Behavioral/Systems/Cognitive

Enduring Reversal of Neuropathic Pain by a Single Intrathecal Injection of Adenosine 2A Receptor Agonists: A Novel Therapy for Neuropathic Pain

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Previous studies of peripheral immune cells have documented that activation of adenosine 2A receptors (A_{2A}Rs) decrease proinflammatory cytokine release and increase release of the potent anti-inflammatory cytokine, interleukin-10 (IL-10). Given the growing literature supporting that glial proinflammatory cytokines importantly contribute to neuropathic pain and that IL-10 can suppress such pain, we evaluated the effects of intrathecally administered A_{2A}R agonists on neuropathic pain using the chronic constriction injury (CCI) model. A single intrathecal injection of the A_{2A}R agonists 4-(3-(6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)prop-2-ynyl)piperidine-1-carboxylic acid methyl ester (ATL313) or 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamido adenosine HCl (CGS21680), 10–14 d after CCI versus sham surgery, produced a long-duration reversal of mechanical allodynia and thermal hyperalgesia for at least 4 weeks. Neither drug altered the nociceptive responses of sham-operated controls. An A_{2A}R antagonist [ZM241385 (4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-a)(1,3,5)triazin-5-ylamino]ethyl)phenol)] coadministered intrathecally with ATL313 abolished the action of ATL313 in rats with neuropathy-induced allodynia but had no effect on allodynia in the absence of the A_{2A}R agonist. ATL313 attenuated CCI-induced upregulation of spinal cord activation markers for microglia and astrocytes in the L4–L6 spinal cord segments both 1 and 4 weeks after a single intrathecal ATL313 administration. Neutralizing IL-10 antibodies administered intrathecally transiently abolished the effect of ATL313 on neuropathic pain. In addition, IL-10 mRNA was significantly elevated in the CSF cells collected from the lumbar region. Activation of A_{2A}Rs after intrathecal administration may be a novel, therapeutic approach for the treatment of neuropathic pain by increasing IL-10 in the immunocompetent cells of the CNS.

Introduction

Neuropathic pain, resulting from nerve injury or inflammation, affects ~4 million people in the United States alone (Taylor, 2006) and remains poorly managed by currently available therapeutics. Most of these therapeutics specifically target neurons. However, spinal glia (astrocytes and microglia) play an important role in facilitating and maintaining neuropathic pain in animal models (Watkins et al., 2007). After the initial injury or inflammation, neuronal central sensitization occurs and normally surveying microglial cells become activated to a reactive state (Hanisch and Kettenmann, 2007). Activated glial cells release proinflammatory cytokines [interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α)], chemokines, and other inflammatory mediators such as prostaglandins, reactive oxygen species, and nitric oxide, contribute to the maintenance of central sensitization (Watkins et al., 2007). Recent studies have identified that decreasing spinal proinflammatory cytokines or increasing anti-inflammatory cytokines is effective in attenuating neuropathy-induced allodynia (DeLeo and Yezierski, 2001; Milligan et al., 2006; Watkins et al., 2007). An ideal pharmacological treatment for neuropathic pain would be to avoid short-term blockade of the downstream effects of glial activation and neuronal hyperexcitability, and instead "reset" activated glia back to their basal, surveying state or to an alternatively activated anti-inflammatory state (Gordon, 2003). As yet, no candidate drug has been identified that induces such changes.

One potential candidate for such a drug may be an agonist at a select adenosine receptor subtype. Adenosine can bind four different receptors: adenosine 1 receptor (A_1R), A_{2A}R, A_{2B}R, and A_3R. Most work investigating the effects of adenosine in pain

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models have used adenosine or nonselective agonists and antagonists, which will target multiple adenosine receptors. In addition, some studies have explored the effect of A_1R agonists, as A_1Rs are found predominantly on neurons (Hasko et al., 2007) and A_1R agonists are antinociceptive in a number of different pain models (Lee and Yaksh, 1996; Yamamoto et al., 2003; Zahn et al., 2007).

A_{2A}R agonists may be of special interest. A growing body of literature is presenting A_{2A}R agonists as having potent anti-inflammatory effects on peripheral immune cells, including suppression of proinflammatory cytokines and enhanced production of the anti-inflammatory cytokine, IL-10 (Hasko and Cronstein, 2004). Such a pattern is consistent with A_{2A}Rs being Gαs-linked receptors that stimulate adenylyl cyclase resulting in increased cAMP production (Hasko et al., 2007). In addition to peripheral immune cells, A_{2A}Rs are found on a wide variety of cell types within the CNS (Dare et al., 2007). Although one cannot rule out the possibility of A_{2A}R agonists exerting at least some of their effects on neurons, microglia are the surveying immunocompetent macrophages of the CNS, and astrocytes have immunogenic properties (Ren and Dubner, 2008). Therefore, it is possible that A_{2A}R activation on microglia and astrocytes may produce anti-inflammatory effects within the spinal cord, thus alleviating allodynia from chronic pain states. The present series of studies was designed to explore this possibility through the use of A_{2A}R agonists.

Materials and Methods

Subjects

Pathogen-free male Sprague Dawley rats (325–350 g; Harlan Laboratories) were used for all experiments. Rats were housed two per cage with standard rat chow and water *ad libitum*. Housing was in a temperature-controlled environment (23 ± 2°C) with a 12 h light/dark cycle (lights on at 7:00 A.M.). All procedures occurred in the light phase. All animals were allowed 1 week of acclimation to the colony rooms before experimentation. The Institutional Animal Care and Use Committee of the University of Colorado at Boulder approved all procedures.

Drugs

The A_{2A}R agonist 4-(3-(6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)prop-2-ynyl)piperidine-1-carboxylic acid methyl ester (ATL313) was a gift from PGxHealth, a division of Clinical Data. The half-life of ATL313 is <30 min (Moore et al., 2008). The A_{2A} agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamido adenosine HCl (CGS21680) and the μ (naloxonazine), κ (nor-binaltrophimine), and δ (naltrindole) selective opioid receptor antagonists were purchased from Sigma-Aldrich. The A_{2A}R selective antagonist 4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-a)(1,3,5)triazin-5-ylamino]ethyl)phenol (ZM24385) was purchased from Tocris Bioscience. All of the adenosine agonists and antagonists were dissolved in DMSO to create 10 mM stock concentrations and stored at −20°C. Fresh aliquots were diluted to the appropriate concentration in sterile endotoxin-free isotonic saline (Abbot Laboratories). The opioid antagonists were made fresh immediately before injections. The vehicle for the adenosine agonists and antagonists was 0.01% DMSO saline solution given the dilution of the drugs from stock was 1:10,000 to yield a 1 μM dose. The vehicle for the opioid antagonists was 0.9% saline. All vehicle injections were administered equivolume to the drugs being tested. Rat IL-10 neutralizing antibodies were raised in sheep at the National Institute of Biological Standards and Control (South Mimms, Hertfordshire, UK) and purified by Avigen. Normal sheep IgG was used as a control (Sigma-Aldrich).

von Frey test for mechanical allodynia

Rats were habituated to the testing apparatus for 4 consecutive days before testing. The von Frey test was performed on the plantar surface of each hindpaw within the region of sciatic nerve innervation, as described previously (Milligan et al., 2000). A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (Stoelting) were sequentially applied (from low- to high-intensity threshold) to the left and right hindpaws in random order, each for 8 s at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by $\log_{10}$(milligrams × 10). The range of monofilaments used in these experiments (0.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity [expressed as $\log_{10}$(milligrams × 10)] (Chaplan et al., 1994). The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function (Harvey, 1986). This method normalizes the withdrawal threshold for parametric analyses (Harvey, 1986). The behavioral testing was performed blind with respect to the drug administration.

