Published in final edited form as:
J Pain. 2012 May ; 13(5): 498–506. doi:10.1016/j.jpain.2012.02.005.

(+)-Naloxone, an opioid-inactive toll-like receptor 4 signaling inhibitor, reverses multiple models of chronic neuropathic pain in rats

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Abstract

Previous work demonstrated that both the opioid antagonist (−)-naloxone and the nonopioid (+)-naloxone inhibit toll-like receptor 4 (TLR4) signaling and reverse neuropathic pain expressed shortly after chronic constriction injury. The present studies reveal that the TLR4 contributes to neuropathic pain in another major model (spinal nerve ligation) and to long established (2–4 mon) neuropathic pain, not just to pain shortly after nerve damage. Additionally, analyses of plasma levels of (+)-naloxone after subcutaneous administration indicate that (+)-naloxone has comparable pharmacokinetics to (−)-naloxone with a relatively short half-life. This finding accounts for the rapid onset and short duration of allodynia reversal produced by subcutaneous (+)-naloxone. Given that TLR2 has also recently been implicated in neuropathic pain, cell lines transfected with either TLR4 or TLR2, necessary co-signaling molecules, and a reporter gene were used to define whether (+)-naloxone effects could be accounted for by actions at TLR2 in addition to TLR4. (+)-Naloxone inhibited signaling by TLR4 but not TLR2. These studies provide evidence for broad involvement of TLR4 in neuropathic pain, both early after nerve damage and months later. Additional, they provide further support for the TLR4 inhibitor (+)-naloxone as a novel candidate for the treatment of neuropathic pain.

Keywords

TLR4; neuropathic pain; chronic constriction injury; spinal nerve ligation; HEK293-TLR4

Neuropathic pain is difficult to treat, with many patients receiving insufficient relief. It is increasingly recognized that the proinflammatory molecules secreted by glial cells following nerve damage contribute to the maintenance of neuropathic pain.[20] However, there are currently no approved treatments that target this response. One mechanism proposed for the proinflammatory activation of glial cells in response to nerve injury is activation of toll-like receptor 4 (TLR4). TLR4 is a pattern recognition receptor shown to be important in the mediation of neuropathic pain,[3, 27] likely through TLR4 detection of damage-associated signals.[24] TLR4 activation by agonists such as lipopolysaccharide (LPS) produces robust proinflammatory responses.[14] TLR4 is found on, at least, microglia in the brain[2] and signals through a receptor complex including CD14 and MD-2, to create proinflammatory responses.[5]

TLR4 signaling can be temporarily inhibited in a number of ways. Two forms of inactive LPS competitively antagonize LPS binding, siRNA against TLR4 can block proinflammatory responses to LPS[28], and the small molecule naloxone can block TLR4 signaling.[10, 11] Notably, inhibition of TLR4 by all four methods has been shown to reduce neuropathic pain expressed shortly following sciatic chronic constriction injury (CCI).[10, 28] However, for TLR4 to be a potentially viable drug target for the treatment of neuropathic pain, TLR4 must remain important in the mediation of neuropathic pain across time and be important in neuropathic pain of varying etiologies. Currently, it is unknown how long following injury TLR4 continues to play a role in maintaining chronic pain, nor whether acute TLR4 antagonism can reverse other models of neuropathic pain.

The inhibition of TLR4 signaling by naloxone is especially intriguing from a clinical perspective. While siRNA and the large lipopolysaccharide molecules are unable to cross the blood-brain barrier, both (+)- and (−)-naloxone have CNS effects following systemic administration, making it a better candidate treatment. While the (−)-naloxone isomer antagonizes opioid receptors, both the (−)- and (+)-naloxone isomers inhibit TLR4 signaling and reverse neuropathic pain from sciatic CCI.[10] (+)-Naloxone, then, is a blood-brain barrier permeable small molecule that inhibits TLR4 receptor signaling without antagonizing classical opioid receptors. (+)-Naloxone has also been screened on a wide range of CNS targets with no known off-target effects.[22]

In order to better characterize the breadth of effect of (+)-naloxone in treating neuropathic pain, we tested (+)-naloxone against allodynia resulting from spinal nerve ligation (SNL), a second neuropathic pain model. Comparisons of CCI and SNL demonstrate a more robust mechanical allodynia in SNL.[12] In addition, SNL has a greater involvement of the sympathetic nervous system, also found clinically. Therefore, it is useful to demonstrate that a novel compound is effective in a second, distinctly different animal model of neuropathic pain. To establish whether TLR4 remains an important mediator of neuropathic pain across time, (+)-naloxone was tested against CCI months after nerve injury, far longer than commonly tested in experimental pain models.

This work seeks to further our understanding of the role of TLR4 in neuropathic pain through the study of naloxone as a TLR4 inhibitor. Specifically, we hypothesize that (+)- and (−)-naloxone will have similar pharmacokinetic profiles and the ability of (+)- and (−)-naloxone to relieve neuropathic pain will generalize to a second major neuropathic pain model, SNL. Additionally, we predict that (+)-naloxone will relieve neuropathic pain of long duration in both SNL and CCI models, findings which would provide the first evidence that TLR4 is important in long-term maintenance of neuropathic pain. Finally, we explore whether (+)-naloxone will also disrupt TLR2 signaling, in addition to TLR4, as TLR2 is a second pattern recognition receptor that has recently been implicated in the mediation of neuropathic pain.[25]

Materials and Methods

Subjects

Adult, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in Experiment 1. Adult, male, pathogen-free Sprague-Dawley rats (Harlan Labs, Madison, WI) were used Experiments 2, 3 and 4. Adult, male, pathogen-free Sprague-Dawley rats (supplied by Laboratory & Animal Services, Waite Campus, University of Adelaide) were used in Experiment 5. Rats were 275–300g at time of injection in Experiment 1; 130–170 g at time of surgery in Experiments 2, 3, and 4; and 325–375 g at time of surgery in Experiment 5. Rats in all locations were housed in temperature (23±3°C) and light (12 h:12 h light:dark) controlled rooms with water and food available *ad libitum*. All habituation and

behavioral testing procedures were performed during the light phase of the daily cycle. Experimental procedure in Experiment 1 was approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Experimental procedures in Experiments 2, 3 and 4 were approved by the University of Colorado, Boulder Institutional Animal Care and Use Committee. The experimental procedure in Experiment 5 was approved by the University of Adelaide Animal Ethics Committee. Each experimental group contains 5–9 rats with the exception of Experiment 1 which contained 3 rats per group.

Drugs

(+)-Naloxone was obtained from the National Institute on Drug Abuse. (−)-Naloxone was obtained from Sigma (St. Louis, MO). For *in vivo* studies, the toll like receptor 4 agonist lipopolysaccharide (LPS, Sigma, St. Louis, MO) and the toll like receptor 2 agonist Pam3CSK4 (Pam; Invivogen, San Diego, CA) were used. The vehicle for all drugs was sterile endotoxin free saline (Hospira, Lake Forest, IL). (+)-Naloxone, (−)-naloxone, Pam and saline were confirmed to be endotoxin-free at the highest concentrations used in the present studies by the limulus amebocyte lysate assay (Lonza, Walkersville, MD), performed according to the manufacturer's instructions.

