 mg/kg on days 2 through 7; all OXY+NTX groups received OXY at 10 mg/kg. Animals receiving Oxytrex showed complete reversal of thermal hypersensitivity (shown as 100% activity) on days 2 and 3. OXY-treated animals displayed a maximal reversal of thermal hypersensitivity on days 1 through 3, with a calculated percent activity of 80%. The calculated percent of antihypersensitivity for noxious stimulation was determined to be statistically different between the 2 groups (Oxytrex and OXY) on days 2 through 4 and 6 ($*P < .05$, ANOVA, FlashCalc). Vehicle and NTX control animals did not

respond in an antihyperalgesic manner throughout the testing period. (C) Dose-dependent reversal of thermal hypersensitivity using OXY and varying doses of ultra-low-dose NTX: The calculated percent activity on day 4 of orally administered OXY+ultra-low-dose NTX showed a dose-dependent reversal of thermal hypersensitivity in nerve-injured animals. The A_{50} value for the dose of NTX to be used with 10 mg/kg OXY was calculated to be 0.05 $\mu\text{g/kg}$ (95% confidence interval, 0.009 to 0.288 $\mu\text{g/kg}$; $P < .02$; 1-way ANOVA, FlashCalc).

Figure 5.

(A) Paw withdrawal thresholds of nerve-injured animals given oral oxycodone (OXY) in the absence or presence of naltrexone (NTX): Antiallodynic effects of oral OXY with or without NTX at various doses. The OXY-alone group received 30 mg/kg on day 1 and 10 mg/kg on days 2 through 7; all OXY+NTX groups received OXY at 10 mg/kg. Statistically significant differences between OXY-treated animals and Oxytrex-treated animals were observed on days 3 and 4 ($P < .05$, Student's t test, FlashCalc [Momentum Engineering Software, Inc., Freeport, TX]) in the mechanical hypersensitivity behavioral test. The peak effect of Oxytrex (Pain Therapeutics, Inc., San Mateo, CA) was observed on day 3. Vehicle and NTX control animals showed no variance from post-spinal nerve ligation baseline paw withdrawal thresholds. (B) Antiallodynic effect of oral OXY in the absence or presence of NTX: Antiallodynic effects of oral OXY with or without NTX at various doses. The OXY-alone group received 30 mg/kg on day 1 and 10 mg/kg on days 2 through 7; all OXY+NTX groups received OXY at 10 mg/kg. Animals receiving Oxytrex showed complete reversal of mechanical hypersensitivity (shown as 100% antiallodynia) on day 3. OXY-treated animals displayed a maximal reversal of thermal hypersensitivity on day 1, with a calculated percent activity of 60%. The calculated percent of antiallodynia for non-noxious stimulation was determined to be statistically different between the 2 groups (Oxytrex and oxycodone) on

days 1, 3, and 4 (* $P < .05$, Student's t test, FlashCalc). Vehicle and NTX-treated animals showed no difference in paw withdrawal thresholds across the 7-day testing period. (C) Dose-dependent reversal of mechanical hypersensitivity using OXY and varying doses of ultra-low-dose NTX: The calculated percent activity on day 1 of orally administered OXY +ultra-low-dose NTX showed a dose-dependent reversal of mechanical hypersensitivity in nerve-injured animals. The A_{50} value for the dose of NTX to be used with 10 mg/kg OXY was calculated to be 0.999 $\mu\text{g/kg}$ (95% confidence interval, 0.669–1.490 $\mu\text{g/kg}$; $P < .02$, 1-way ANOVA, FlashCalc).