Modified Hargreaves test for thermal hyperalgesia

Thresholds for behavioral response to heat stimuli applied separately to the tail and each hindpaw were assessed using a modified Hargreaves test (Hargreaves et al., 1988). Baseline withdrawal values were calculated from an average of three consecutive withdrawal latencies of the tail and left and right hindpaws. A cutoff time of 20 s was imposed to avoid tissue damage. As with the von Frey test, this behavioral testing was performed blind with respect to the drug administration.

Surgery

Chronic constriction injury (CCI) (Bennett and Xie, 1988) of the left sciatic nerve was aseptically performed under isoflurane anesthesia. Four ligatures of 4-0 chromic gut were tied loosely around the left sciatic nerve at the level of the midthigh, as described previously (Milligan et al., 2004).

Acute intrathecal injections

Animals were lightly anesthetized with isoflurane. The lumbar region was shaved and cleaned. An 18 gauge guide needle, with the hub removed, was inserted into the L5/6 intervertebral space. A PE-10 catheter was inserted into the guide needle, premarked such that the proximal end of the PE-10 tubing rested over the L4–L6 lumbar spinal cord. All drugs were administered over 20 s (1 μl of drug followed by 2 μl of sterile saline flush) with a 30 s delay before removing the catheter and guide needle. Each animal was anesthetized for a maximum of 5 min, and none incurred observable neurological damage from the procedure.

Immunohistochemistry

The 0.6 mm pieces of the L4–L6 lumbar spinal cord were sectioned (20 μm), mounted onto gelatin-subbed slides, and treated with 0.03% H_2O_2 in Tris-buffered saline for 15 min. Sections were incubated overnight in primary antibodies for monoclonal mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen) and monoclonal mouse anti-rat glial fibrillary acidic protein (GFAP) (1:100; ImmunO). After washing, the sections were incubated with biotinylated secondary antibodies (1:200; Jackson ImmunoResearch) for 2 h at room temperature. The sections were washed followed by 2 h incubation in ABC (1:400; Vector Laboratories), washed, and reacted with 3,3′-diaminobenzadine tetrahydrochloride (DAB) (Sigma-Aldrich). Glucose oxidase and D-glucose were used to generate hydrogen peroxide. Nickeleous ammonium sulfate was used with the DAB reaction to optimize the reaction product. Sections were dried overnight and then dehydrated with graded alcohol, cleared in Histoclear, and coverslipped with Permount. From each animal's spinal cord, five to seven sections within the L4–L6 region were included in the analysis. The ipsilateral and contralateral dorsal horn to the side of injury, of each section, was captured at 10× magnification as a tiff file. Each image was analyzed, under blinded conditions, using NIH ImageJ using a gray scale. The signal pixels within the dorsal horn were identified above 3.5 SDs beyond a control region (lateral column of the cord). The integrated densitometry was calculated as the number of pixels and the average gray scales above the set background (Chacur et al., 2004).

RNA isolation and cDNA synthesis

Total RNA from the lumbar spinal cord was extracted using the standard phenol/chloroform extraction with TRIzol Reagent (Invitrogen) accord-

Table 1. Primer sequences

Gene	Primer sequence (5′–3′)	GenBank accession no.
β-actin	AGAGGCATCCTGACCCTGAA (forward)	NM_031144
	GCTCATTGTAGAAAGTGTGGT (reverse)	
β-actin (i-s)	TTCCTTCCTGGGTATGGAAT (forward)	
	GAGGAGCAATGATCTTGATC (reverse)	
TNF-α	CTTCAAGGGACAAGGCTG (forward)	D00475
	GAGGCTGACTTTCTCCTG (reverse)	
TNF-α (i-s)	CAAGGAGGAGAAGTTCCCA (forward)	
	TTGGTGGTTTGCTACGACG (reverse)	
IL-10	TAAGGGTTACTTGGGTTGCC (forward)	NM_012854
	TATCCAGAGGGTCTTCAGC (reverse)	
IL-10 (i-s)	GGACTTTAAGGGTTACTTGGG (forward)	
	AGAAATCGATGACAGCGTCG (reverse)	

i-s, Intron-spanning primers generated to reduce genomic interference for the CSF cells that did not receive DNAse treatment.

ing to the manufacturer's guidelines. Samples were treated with DNase to remove any contaminating DNA (Ambion). Total RNA was reverse transcribed into cDNA using Superscript II First-Strand Synthesis System (Invitrogen). First-strand cDNA was synthesized using total RNA, random hexamer primer (5 ng/μl) and 1 mM dNTP mix (Invitrogen) and incubated at 65°C for 5 min. After 2 min incubation on ice, a cDNA synthesis buffer [5× reverse transcription (RT) buffer; Invitrogen] and dithiothreitol (10 mM) was added and incubated at 25°C for 2 min. Reverse transcriptase (Superscript III; 200 U; Invitrogen) was added to a total volume of 20 μl and incubated for 10 min at 25°C, 50 min at 42°C, and deactivating the enzyme at 70°C for 15 min. cDNA was diluted twofold in nuclease-free water and stored at −80°C. The cells within the CSF were processed into cDNA using a Cells-Direct III cDNA synthesis kit (Invitrogen) according to the manufacturer's guidelines. Briefly, after washing the cells in 100 μl of Dulbecco's PBS, the CSF cells were lysed in 11 μl of lysis buffer and lysis solution for 10 min on ice. Ten microliters of cell lysate were added to 1 μl of RNase inhibitor and incubated at 75°C for 10 min. First-strand cDNA was synthesized by adding 2 μl of oligo-dT, 1 μl of dNTP, and 7.8 μl of nuclease-free water to the cell lysate and incubating at 70°C for 5 min. After 2 min on ice, 6 μl of 5× RT buffer, 1 μl of RNase inhibitor, 1 μl of Superscript III, and 1 μl of dithiothreitol was added to the cell lysate and incubated at 50°C for 50 min, 5 min for 85°C. Finally, 1 μl of RNase H was added and incubated at 37°C for 20 min. All cDNA was stored at −80°C until real-time PCR (RT-PCR) was performed.