Measurement of (+)-naloxone content in plasma samples

A protein precipitation method was used to measure (+)-naloxone content in collected plasma samples, using a blinded procedure. An aliquot (20 μL) of rat plasma and additional 20 μL of 50% acetonitrile were mixed prior to the precipitation with 75 μL of acetonitrile (ACN) containing the isotope-labeled naloxone (naloxone-d5, the internal standard, 5 ng/mL). The mixture was vortexed for 1 min. After centrifugation (3000 r.p.m., 10 min), the supernatant was transferred an autosampler vial, and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). All analytes were separated on a Synergi Polar-RP column (4.6×75 mm, 4 μm, Phenomenex, Torrance, CA) with a flow rate at 1 mL/min. A mobile phase system consisting of methanol: water: formic acid (28: 72: 0.2, v/v/v) with 5 mM ammonium formate was used to achieve the separation. Mass spectrometric detection (Micromass Ultima) was equipped with positive ionization by ESI (electrospray ionization) and mass scanning by MRM (Multiple Reaction Monitoring, m/z 328 → 310 for (+)-naloxone, m/z 333 → 315 for naloxone-d5) analysis. The standard curve range is 1.00 ng/mL to 400 ng/mL and the lower limit of quantitation is 1.00 ng/mL. The LC-MS/MS method was validated with three inter-day assays (N = 12), one intra-day assay (N = 6), all precision values (coefficient of variation, CV) and accuracy values (relative error) were within 15%, suggesting that the method was sufficiently reproducible for analysis of study samples.

Spinal nerve ligation (SNL)

In Experiments 2, 3 and 4, neuropathic pain was induced through ligation of the fifth and sixth lumbar (L) spinal nerves, as described previously.[10, 12, 13] Briefly, under isoflurane anesthesia, the left L5 and L6 spinal nerves were isolated and each nerve root tightly ligated with 6-0 silk suture. Sham controls had an identical surgery procedure except without the ligation of the spinal nerves.

Chronic constriction injury (CCI) of the sciatic nerve

A second model of neuropathic pain used in Experiment 5 was the CCI model of partial sciatic nerve injury.[1] CCI was performed at the mid-thigh level of the left hindleg as previously described.[16] In brief, under isoflurane anesthesia, the sciatic nerve was isolated and loosely tied with four sterile chromic gut sutures (4-0 chromic gut, Ethicon, Somerville, NJ, USA).

Von Frey test for tactile allodynia

All behavioral testing was conducted by an experimenter blind to group assignment. Rats received at least four 1 h habituations to the testing apparatus and environment prior to baseline testing, as in previous studies.[15] Baseline response thresholds were determined prior to nerve injury and rats tested periodically from 3 days post-injury until time of drug administration.

The response threshold to light touch on both plantar hind paws was measured using calibrated microfilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA), as described previously.[17] Briefly, a logarithmic range of hairs from 0.406–15.136 g force were used, allowing both analgesia and allodynia to be measured, following the standard procedures previously described.[4] Responses were fitted to a Gaussian integral psychometric function using a maximum-likelihood fitting method as described previously.[17]

In vitro assay for TLR4 signaling

Two human embryonic kidney-293 (HEK 293) cell lines were used in Experiment 6. One cell line was stably transfected by Invivogen (San Diego, CA) to over-express human TLR4 and co-receptor molecules (MD-2, CD14) (referred to here as HEK-TLR4). The second cell line was stably transfected by Invivogen (San Diego, CA) to overexpress human TLR2 and its coreceptor CD14. In addition, both cell lines stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors activated as part of the TLR2 and 4 signaling cascades. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

Both HEK-TLR4 and HEK-TLR2 cell lines were grown at 37°C (5% CO_2; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selecton [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocine [Invivogen], and 200 nM L-Glutamine [Invitrogen]). The cells were then plated for 48 h in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; 5×10^3 cells/well) with the same media. After 48 h, supernatants were removed and replaced with fresh media. Drugs tested were added in concentrations indicated and incubated for 24 h. Supernatants (15 μl) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 μl test samples were diluted in 45 μl of 1× dilution buffer, transferred to 96-well plates (Thermo, Walthma, MA, USA), and heated at 65°C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min, then cooled on ice to room temperature. Assay buffer (50 μl/well) was added and, 5 min later, reaction buffer (50 μl/well) is added and allowed to incubate for 20 min at room temperature. The light output is then measured in a microplate luminometer (Dynex Technologies, #IL213.1191, Chantilly, VA, USA).

Statistics

Pharmacokinetic parameters in Experiment 1 were calculated using WinNonlin software (Pharsight Corporation, Mountain View, CA). GraphPad Prism (version 5 for Windows, San Diego, CA) software was used for all other statistical analyses. Two-way repeated-measures ANOVAs with Bonferroni post-hoc tests when appropriate were used to determine statistical significance between behavioral measures, with the exception of the sham rats in

Experiment 2, which were analyzed with a 1-way repeated measures ANOVA. One-way ANOVAs with appropriate Bonferroni post hocs were used to compare experimental groups on *in vitro* experiments and to confirm that there were no baseline differences on behavioral measures. For all analyses, $p<0.05$ was considered significant.

Experimental Procedures

Experiment 1: What is the pharmacokinetic profile of systemic (+)-naloxone?

All rats had surgically implanted jugular vein cannula (single-side jugular vein cannula). All animals were fasted 16 hr prior to dosing. (+)-Naloxone in sterile phosphate buffered saline at 10 mg/mL was administered orally (p.o.) and subcutaneously (s.c.) at a dose of 50 and 10 mg/kg, respectively. Blood samples (approximately 0.2 mL) were collected via the jugular vein catheter at 0.25, 0.5, 1, 2, 4, and 8 hr for both treatments. Blood samples were centrifuged at approximately 10,000 rpm for 5 min. at 4°C to obtain plasma. All plasma samples were stored at −20°C until analysis, described previously.

Experiment 2: What dose of (−)-naloxone is effective on neuropathic SNL pain?

All rats underwent baseline (BL) von Frey testing followed by SNL surgery. Two weeks following surgery a robust and stable allodynia had developed. Rats were given (−)-naloxone doses alternating with saline doses in a within-subjects design. Group 1 received saline, 10 mg/kg s.c. (−)-naloxone, and saline in weeks 2, 3 and 4 post-surgery. Group 2 received 1 mg/kg s.c. (−)-naloxone, saline and 100 mg/kg s.c. (−)-naloxone in weeks 2, 3 and 4 post-surgery. This design was utilized to minimize rat numbers and duration of time spent in a painful condition while still providing blinded testing each week. Tactile sensitivity was assessed prior to and 1 and 3 h following each subcutaneous injection. These time points were selected based on pilot data that showed a maximal effect at 1 h that had returned to baseline by 3 h following injection.

Experiment 3: Does (+)-naloxone reverse neuropathic pain 2 weeks following SNL?

All rats underwent baseline (BL) von Frey testing followed by SNL surgery, as in Experiment 2. Two weeks following SNL surgery, after robust and stable tactile allodynia had developed, rats were given 100 mg/kg (+)-naloxone subcutaneously (s.c.) or equivolume saline vehicle in a between-subjects design. An additional group of rats (n=6) were given sham surgery followed by 100 mg/kg (+)-naloxone s.c. and equivolume saline in a counter-balanced, within-subjects design with a 1-week washout between drugs. For all groups, tactile thresholds were tested prior to and 1 and 3 h following s.c. injection.

Experiment 4: Does (+)-naloxone reverse neuropathic pain at 8 wk after SNL?

Eight weeks following SNL surgery, the efficacy of s.c. (+)-naloxone in increasing tactile thresholds was again tested. Six rats from Experiment 3 were used here, along with 6 sham rats from another, similar experiment. All rats underwent BL von Frey testing and then SNL or sham surgery. Beginning 8 weeks following surgery, rats were again assessed for their von Frey response and were then given (+)-naloxone (100 mg/kg, s.c.) or equivolume saline vehicle in a counterbalanced within-subjects design with a one-week washout period between injections. Tactile thresholds were assessed prior to and 1 and 3 h following each s.c. injection.

Experiment 5: Does (+)-naloxone reverse neuropathic pain 16 wk after CCI?