RT-PCR

Primer sequences were obtained from the GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and are displayed in Table 1. Primers were generated to span an intron to eliminate genomic interference in the CSF samples that did not receive DNase treatment. The CSF samples were approached in this manner as the RNA isolation through to cDNA synthesis is completed in the same tube. Therefore, genomic variability was minimized by not doing a DNase treatment but rather designing primer sequences that spanned an intron. Amplification of the cDNA was performed, in a blinded procedure, using Quantitect SYBR Green PCR kit (QIAGEN) in iCycler iQ 96-well PCR plates (Bio-Rad) on a MyiQ single Color Real-Time PCR Detection System (Bio-Rad). The reaction mixture (26 μl) was composed of QuantiTect SYBR Green (containing fluorescent dye SYBR Green I, 2.5 mM $MgCl_2$, dNTP mix, and Hot Start Taq polymerase), 10 nM fluorescein, 500 nM each forward and reverse primer (Invitrogen), nuclease-free water, and 1 μl of cDNA from each sample. Each sample was measured in duplicate. The reactions were initiated with a hot start at 95°C for 25 min, followed by 40 cycles of 15 s at 94°C (denaturation), 30 s at 55–60°C (annealing), and 30 s at 72°C (extension). Melt curve analyses were conducted to assess uniformity of product formation, primer–dimer formation, and amplification of nonspecific products. The PCR product was monitored in real-time, using the SYBR Green I fluorescence, using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log-linear phase of amplification and the threshold cycle (C_T) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene (*β-actin*) using the comparative C_T (ΔC_T) method. The expression of *β-actin* was not significantly different between treatments.

Statistical analysis

Behavioral measures were normalized as described above and analyzed using repeated-measures two-way ANOVA with time and treatment as main effects. The integrated density from the histology and RT-PCR data were analyzed using a two-way ANOVA with surgery and drug administration as main effects. Bonferroni's *post hoc* tests were used where appropriate, and $p < 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

Experimental procedures

Experiment 1: effect of A_{2A}R agonists on peripheral neuropathy-induced mechanical allodynia and thermal hyperalgesia. Baseline behavioral measures were recorded after 4 d of 40 min/d habituation to the testing environment. CCI or sham surgery was then conducted, and behavioral responses to mechanical stimuli or thermal stimuli were tested, in separate groups of rats, at 4 and 10 d after surgery. At 10–14 d after surgery, an acute intrathecal administration of ATL313 (0.1 or 1 μM in 1 μl), CGS21680 (1 or 10 μM in 1 μl), or equivolume vehicle was given (n = 6–7 rats per group) in groups tested for mechanical allodynia. Based on these results, an acute intrathecal administration of either ATL313 (1 μM) or vehicle was given to CCI and sham groups tested for thermal hyperalgesia. For both the mechanical and thermal testing, behavioral responses were measured 4, 24, 72 h, and weekly for 6 weeks after intrathecal drug administration.

Experiment 2: effect of A_{2A}R antagonism on the A_{2A}R agonist effect. As in experiment 1, baseline behavioral measures were performed after 4 d of 40 min/d habituation to the testing environment. CCI was conducted and behavioral responses to mechanical stimuli were tested 4 and 10 d after surgery. At 10–14 d after surgery, an acute intrathecal administration of ATL313 (1 μM) or vehicle (1 μl) was given with either ZM241385 (10 μM; A_{2A}R antagonist) or vehicle (1 μl; n = 6 rats per group). A 10-fold higher dose of the antagonist was used to ensure complete blockade of A_{2A}Rs. Behavioral responses were measured 1, 2, 3, 4, 6, and 24 h after drug administration. In a second group of animals, CCI surgery and behavioral measures were conducted as described above. At 10–14 d after surgery, ATL313 (1 μM) was administered intrathecally. One week after ATL313 administration, ZM21385 (10 μM) or equivolume vehicle (1 μl) was administered intrathecally. Behavioral responses were measured 24 h after ATL313 administration, before ZM241385 and 1, 2, 3, 4, 6, and 24 h after drug administration.

Experiment 3: effect of repeated dosing of an A_{2A}R agonist on CCI allodynia. CCI surgery and behavioral measures were conducted as described in experiment 1. At 10–14 d after surgery, a single intrathecal injection of ATL313 (1 μM) or equivolume vehicle was administered. The rats then received additional injections, the same as that received on the first injection, once every 4 weeks after the first injection for a total of three injections. The behavioral testing was conducted before surgery, before each injection, 24 h after each injection, and weekly thereafter for 14 weeks after the first injection (n = 6/CCI group and n = 6/sham group).

Experiment 4: effect of opioid antagonists on the A_{2A}R agonist-mediated reversal of CCI-allodynia. As previous reports have implicated endogenous opioids in some A_{2A}R-mediated effects at supraspinal sites (Schiffmann et al., 2007), a mixture of selective opioid receptor antagonists was used to explore whether endogenous opioids account for the reversal of CCI-induced allodynia by ATL313. CCI surgery and behavioral measures were conducted as described in experiment 2. At 10–14 d after surgery, ATL313 (1 μM) was administered intrathecally. Once the CCI-induced mechanical allodynia was stably reversed by ATL313, a combination of selective opioid receptor antagonists: μ (naloxonazine; 1 μM in 1 μl), κ (nor-binaltrophimine; 1 μM in 1 μl), and δ (naltrindole; 1 μM in 1 μl) or equivolume vehicle (3 μl) was administered intrathecally (n = 6/group). These doses were chosen to ensure adequate treatment, as they are each 10-fold higher than those known to be effective in abolishing the effects of the opioid agonists *in vivo* (Lu et al., 2004; Nielsen et al., 2007). Behav-

ioral responses were measured 24 h after ATL313 administration, before opioid antagonist/vehicle administration and 0.5, 1, 2, and 3 h after drug administration.

Experiment 5: effect of neutralizing IL-10 antibody on the A_{2A}R agonist effect in CCI allodynia. Chronic constriction injury was conducted and behavioral responses to mechanical stimuli were tested as described in experiment 2. At 10–14 d after surgery, an acute intrathecal administration of ATL313 (1 μM) or equivolume vehicle (1 μl) was coadministered with either sheep anti-rat neutralizing IL-10 IgG antibodies (0.2 μg/ml; 10 μl) or equivolume and equidose sheep IgG (0.2 μg/ml; 10 μl) was coadministered ($n = 6$ rats per group). Behavioral responses were measured 3, 6, 24, 48 h, and 1 week after drug administration. A second injection of sheep anti-rat neutralizing IL-10 IgG antibodies (0.2 μg/ml; 10 μl) or equivolume and equidose sheep IgG was injected intrathecally 1 week later, and behavioral responses to mechanical stimuli were measured before drug administration and 1, 2, 3, 4, 6, 24, 48 h, and 1 week after drug administration.