All rats underwent BL von Frey testing and then CCI surgery. Four months later, (+)-naloxone (60 mg/kg, s.c.) or equivolume saline vehicle was given. This lowered dose, relative to preceding experiments was based both on pilot testing and necessitated by

constraints on (+)-naloxone availability at the time that this study was underway. Tactile thresholds were assessed prior to and 30, 60, 90, 120 and 180 min following s.c. injection.

Experiment 6: Does (+)-naloxone antagonize HEK-TLR4 or HEK-TLR2 cell SEAP expression?

HEK-TLR4 cells were stimulated with 0.1 ng/ml LPS to produce a robust increase in SEAP signal. The efficacy of 1, 10 and 100 μM (+)-naloxone to inhibit the LPS signal was tested by coincubating each (+)-naloxone dose with 0.1 ng/ml LPS. Following a 24 h coincubation, supernatants were then assayed for SEAP expression.

Similarly, HEK-TLR2 cells were stimulated with 1 ng/ml Pam to produce a robust increase in SEAP signal of a similar magnitude to the LPS signal investigated in the HEK-TLR4 cells. The efficacy of 1, 10 and 100 μM (+)-naloxone to inhibit the Pam signal was tested by coincubating each (+)-naloxone dose with 1 ng/ml Pam. Following a 24 h coincubation, supernatants were then assayed for SEAP expression.

Results

Experiment 1: (+)-Naloxone has a brief half-life in blood in both oral and subcutaneous routes of administration

(−)-Naloxone is known to have a brief half-life in blood of approximately 0.5–0.67 h.[9, 21] As it is unknown whether the pharmacokinetics of (+)-naloxone would be the same, the timecourse of blood levels of (+)-naloxone was assessed in rats after s.c. administration so to guide the route of (+)-naloxone administration and appropriate time points to test for reversal of allodynia in the following studies. The pharmacokinetics of (+)-naloxone was assessed in rats after oral (50 mg/kg) and s.c. (10 mg/kg) administration. A higher oral dose was used as a low absorption would be anticipated if (+)-naloxone were to prove to be similar to (−)-naloxone in this regard. As shown in Figure 1, (+) naloxone was absorbed very rapidly after p.o. and s.c. administration. The half-life values (mean ± standard deviation) of (+) naloxone were 2.33 ± 1.08 h and 1.57 ± 0.784 h for p.o. and s.c. administration, respectively. The $AUC_{0\rightarrow\infty}$ (area under the curve) of p.o. (50 mg/kg) and s.c. (10 mg/kg administration) were 82.7 and 1825 hr*ng/mL, respectively. The relative bioavailability of (+)-naloxone (p.o. vs s.c.) was only 0.9%. The low oral bioavailability of (+)-naloxone in this study was consistent with (−)-naloxone in the literature (< 1% oral bioavailability).[9]

Given this pharmacokinetic profile, behavioral measures were assessed in subsequent studies at 1 and 3 h following subcutaneous administration, with the prediction of greater drug efficacy at 1 h.

Experiment 2: (−)-Naloxone reverses neuropathic pain at 100 mg/kg but not at 10 or 1 mg/kg

For all *in vivo* behavioral studies, no differences in tactile thresholds were detected between the ipsilateral and contralateral paws prior to or following drug administration; thus, results here and subsequent behavioral studies are reported as the average of both paws.

Hutchinson et al.[10] demonstrated that a single 100 mg/kg (s.c.) dose of either (+)- or (−)-naloxone reversed neuropathic pain induced by CCI. To define whether TLR4 activation may contribute to neuropathic pain beyond just that from CCI, the effects of naloxone were tested on a second major neuropathic pain model. SNL was chosen for study here as it has been reported to be a more robust inducer of mechanical allodynia[12], less inflammatory, as well as being mediated by mechanisms distinct from those of CCI. Here we assessed

whether naloxone also was effective in SNL. (+)-Naloxone is not commercially available and in very limited supply, from NIDA. Given availability constraints of (+)-naloxone and the previously demonstrated parity between (+)- and (−)-naloxone efficacy on *in vitro*[10], *in vivo*[10], and *in silico*[11] tests on blockade of TLR4, the effective dose of (−)-naloxone, rather than (+)-naloxone, on SNL-induced pain was first determined as a prediction of what (+)-naloxone dose to test in Experiment 3.

The dose-response function for the (−)-naloxone reversal of SNL pain was determined using a within-subjects design that took place between 2–4 weeks post-surgery, as described in methods. Rats were habituated and baseline testing conducted as described in the methods. Baselines are lower than typically reported for CCI due to the particularly young and small rats used in the SNL model (~150 g at time of surgery) and were consistent across Experiments 2, 3 and 4. All rats developed allodynia following SNL. There was a significant interaction between treatment group and pain thresholds ($F_{(9, 9)}$=9.37, $p<0.05$, Figure 2), and post hoc tests showed (−)-naloxone significantly reversed allodynia at the 100 mg/kg dose, but not at the 10 or 1 mg/kg doses, one hour following injection. In all cases, rats were at stable allodynia levels prior to (−)-naloxone administration and returned to baseline thresholds by the 3 h timepoint. This result indicated that the effect of (−)-naloxone generalized beyond neuropathic pain from CCI to a second major neuropathic pain model and that the most effective dose of those tested for reversal of SNL-induced neuropathic pain by (+)-naloxone in Experiment 3 was 100 mg/kg.

Experiment 3: (+)-Naloxone reverses SNL-induced neuropathic pain

Experiment 2 showed that 100 mg/kg (−)-naloxone s.c. was sufficient to reverse SNL-induced neuropathic pain. Prior research has shown that the TLR4 antagonists (+)-naloxone and (−)-naloxone were effective at reversing CCI-induced neuropathic pain[10] at the same dose. Therefore, 100 mg/kg (+)-naloxone was selected for subsequent studies of TLR4 mediation of SNL-induced neuropathic pain. Testing (+)-naloxone, rather than just (−)-naloxone, is important given that the effects of the two stereoisomers are distinct in that (+)-naloxone fails to bind classical opioid receptors.

Whether (+)-naloxone produced a similar reversal of SNL-induced neuropathic pain as (−)-naloxone produced in Experiment 2 was tested here. Baseline tactile sensitivity was measured followed by SNL or sham surgery. All rats that underwent SNL surgery developed significant allodynia by fourteen days after SNL, while sham rats failed to develop allodynia. There were no significant differences between groups in baseline tactile sensitivity ($F_{(2,16)}$=1.21, $p>0.05$). Sham and SNL rats were run in at separate times and in different experimental designs and are thus analyzed separately to determine the effect of (+)-naloxone on each surgery. Rats that received SNL surgery did not differ in the established allodynia prior to injection (t_{11}=0. 56, $p>0.05$). In the SNL groups, 100 mg/kg (+)-naloxone significantly reversed the allodynia compared to saline controls ($F_{(3, 11)}$=5.80, $p<0.05$, Figure 3), with post-hoc comparisons showing significant reversal at 1 h after injection (t_{11}=3.83, $p<0.05$), but not at 3 h (t_{11}=0.09; $p>0.05$). In the sham rats, there was no effect of (+)-naloxone on von Frey behaviors ($F_{(6,5)}$=1.20, $p>0.05$).

Taken together, the results of Experiments 2 and 3 support that blockade of TLR4 by both (−)-naloxone and (+)-naloxone, at a dose of 100 mg/kg s.c., was sufficient to reverse established SNL-induced neuropathic pain two weeks following nerve injury while failing to alter pain thresholds in sham rats. Additionally, the magnitude of (+)- and (−)-naloxone reversal of SNL-induced allodynia was comparable, providing further evidence for the non-stereoselective nature of naloxone reversal of neuropathic pain reported previously[10] and suggestive that endogenous opioids are not tempering the expression of SNL allodynia, as

one would have anticipated a pro-nociceptive effect of (−)-naloxone actions via classical opioid receptor binding had that been the case.