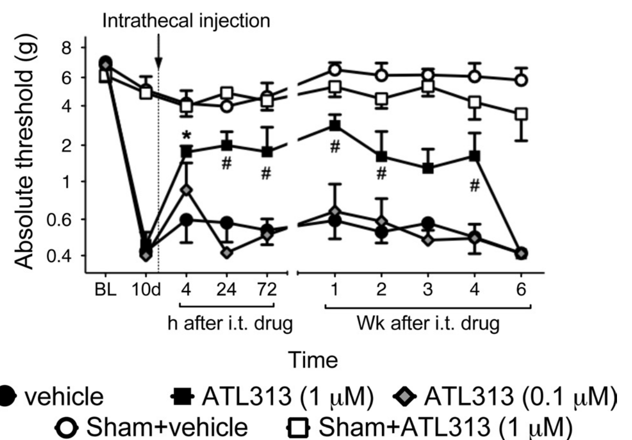
Experiment 6: effect of A_{2A}R agonist on glial activation markers in the lumbar spinal cord. Groups of rats received either CCI or sham surgery, followed 10–14 d after surgery with either ATL313 (1 μM) or equivolume vehicle intrathecally. One week and 4 weeks after drug administration, the animals were injected intraperitoneally with a terminal dose of sodium pentobarbital ($n = 3$–4/group). Animals were transcardially perfused with ice-cold heparinized saline followed by 4% paraformaldehyde/0.1 M PBS. Lumbar spinal cord sections were dissected and postfixed overnight in 4% paraformaldehyde. The lumbar spinal cord was cryoprotected in 30% sucrose solution and processed for microglial and astrocytes activation using immunohistochemistry.

Experiment 7: effect of A_{2A}R agonist on gene expression. Groups of animals ($n = 8$–10/group) received either CCI or sham surgery, followed 10–14 d after surgery with either ATL313 (1 μM) or equivolume vehicle intrathecally. One week after drug administration, rats were deeply anesthetized with sodium pentobarbital (intraperitoneal injection, 0.8 ml). CSF was aspirated via acute lumbar technique as described for the acute lumbar intrathecal injections. The CSF was centrifuged at 1000 × *g* for 10 min at 4°C to pellet the cells. The CSF cells were processed into cDNA as described above. Animals were then transcardially perfused with ice-cold saline for 2 min. The lumbar spinal cord (L4–L6 region) was dissected out. The meninges and lumbar tissue were processed together for gene expression. In a separate group of animals, the overlying meninges were separated from the spinal tissue and processed for gene expression. The CSF was collected on both groups of animals for mRNA analysis. All tissues were flash frozen in liquid nitrogen and stored at −80°C until additional analysis.

Results

Experiment 1: effect of A_{2A}R agonists on peripheral neuropathy-induced mechanical allodynia and thermal hyperalgesia

We examined the effects of two structurally different A_{2A}R agonists on CCI-induced mechanical allodynia to identify whether intrathecal administration of A_{2A}R agonists produces comparable results with that observed *in vitro* (Hasko et al., 1996). Figure 1 shows that the mechanical allodynia induced after CCI surgery remains stable from 10 d throughout the duration of the study (8 weeks after surgery). A single bolus dose of ATL313 (1 μM), administered intrathecally between 10 and 14 d after surgery, induced a significant attenuation of the mechanical allodynia induced by CCI surgery for 4 weeks in both the ipsilateral and contralateral hindpaw ($p < 0.01$; interaction, $F_{(9,160)} = 2.176$; $n = 6$–7/group). Sham surgery had no significant impact on behavior throughout the study. Additionally, ATL313 (1 μM) had no significant effect on behavioral responses of sham-operated rats ($p > 0.05$) (Fig. 1). The lower dose of ATL313 (0.1 μM) administered to allodynic rats had no significant effect on the mechanical allodynia at any time tested ($p > 0.05$; main effect of drug, $F_{(1,9)} = 0.025$) (Fig. 1).



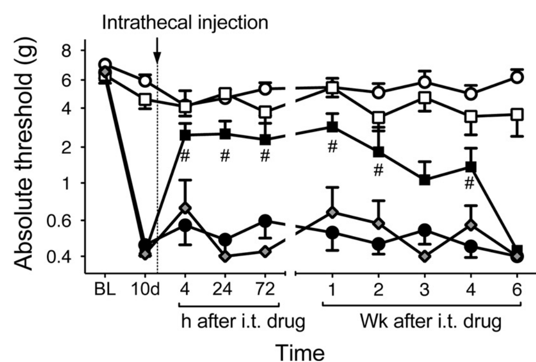


Figure 1. Mechanical allodynia is induced by CCI surgery but not in sham-operated animals. Mechanical thresholds were as assessed by von Frey testing. Intrathecal A_{2A} agonist, ATL313 (A_{2A}R agonist), given 10–14 d after CCI surgery, reverses CCI-induced mechanical allodynia for 4 weeks after administration (solid squares) in both the ipsilateral hindpaw (***A***) and contralateral hindpaw (***B***) (main effect of drug, $F_{(3,19)} = 47.45$, $p < 0.0001$; main effect of time, $F_{(7,133)} = 2.68$, $p = 0.013$; interaction, $F_{(21,133)} = 2.176$, $p = 0.004$; $n =$ 6–7/group). ATL313 has no effect on sham-operated animals. ATL313 reverses bilateral CCI-induced allodynia at 1 μM but has no effect at 0.1 μM on either the ipsilateral (***A***) or contralateral (***B***) hindpaw (main effect of drug, $F_{(3,20)} = 336.4$, $p < 0.0001$; main effect of time, $F_{(9,200)} =$ 11.68, $p < 0.0001$; interaction, $F_{(27,200)} = 11.04$, $p < 0.0001$). Data are presented as mean ± SEM. Solid squares, CCI plus ATL313 (1 μM); gray diamond, CCI plus ATL313 (0.1 μM); solid circles, CCI plus vehicle; open squares, sham plus ATL313 (1 μM); open circles, sham plus vehicle. $^{*}p <$ 0.05, $^{\#}p < 0.01$, drug against vehicle at the respective time point.

Based on these results, the effect of 1 μM ATL313 was tested for its effects on CCI-induced thermal hyperalgesia (Fig. 2). Comparable results were obtained against thermal hyperalgesia as that against mechanical allodynia; that is, ATL313 significantly reversed CCI-induced thermal hyperalgesia for 4+ weeks, with no effect of the drug on the responses of sham controls ($p <$ 0.0001; interaction, $F_{(27,200)} = 11.04$; $n = 6$/group).

To begin to provide converging lines of evidence that A_{2A}R agonism underlies the effects observed above, a second, structurally distinct A_{2A}R agonist, CGS21680, was also tested and found to produce the same effect as that of ATL313 in allodynic rats at a 10-fold higher dose ($p < 0.001$; interaction, $F_{(9,160)} = 2.865$; $n = 6$/group) (Fig. 3), which is consistent with their relative receptor binding affinities. Therefore, both ATL313 and CGS21680 produced a reduction of neuropathic-induced allodynia for 4+ weeks after a single intrathecal administration.