Experiment 4: (+)-Naloxone reverses long established SNL-induced neuropathic pain

Long-established neuropathic pain may involve different underlying mechanisms than neuropathic pain expressed shortly after injury. To test whether TLR4 remains important for the mediation of long-established neuropathic pain, rats 8 weeks following SNL or sham surgery were given (+)-naloxone (100 mg/kg, s.c.) or equivolume saline vehicle in a within-subjects design. There were no significant differences in baseline thresholds (t_{12}=1.8, p>0.05). (+)-Naloxone significantly reversed established allodynia in rats with SNL one hour following injection, but did not alter the tactile sensitivity of rats that underwent the sham surgery ($F_{(5,12)}$=19.2 p<0.05, Figure 4). This finding is the first to demonstrate that TLR4 remains importantly involved in the maintenance of long-established neuropathic pain. Additionally, this experiment confirmed that TLR4 blockade had no effect on tactile thresholds of rats that underwent sham surgery 8 weeks prior to testing when compared to sham plus vehicle.

Experiment 5: (+)-Naloxone reverses long established CCI-induced neuropathic pain

Experiment 4 showed that (+)-naloxone was capable of reversing long-established SNL pain. To determine if this ability to reverse long-established neuropathic pain generalized to other models, and to test this in neuropathic pain of even longer duration[18, 19], rats 4 months post-CCI surgery were given (+)-naloxone (60 mg/kg) or equivolume saline vehicle. As noted above, a 60 mg/kg dose was used here due to severely limited (+)-naloxone availability while this study was underway. There were no baseline differences between groups (t_{10}=0.05 p>0.05) and no difference post-operatively prior to injection. (+)-Naloxone caused a significant reversal of CCI-induced allodynia ($F_{(3, 10)}$=26.1, p<0.05. Figure 5). Post-hoc tests showed a significant reversal at 1 h, but not 3 h, following injection.

Experiment 6: (+)-Naloxone antagonizes stimulated HEK-TLR4 cell SEAP expression, but not stimulated HEK-TLR2 cell SEAP expression

While TLR4 has been the TLR most studied in prior studies of neuropathic pain, it is now clear that it is not the only TLR involved. TLR2 has also recently been recognized as a key TLR for the recognition of endogenous danger signals released by stressed, damaged and dying cells. This led to TLR2 being explored for its potential relevance to neuropathic pain. TLR2 does indeed appear to be involved in the mediation of neuropathic pain given that mechanical allodynia is reduced in TLR2 knock-out mice.[25] While previous evidence supports that (+)-naloxone can inhibit TLR4 signaling induced by the classical TLR4 agonist, LPS.[10], these data do not negate a potential action of (+)-naloxone at TLR2 as well. TLR2, in fact, shares cosignaling molecules with TLR4, including CD14[7] and sometimes MD-2[7] as well as a similar downstream intracellular cascade[23] including TIRAP[29]. Because the exact site of (+)-naloxone action along the TLR4 signaling cascade is not yet known, (+)-naloxone has the potential to inhibit TLR2 function as well, given shared cell signaling steps. If this were true, this would have important implications for how (+)-naloxone effects could be interpreted. To investigate whether (+)-naloxone is a TLR2 inhibitor in addition to being a TLR4 inhibitor, HEK-TLR4 and HEK-TLR2 cells were stimulated with a LPS or Pam, respectively, and the ability of (+)-naloxone to inhibit the agonist response measured.

In HEK-TLR4 cells (Figure 6A), (+)-naloxone significantly inhibited TLR4 signaling in response to LPS ($F_{(5, 10)}$=8.04, p<0.05). Post-hoc tests showed that when 1, 10 and 100 μM (+)-naloxone was coincubated with LPS, the SEAP reporter proteins expression was significantly reduced compared to LPS alone (1 uM: (t_{10}=4.38, p<0.05); 10 uM: (t_{10}=4.83, p<0.05); 100 uM: (t_{10}=5.51 p<0.05)), while there were no significant differences between

the 3 doses of (+)-naloxone used (each $p>0.05$) (Figure 6A). In HEK-TLR2 cells (Figure 6B), there was also a significant difference between groups ($F_{(5,21)}=24.1$, $p<0.05$). However, post hoc tests determined that neither 1, 10 nor 100 μM (+)-naloxone coincubated with Pam was significantly different from Pam alone (each $p>0.05$) (Figure 6B). Pam stimulated SEAP expression was significantly higher than vehicle ($t_{21}=6.87$, $p<0.05$) or 100 μM (+)-naloxone alone ($t_{21}=5.77$, $p<0.05$). In summary, (+)-naloxone was able to block the HEK-TLR4 effects of LPS, but not the HEK-TLR2 effects of Pam.

Discussion

The studies presented here expand our knowledge of the TLR4 inhibitors (+)-and (−)-naloxone and their ability to reverse neuropathic pain behaviors. The ability of TLR4 inhibition by (+)- and (−)-naloxone to reverse neuropathic pain in models beyond just short-duration chronic constriction injury (CCI) was tested using a spinal nerve ligation (SNL) model of neuropathic pain. A dose-response study showed (−)-naloxone was able to reverse SNL-induced neuropathic pain at a dose of 100 mg/kg, but not 10 mg/kg or 1 mg/kg, 1 h following s.c. injection. The opioid-inactive isomer, (+)-naloxone, was similarly effective at this dose on SNL-induced neuropathic pain, further evidence that that pain reversal by naloxone is nonstereoselective. This behavioral profile of efficacy at 1 h, but not 3 h, is parsimonious with the pharmacokinetics of (+)-naloxone defined here; namely, that s.c. (+)-naloxone in rat has a half-life of 1.57 ± 0.784 h. Our results with SNL provide the first evidence for TLR4 mediation of a second major neuropathic pain model mechanistically distinct from CCI and further support that naloxone can reverse neuropathic pain in a nonstereoselective manner. Furthermore, we present the first evidence that TLR4 is involved not only in newly expressed neuropathic pain but, importantly, continues to be important in the mediation of even long-standing neuropathic pain. (+)-Naloxone reversed the allodynia expressed either 2 months following SNL or 4 months following CCI. This supports TLR4 as a potential therapeutic target given its enduring mediation of these 2 major neuropathic pain models. Finally, utilizing HEK cells stably transfected to express either TLR4 or TLR2, the ability of (+)-naloxone to inhibit TLR4 and TLR2 signaling was tested, given that: (a) TLR2 has recently been implicated in neuropathic pain[25] and (b) TLR2 and TLR4 are now known to share many elements of their signaling pathways,[7, 23, 29] raising the potential that (+)-naloxone may potentially disrupt signaling from both. If so, such an action would importantly alter interpretation of (+)-naloxone effects. Here, (+)-naloxone inhibited LPS-induced increases in TLR4 reporter protein, as we have published previously,[10] but did not inhibit the TLR2 agonist Pam increase in TLR2 reporter protein, providing the first evidence that the *in vivo* results with (+)-naloxone in CCI and SNL can be appropriately interpreted as reflecting involvement of TLR4 rather than TLR2 as well as adding to the growing lines of evidence of (+)-naloxone's specificity as a TLR4 antagonist given its lack of non-TLR4 actions at other receptors, second messengers, or enzymes.

The effectiveness of (+)-naloxone in reversing neuropathic pain up to 4 months after nerve injury provides additional support for a role of TLR4 in perpetuating neuropathic pain. Previous work has shown that both TLR4 knockout and co-receptor CD14 knockout mice developed reduced analgesia and hyperalgesia up to 14 days following L5 spinal nerve transection.[3, 27] Additionally, siRNA blockade of TLR4 given at time of surgery was also able to reduce the development of CCI neuropathic pain, up to 14 days following nerve injury.[28] (+)-Naloxone, and the LPS-based TLR4 antagonists mutant LPS and LPS-RS were able to reverse neuropathic pain 14 days following nerve injury, as shown here and in Hutchinson et al.,[10] suggestive that TLR4 is being continually stimulated during these neuropathic pain conditions and contributing to the ongoing pain at this time point. The studies here provide new evidence that TLR4 continues to contribute to neuropathic pain for months after injury, far beyond the 14 day period tested in previous work.

Given the role of TLR4 in ongoing neuropathic pain, it is a potential target for novel pharmacological treatment. TLR4 is principally found on microglia in the central nervous system,[2] but has been shown to be upregulated on neurons following ischemic injury.[26] TLR4 activation induces the production and release of proinflammatory cytokines, including interleukin-1β and tumor necrosis factor-α.[5] (+)-Naloxone can reduce the production of proinflammatory cytokines *in vitro* and reduce the microglial activation marker CD11b *in vivo*.[10] This finding suggests that glial activation inhibition and reduced proinflammatory cytokine production are potential mechanisms for (+)-naloxone reversal of neuropathic pain.

(+)-Naloxone provides a blood-brain barrier permeable, small molecule, TLR4 inhibitor that reverses neuropathic pain in multiple models, months after onset, making it an intriguing potential treatment. While (−)-naloxone is similarly effective on neuropathic pain (here and [10]), and *in silico* ligand-receptor modeling indicates that (−)-naloxone docks similarly to the TLR4-MD2 complex as does (+)-naloxone[10], (−)-naloxone has the disadvantage of also inhibiting the endogenous opioid system, which reduces its applicability. One limitation of (+)-naloxone is its short duration of effect, having a half-life in blood of 1.57 h after s.c. dose in the rat. In keeping with this pharmacokinetic profile, the behavioral efficacy of s.c. (+)-naloxone was apparent at 1 hour but gone by 3 hours here and in prior studies.[10] One potential candidate to improve upon the pain reversal of (+)-naloxone is (+)-naltrexone. (+)-Naltrexone is a small molecule that, similar to (+)-naloxone, is the non-opioid active isomer of the opioid receptor antagonist (−)-naltrexone. In humans, (−)-naltrexone has a half life of about 4–6 hours, significantly longer half-life than the 1–1.5 hour half-life of naloxone, and is also blood-brain barrier permeable.[6] (+)-Naltrexone inhibits the LPS-induced increase in HEK-TLR4 cell reporter gene expression, reverses neuropathic pain behaviors when given intrathecally[10], and docks to the TLR4 co-receptor MD-2 *in silico*.[11] However, the lack of commercial availability is presently a serious impediment to investigating the effects of both (+)-naloxone and (+)-naltrexone.

Current evidence suggests that (+)-naloxone is relatively specific to TLR4 receptor activity. In a screen of central nervous system targets other than TLR4, including neuronal neurotransmitter receptors, neurotransmitter transporters, hormone receptors, and enzymes, (+)-naloxone did not have a significant binding affinity to any target.[22] As TLR2 has recently been implicated in neuropathic pain and has overlapping signaling cascades with TLR4, (+)-naloxone was tested for potential cross-reactivity to TLR2 as actions here would impact interpretation of (+)-naloxone effects. (+)-Naloxone did not affect TLR2 activation by an agonist, Pam. However, the HEK-TLR2 cell line was not transfected with MD-2, while the HEK-TLR4 cell line was. While MD-2 is not required for TLR2 signaling, it has been shown to enhance TLR2 responses to the classic TLR4 ligand LPS, among other compounds, while having no effect on signaling following other TLR2 agonists.[8] It remains possible that (+)-naloxone may effect TLR2 signaling in situations where MD-2 is involved. A lack of off-target effects decreases the chances that (+)-naloxone will have negative side effects. Additionally, (−)-naloxone has been used clinically for years to reverse opioid actions, such as respiratory depression, with a good safety profile. Given that chronic neuropathic pain is a widespread condition that often remains resistant to current treatments, the effectiveness of (+)-naloxone on neuropathic pain and ease of administration make it and like compounds intriguing compounds for future study.

Perspective

These studies demonstrated that (+)-naloxone, a systemically available, blood-brain barrier permeable, small molecule TLR4 inhibitor can reverse neuropathic pain in rats, even months after nerve injury. These findings suggest that (+)-naloxone, or similar

compounds, be considered as a candidate novel, first-in-class treatment for neuropathic pain.

Acknowledgments

Disclosures

This work was funded in part by NIH Grants DA024044, DE017782, DA023132 and NIDA contract N01DA-9-8883. This work was also supported in part by the NIH Intramural Research Programs of NIDA and NIAAA. Mark R. Hutchinson is a NHMRC CJ Martin Fellow (ID 465423; 2007-2010) and an Australian Research Council Research Fellow (DP110100297). HEK-TLR4 cells were gifted from Avigen.

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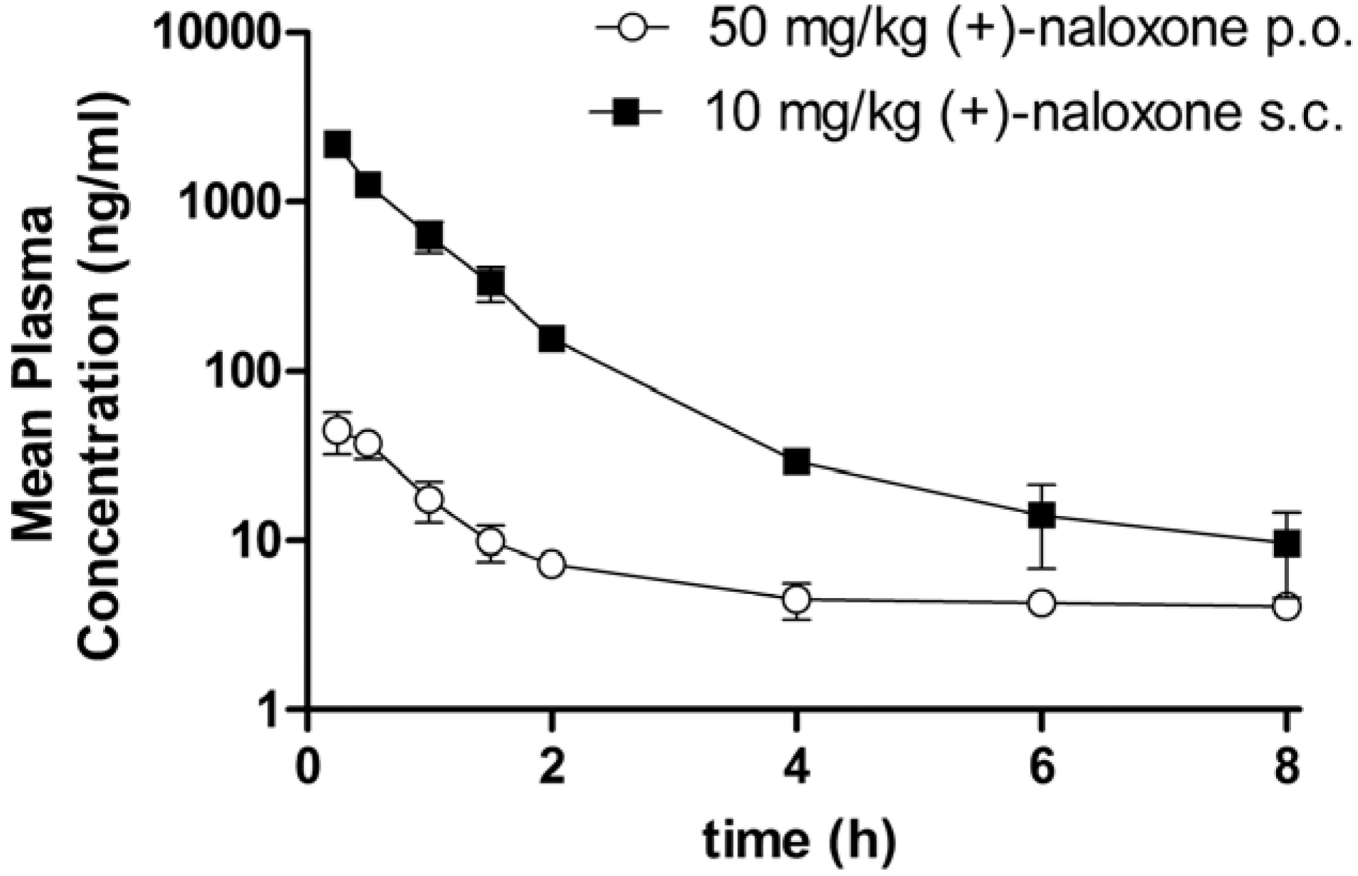

Figure 1.