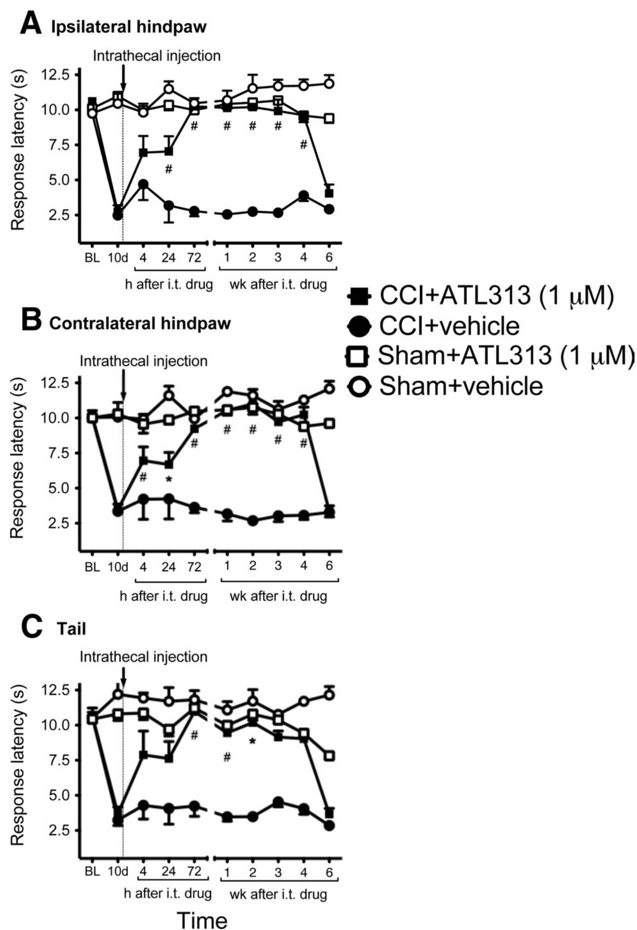


Figure 2. Intrathecal $A_{2A}R$ agonist, ATL313 (1 μM), given 10–14 d after CCI surgery, reverses CCI-induced thermal allodynia for 4 weeks after administration in both the ipsilateral hindpaw (***A***), contralateral hindpaw (***B***), and tail (***C***). ATL313 has no effect on sham-operated animals (main effect of drug, $F_{(3,20)} = 336.4$, $p < 0.0001$; main effect of time, $F_{(9,200)} = 11.68$, $p < 0.0001$; interaction, $F_{(27,200)} = 11.04$, $p < 0.0001$; $n = 6$ per group). We assessed thermal thresholds by modified Hargreaves. Data are presented as mean ± SEM. Solid squares, CCI plus ATL313 (1 μM); solid circles, CCI plus vehicle; open squares, sham plus ATL313 (1 μM); open circles, sham plus vehicle. $^{*}p < 0.05$, $^{\#}p < 0.01$, drug against vehicle at the respective time point.

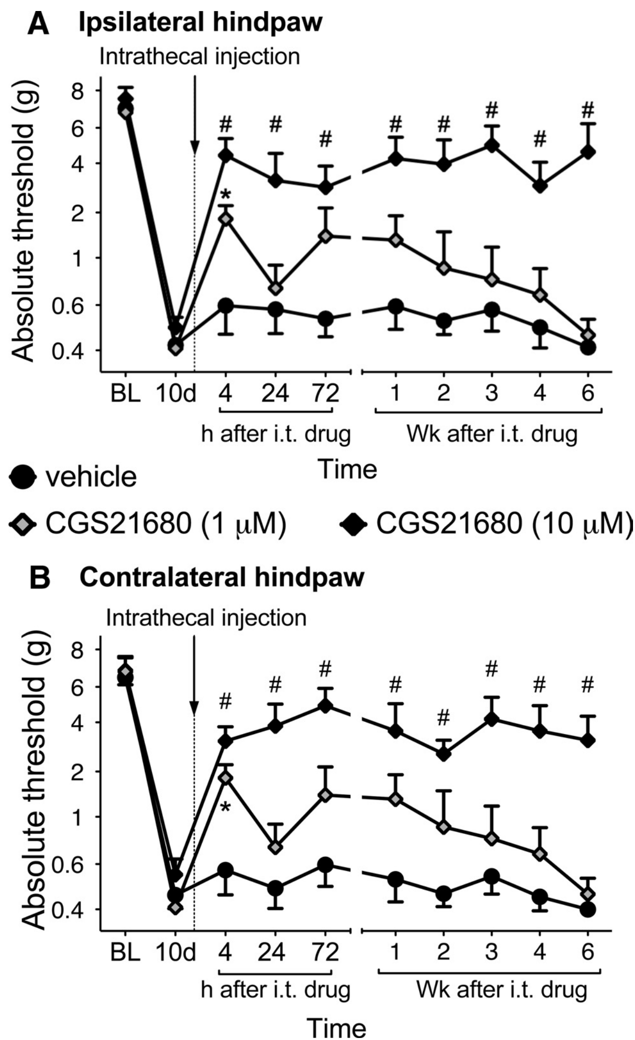


Figure 3. CGS21680 (10 or 1 μM in 1 μl), another A_{2A} agonist, dose-dependently reverses CCI-induced mechanical allodynia (assessed by von Frey filaments) in both the ipsilateral (***A***) and contralateral (***B***) hindpaw but requires a 10-fold higher dose than ATL313 in CCI-induced allodynic animals to produce the same 4 week reversal of allodynia (main effect of drug, $F_{(2,9)} = 93.03$, $p < 0.0001$; main effect of time, $F_{(9,160)} = 23.19$, $p < 0.0001$; interaction, $F_{(18,160)} = 2.87$, $p < 0.01$; $n = 6$ per group). Data are presented as mean ± SEM. Solid diamonds, CCI plus CGS21680 (10 μM); gray diamonds, CCI plus CGS21680 (1 μM); solid circles, CCI plus vehicle. $^{*}p < 0.05$, $^{\#}p < 0.01$, drug against vehicle at the respective time point.

Experiment 2: effect of $A_{2A}R$ blockade on the $A_{2A}R$ agonist effect

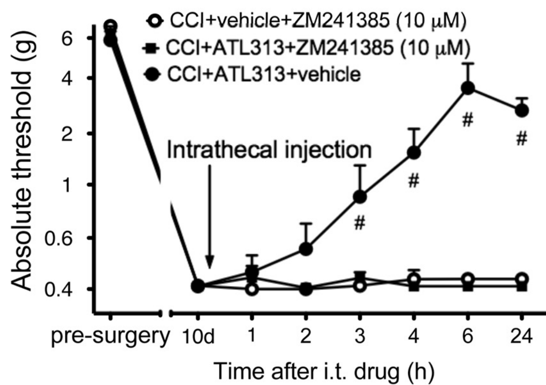
As a second test to validate that the effect of ATL313 is indeed $A_{2A}R$ mediated, a single intrathecal injection of an $A_{2A}R$ selective antagonist (ZM23185) was coadministered with a single intrathecal injection of ATL313 10–14 d after CCI surgery. Coadministration of the $A_{2A}R$ agonist (ATL313) and $A_{2A}R$ antagonist (ZM23185; 10 μM) abolished the effect of the $A_{2A}R$ agonist alone in reversing neuropathic-induced allodynia (Fig. 4*A*) ($p < 0.001$; interaction, $F_{(6,93)} = 12.67$; $n = 6$/group). The $A_{2A}R$ antagonist (ZM23185) administered alone produced no effect on CCI-induced allodynia ($p > 0.05$). Therefore, the initiation of the effect of an $A_{2A}R$ agonist on neuropathic pain is mediated by $A_{2A}R$s. To assess whether the sustained effect is also mediated by $A_{2A}R$, we administered the $A_{2A}R$ antagonist 1 week after the administration of the $A_{2A}R$ agonist (ATL313). There was no significant effect of the $A_{2A}R$ antagonist, ZM23185, on the established ATL313-induced reversal of CCI-induced allodynia ($p = 0.76$; main effect of drug, $F_{(1,9)} = 0.696$; $n = 6$/group) (Fig. 4*B*).

Experiment 3: effect of repeated dosing of an $A_{2A}R$ agonist on CCI allodynia

To assess whether tolerance to the $A_{2A}R$ agonist would occur with repeated dosing, we gave the $A_{2A}R$ agonist once every 4 weeks, when the drug effect was still apparent (Fig. 5). The rats did not develop tolerance to ATL313 when given once every 4 weeks as the reversal of allodynia was maintained for 11 weeks after the first injection, compared with CCI plus vehicle ($p < 0.0001$; interaction, $F_{(39,260)} = 6.439$; $n = 6$/group). The reversal of allodynia may have lasted longer except that the allodynia induced by the CCI was resolving by 11 weeks after intrathecal injection.

Experiment 4: effect of opioid antagonists on the $A_{2A}R$ agonist-mediated reversal of CCI allodynia

Given the interaction between adenosine receptors and opioid receptors, we assessed whether the long-term reduction in allodynia after ATL313 administration in neuropathic rats was opioid receptor mediated. To ensure that we blocked all opioid



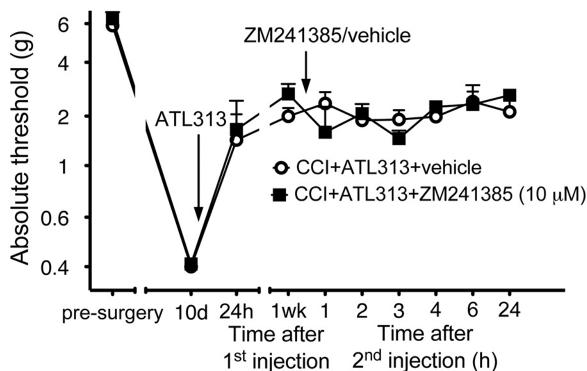
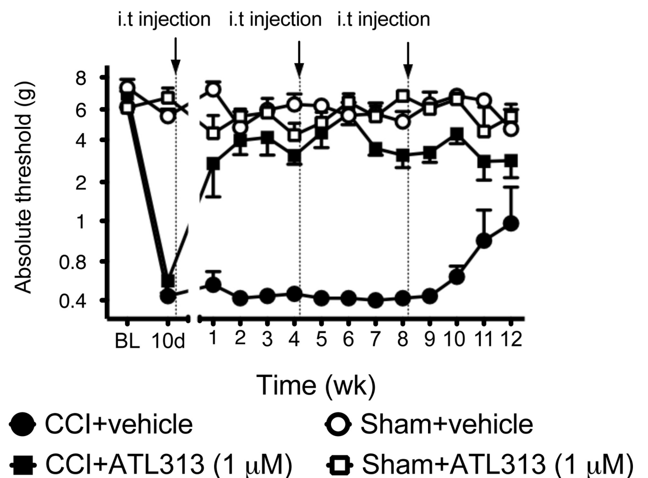


Figure 4. An A_{2A} agonist (1 μM) coadministered with an A_{2A}R selective antagonist (ZM23185; 10 μM) completely abolished the effect of the A_{2A}R agonist in CCI-induced allodynic animals (***A***) (main effect of drug, $p < 0.0001$, $F_{(2,6)} = 110.3$; main effect of time, $p < 0.0001$, $F_{(6,93)} = 13.49$; interaction, $p < 0.0001$, $F_{(12,93)} = 12.67$; $n = 6$ per group). When the antagonist (ZM23185; 10 μM) was administered 1 week after the administration of the A_{2A} agonist (ATL313; 1 μM), in allodynic animals, there was no significant effect of the antagonist on the animals reversed by the agonist (***B***) (main effect of drug, $p = 0.696$, $F_{(1,9)} = 0.153$; $n = 6$ per group). Data are presented as mean ± SEM. Solid circles, CCI plus ATL313 plus vehicle; open circles, CCI plus vehicle plus ZM23185; solid squares, CCI plus ATL313 plus ZM231865. $^{\#}p < 0.01$, ATL313 plus antagonist against ATL313 plus vehicle at the respective time point.



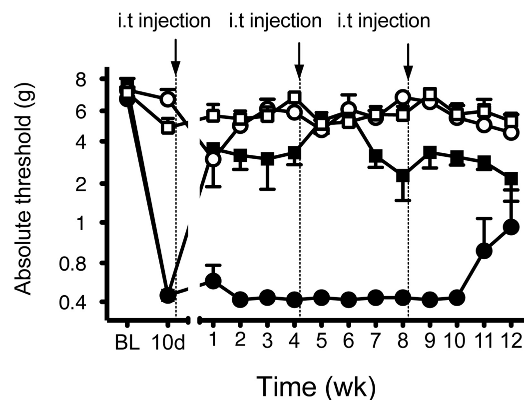


Figure 5. An A_{2A}R agonist (ATL313 1 μM) intrathecally administered every 4 weeks (total, 3 injections) in rats with CCI-induced allodynia is able to sustain the reversal of allodynia for at least 12 weeks (data ongoing), as measured by von Frey testing in both the ipsilateral hindpaw (***A***) and contralateral hindpaw (***B***). The first injection was administered 10 d after CCI or sham surgery (main effect of drug, $F_{(3,20)} = 239.9$, $p < 0.0001$; main effect of time, $F_{(13,260)} = 10.69$, $p < 0.0001$; interaction, $F_{(39,260)} = 6.349$, $p < 0.0001$; $n = 6$ per group). Data are presented as mean ± SEM. Solid squares, CCI plus ATL313 (1 μM); solid circles, CCI plus vehicle; open squares, sham plus ATL313 (1 μM); open circles, sham plus vehicle.