(+)-Naloxone has a short plasma half-life when given s.c. (10 mg/kg) or p.o. (50 mg/kg) in rats (n=3 per group). The half-life values of (+) naloxone were 2.33±1.08 h and 1.57 ± 0.784 h for p.o. and s.c. administration. The $AUC_{0\rightarrow\infty}$ (area under the curve) of p.o. (50 mg/kg) and s.c. (10 mg/kg administration) were 82.7 and 1825 hr*ng/mL, respectively. The relative bioavailability of (+)-naloxone (p.o. vs s.c.) was only 0.9%. The data are presented as mean ± S.D.

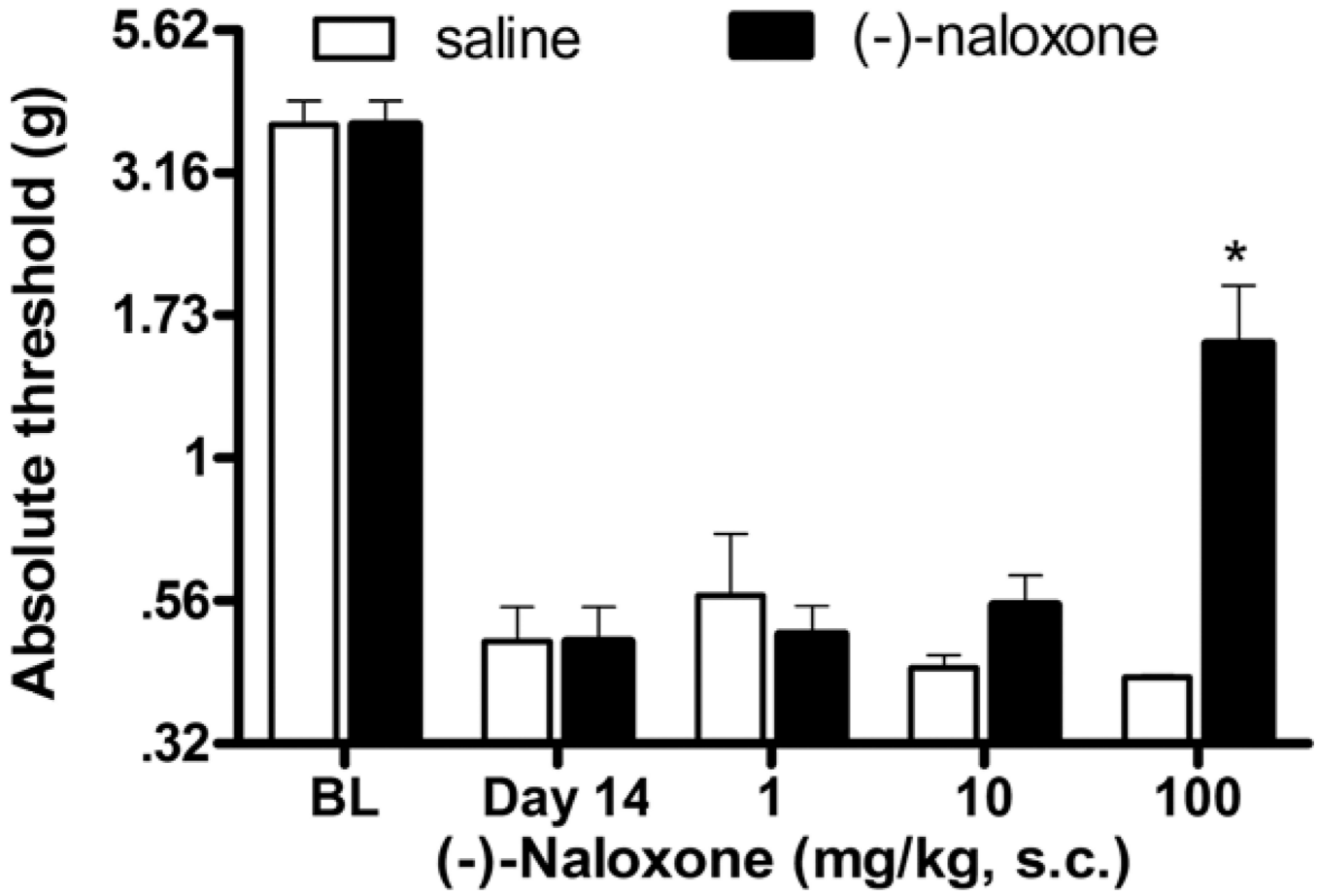

Figure 2.

(−)-Naloxone significantly reversed SNL pain at a dose of 100 mg/kg, but not at lower doses. Data shown are from 1 hour following s.c. injection as all rats at each dose had returned to baseline 3 hours following s.c. (−)-naloxone injection. There were no baseline differences between groups and all rats developed significant allodynia in this within-subjects design (n=12). A star (*) indicates a significant ($p<0.05$) between (−)-naloxone and saline treatments, as indicated by Bonferroni post-hoc tests.