receptors effectively, we used a 10-fold higher dose than that used previously and used antagonists for μ, κ, and δ opioid receptors. After intrathecal ATL313 stably reversed CCI-induced mechanical allodynia for 1 week, a combination of κ (1 μM nor-binaltrophimine), δ (1 μM naltrindole), and μ (1 μM naloxonazine) opioid receptor antagonists were coadministered intrathecally. Before administration of the opioid antagonists or vehicle, there was no significant difference between the groups (Fig. 6). After the administration of the opioid antagonists, there was no significant effect of the opioid antagonists on the A_{2A}R-mediated reversal of neuropathic allodynia compared with vehicle injections ($p = 0.73$; main effect of drug, $F_{(1,7)} = 0.120$; $n = 6$ per group). Therefore, although we have not tested in involvement of opioids in the onset of the A_{2A}R drug effect, the sustained A_{2A}R reversal of mechanical allodynia does not appear to involve endogenous opioids.

Experiment 5: effect of neutralizing IL-10 antibody on the A_{2A}R agonist effect in CCI allodynia

Previous studies of A_{2A}R effects on peripheral immune cells have implicated increased release of IL-10 as importantly contributing to the anti-inflammatory effects of A_{2A}R agonists (Hasko et al., 1996; Csoka et al., 2007b). No previous studies have explored whether IL-10 may be involved in A_{2A}R effects centrally. Therefore, we assessed whether neutralizing IL-10 in the intrathecal space, using IL-10 antibodies, would abolish the effect of the A_{2A}R agonist. After establishment of CCI-induced mechanical allodynia, we administered intrathecal ATL313 together with neutralizing IL-10 IgG versus control IgG (Fig. 7). There was no significant effect of the IL-10 antibodies when coadministered with the ATL313, suggesting that the initial effect of the A_{2A}R agonist is not via IL-10 ($p = 0.314$; main effect of drug, $F_{(3,20)} = 1.263$). One week after the first intrathecal injection, the rats were injected with a second dose each of either neutralizing IL-10 IgG or control IgG, according to the same grouping as the first injection. Now, neutralizing IL-10 reversed the enduring effect of the A_{2A}R agonist ($p < 0.05$; main effect of drug, $F_{(3,20)} = 4.001$; $n = 6$ per group). Interestingly, the effect of the A_{2A}R agonist returned by 48 h after the neutralizing IL-10 antibodies had been administered, suggesting that the A_{2A}R agonist induces sustained release of IL-10 contributing to the long-lasting effect of the drug.

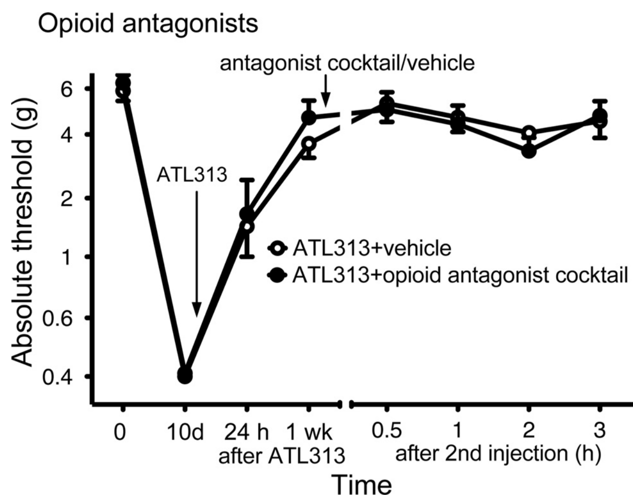
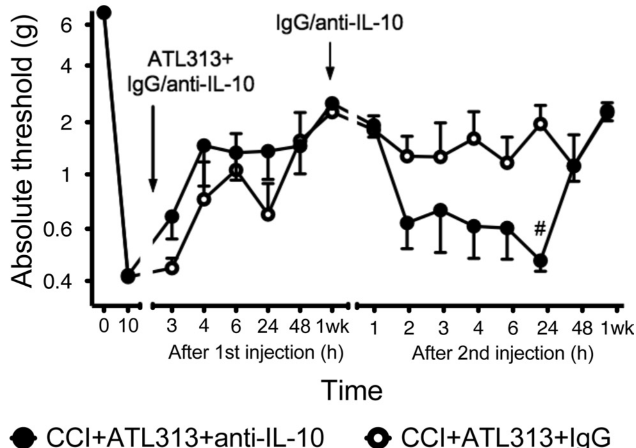


Figure 6. The effect of ATL313 in reversing CCI-induced mechanical allodynia is not mediated by opioid receptors as assessed by von Frey testing. ATL313 (1 μM in 1 μl) was administered intrathecally 10–14 d after CCI surgery. Once the neuropathic pain was stably reversed by ATL313 (at least 1 week), a combination of opioid antagonists selective for κ (1 μM nor-binaltorphimine), δ (1 μM naltrindole), and μ (1 μM naloxonazine) had no effect on established reversal of CCI-induced allodynia by ATL313 measured 30 min, 1, 2, and 3 h after drug administration (1 μM; main effect of drug, $F_{(1,7)} = 0.120$, $p = 0.731$; $n = 6$ per group). Data are presented as mean ± SEM. Solid circle, CCI plus ATL plus opioid antagonist mixture; open circle, CCI plus ATL plus vehicle.

However, given the rats received the IL-10 neutralizing antibodies in both the first and second injection, it is not certain whether the first injection of IL-10 neutralizing antibody may have potentially impacted the effect of the second.

Experiment 6: effect of A_{2A}R agonist on glial activation markers in the lumbar spinal cord

Previous studies of peripheral immune cells have documented that A_{2A}R agonists can suppress the expression of activation markers on monocytes/macrophages (Mantovani et al., 2002; Kreckler et al., 2006; Hasko et al., 2007). No previous studies have explored whether similar effects could be produced centrally. Therefore, we assessed the effect of ATL313 on the predominant immunocompetent cells within the CNS by measuring immunohistochemical indices of glial activation, known to occur in spinal cord dorsal horn, in both the ipsilateral and contralateral sides, in response to CCI (Figs. 8, 9). On the ipsilateral side, microglial activation (measured by OX-42 labeling) in CCI rats was significantly lower 1 week ($p < 0.001$; $F_{(3,9)} = 14.85$) and 4 weeks ($p < 0.01$; $F_{(3,9)} = 7.37$) after a single intrathecal dose of ATL313, compared with rats receiving CCI plus vehicle, sham surgery plus vehicle, or sham surgery plus ATL313. In addition, there was a significant attenuation of the astrocyte activation marker, GFAP, on the ipsilateral side, in CCI rats at 1 week ($p < 0.01$; $F_{(3,11)} = 8.32$) and 4 weeks ($p < 0.05$; $F_{(3,12)} = 4.14$) after a single intrathecal dose of ATL313, compared with rats receiving CCI plus vehicle. On the contralateral side, there was no significant differences in microglial activation markers between any of the groups at either 1 or 4 weeks after drug administration ($F_{(3,8)} = 2.090$; $p = 0.18$). Astrocyte activation marker, GFAP, was significantly increased in the contralateral dorsal horn after CCI surgery compared with sham plus ATL313 at 1 week ($p < 0.05$) and both sham controls at 4 weeks ($p < 0.05$). The increase in astrocytes activation observed in CCI was significantly attenuated after administration of ATL313 at both 1 week ($p < 0.001$; $F_{(3,11)} = 13.96$) and 4 weeks ($p < 0.01$; $F_{(3,10)} = 10.28$).