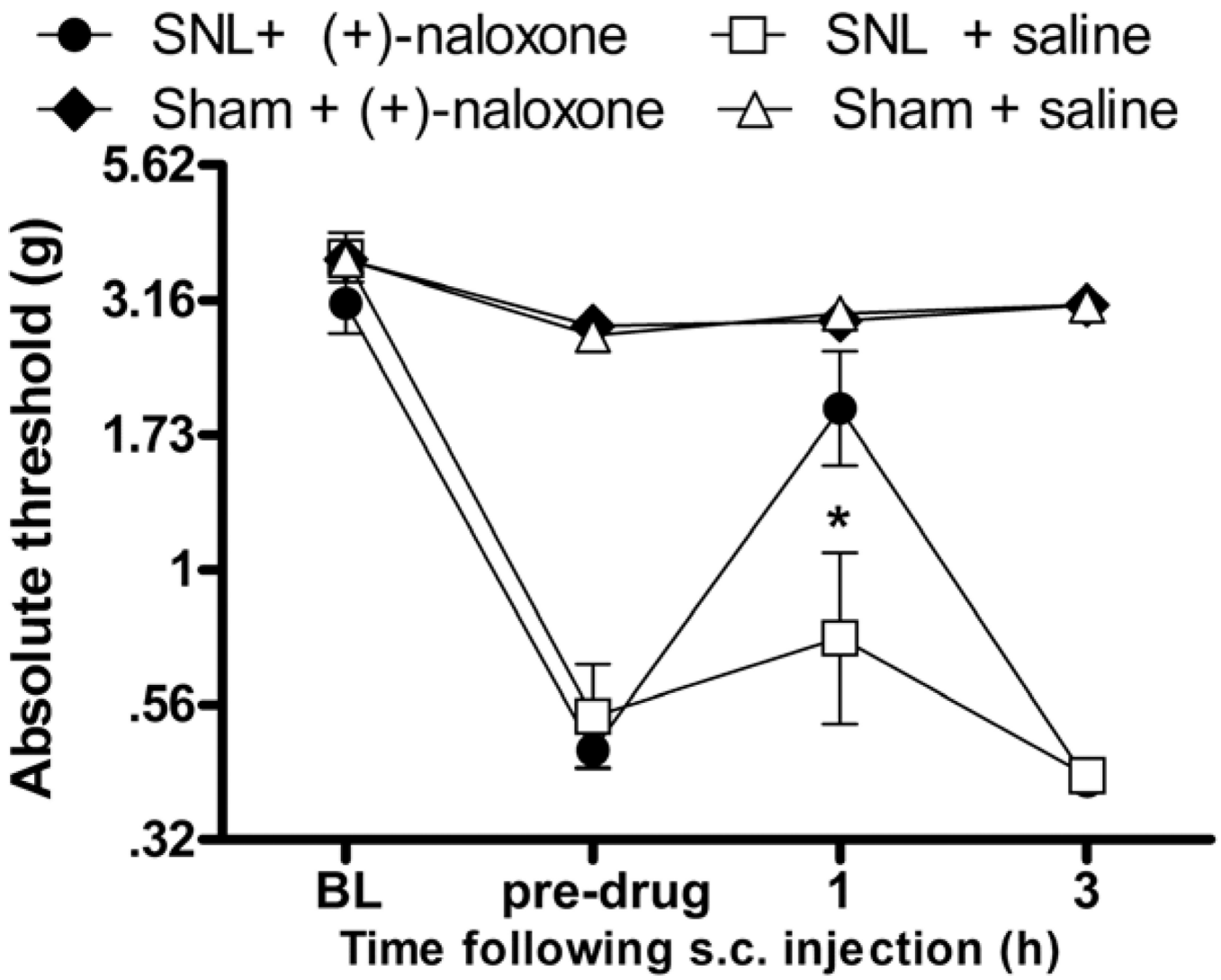

Figure 3.

(+)-Naloxone reverses SNL pain 2 weeks following injury. There were no baseline differences between groups. All rats developed significant allodynia following SNL surgery which was significantly reversed 1 h, but not 3 h, following 100 mg/kg s.c. (+)-naloxone. (+)-Naloxone had no effect on response thresholds in sham operated rats, compared to saline (n=6). A star (*) indicates a significant difference ($p<0.05$) between (+)-naloxone (n=7) and saline (n=6) treated SNL rats, as indicated by Bonferroni post-hoc tests.

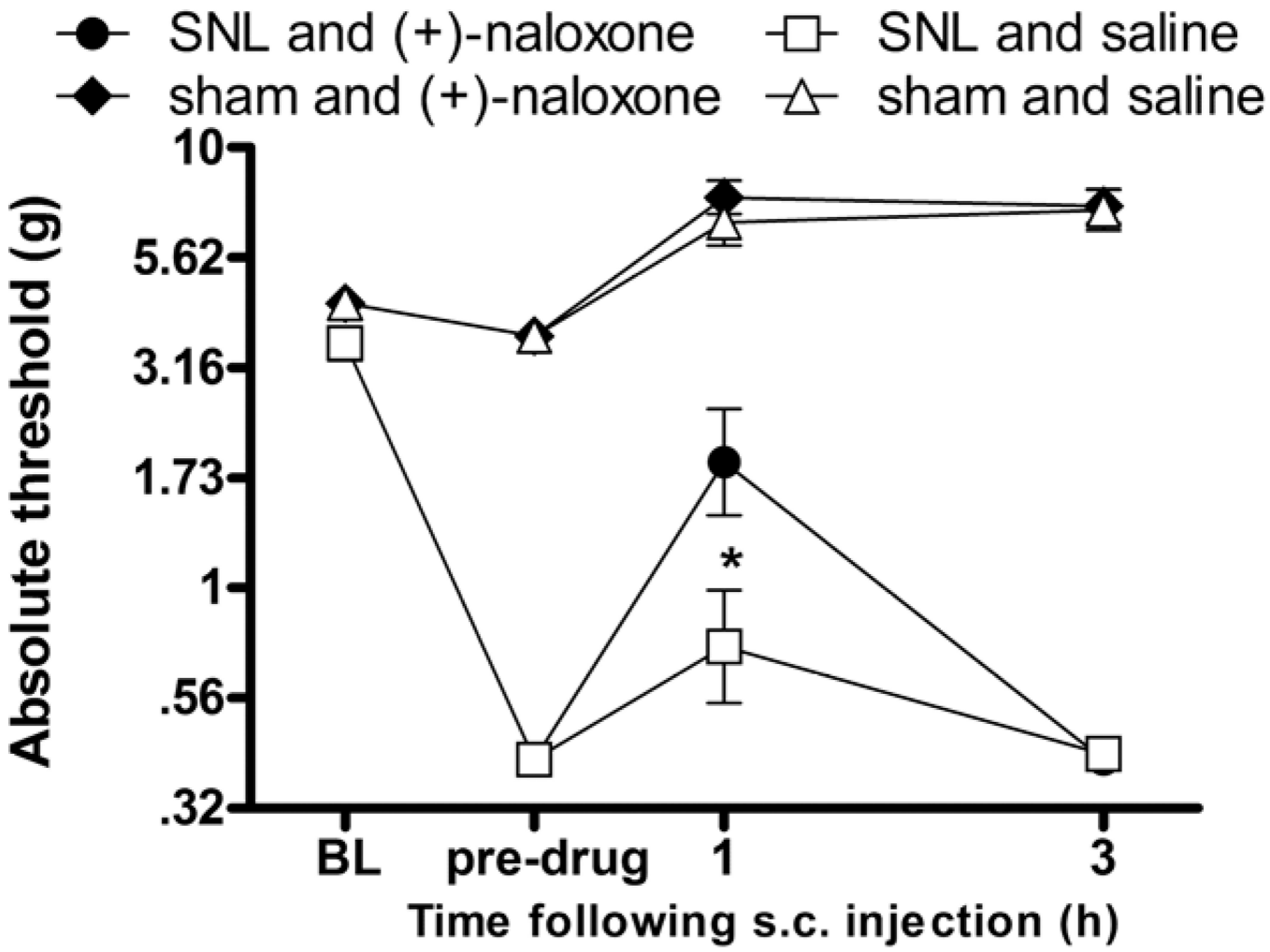

Figure 4.

(+)-Naloxone reverses SNL pain 8 weeks following injury. Rats with SNL surgery (n=9) developed significant allodynia while rats which underwent a sham surgery (n=5) did not. SNL-induced allodynia was significantly reversed 1 h following a 100 mg/kg s.c. dose of (+)-naloxone, but saline vehicle did not significantly change SNL allodynia in this within-subjects design. Neither (+)-naloxone nor saline altered the response thresholds of the rats who received sham surgery. A star (*) indicates a significant difference ($p<0.05$) between (+)-naloxone and saline treatments in the SNL rats.

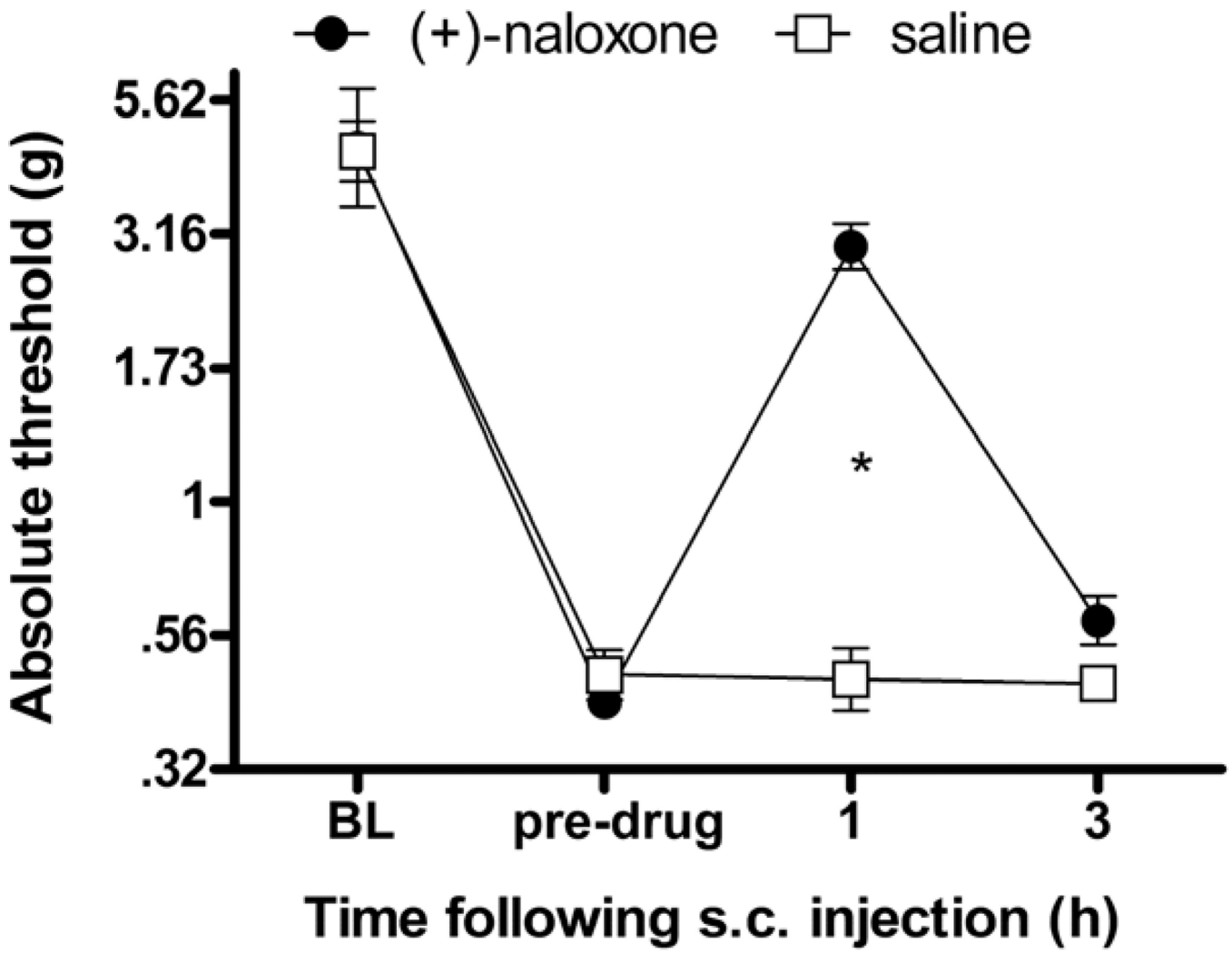

Figure 5.

(+)-Naloxone reversed CCI pain of 4 months duration. There were no significant differences at baseline testing between groups. All rats developed chronic allodynia following CCI surgery. (+)-Naloxone (60 mg/kg, s.c., n=6) significantly reversed allodynia at 1 h, but not 3 h, following injection compared to saline injected rats (n=6). A star (*) indicates a significant difference ($p<0.05$) between (+)-naloxone and saline treated rats.

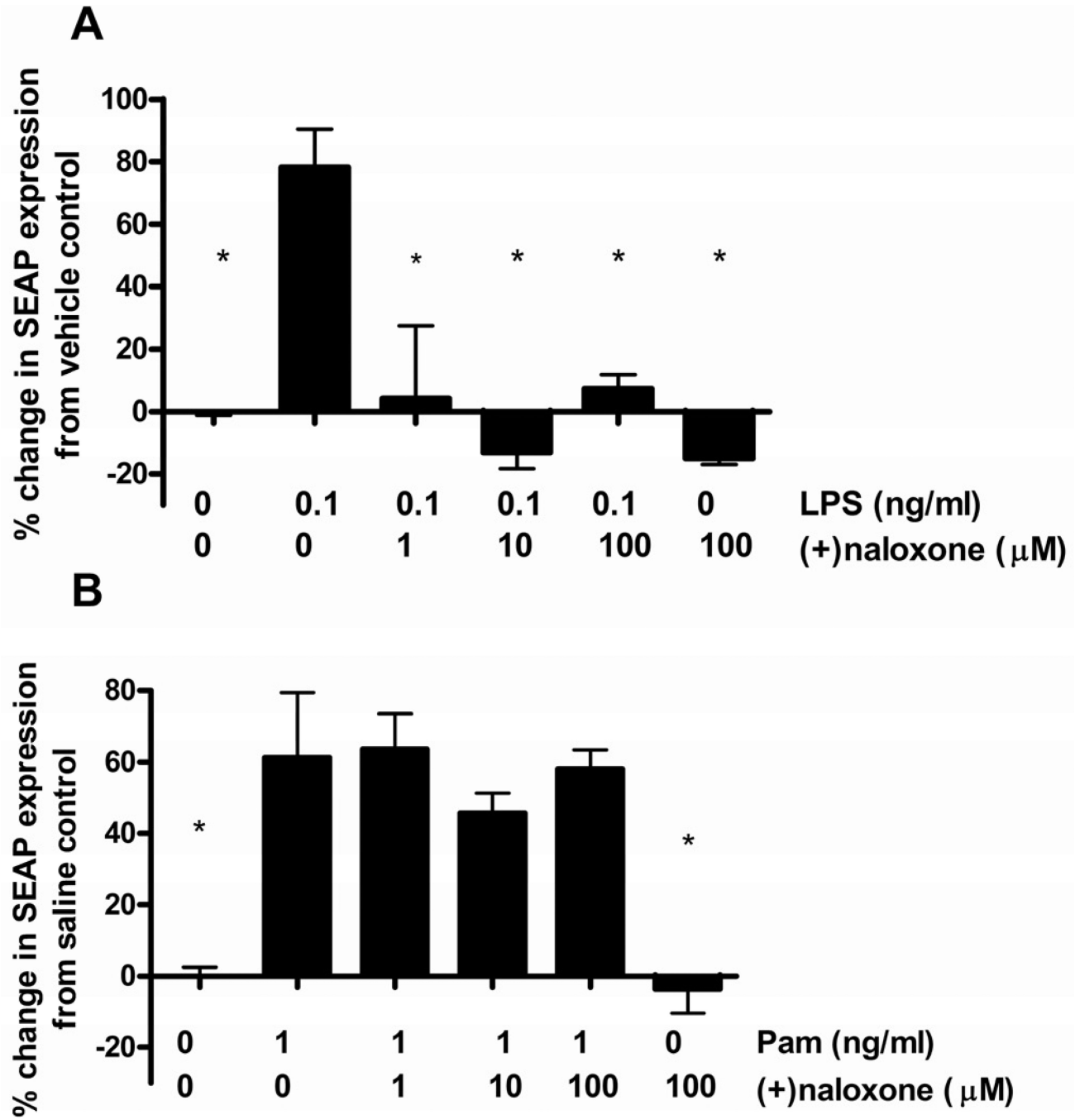

Figure 6.

(+)-Naloxone antagonizes stimulated HEK-TLR4 cell SEAP expression, but not stimulated HEK-TLR2 cell SEAP expression. The increase in SEAP expression seen in LPS stimulated HEK-TLR4 cells was significantly blocked by coincubation with 1, 10 or 100 μM (+)-naloxone. The increase in SEAP expression caused by Pam in HEK-TLR2 cells was unaffected by coincubation with 1, 10 or 100 μM (+)-naloxone. A star (*) indicates a significant decrease ($p<0.05$) from vehicle/agonist (LPS (A) or Pam (B)) group on Bonferroni post-hoc tests.