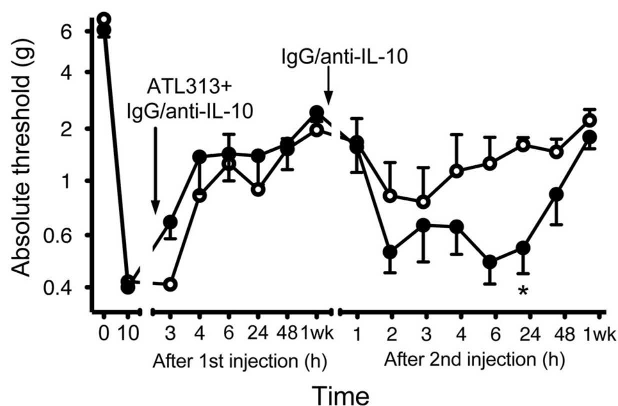


Figure 7. Ten to 14 d after CCI, ATL313 (1 μM) was coadministered intrathecally with either IL-10 neutralizing antibodies (0.2 μg in 10 μl) or equivolume IgG. The behavioral response to mechanical stimuli, assessed by von Frey testing, was measured before surgery, before drug administration, and after drug administration in the ipsilateral (***A***) and contralateral (***B***) hindpaw. No significant effect was found when the drugs were coadministered (main effect of drug, $p = 0.31$, $F_{(3,20)} = 1.263$). Allowing 1 week washout, a second injection of the IL-10 neutralizing antibodies (0.2 μg in 10 μl) or equivolume IgG was administered. The IL-10 neutralizing antibodies reversed the effect of ATL313 at 24 h but had returned within 48 h to that comparable before neutralizing antibody administration (main effect of drug, $p < 0.05$, $F_{(1,8)} = 6.339$; main effect of time, $p < 0.0001$, $F_{(8,80)} = 5.921$; interaction, $p < 0.001$, $F_{(8,80)} = 2.975$; $n = 6$ per group). Data are presented as mean ± SEM. Solid circle, CCI plus ATL plus IL-10 neutralizing antibodies; open circle, CCI plus ATL plus IgG vehicle. $^{*}p < 0.05$, $^{\#}p < 0.01$ antagonist against vehicle at the respective time point.

Experiment 7: effect of A_{2A}R agonist on gene expression

Previous studies of peripheral immune cells have documented that A_{2A} agonists can increase *IL-10* gene expression and suppress the proinflammatory cytokine *TNF-α* gene expression in monocytes/macrophages (Hasko et al., 1996; Csoka et al., 2007a). No previous studies have explored whether similar effects could be produced centrally. Here, tissues (CSF cells, L4–L6 lumbar spinal cord with overlying meninges, or meninges alone; $n = 6–8$/group) were collected after stable reversal of CCI-induced mechanical allodynia by ATL313. As shown in Figure 10, there was a significant increase in *IL-10* gene expression in cells within the CSF (two-way ANOVA with surgery and drug as main effects, drug effect: $p < 0.05$, $F_{(1,22)} = 159.5$). There was no significant difference in *IL-10* mRNA in the meninges between groups (drug effect: $p = 0.33$, $F_{(1,22)} = 0.976$) or in the lumbar tissue with the

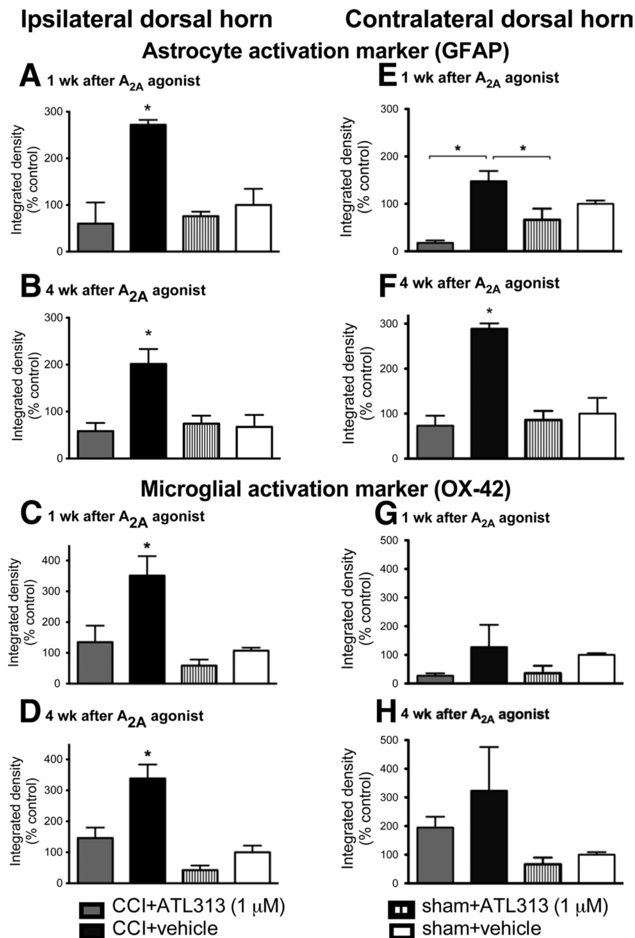


Figure 8. ATL313 administered in animals with CCI attenuates expression of the microglial activation marker (OX-42) and the astrocytes activation marker (GFAP) in lumbar L4–L6 spinal cord sections of the ipsilateral and contralateral dorsal sides. Tissue was collected 1 week (***A***, ***C***, ***E***, ***G***) and 4 weeks (***B***, ***D***, ***F***, ***H***) after intrathecal administration of ATL313 (1 μM) or vehicle in both CCI and sham-operated animals ($n = 3$–4/group) for both GFAP (***A***, ***B***, ***E***, ***F***) and OX-42 (***C***, ***D***, ***G***, ***H***) immunohistochemistry. The integrated density of an average of four to six sections per animal using NIH ImageJ with the signal pixels within the dorsal horn identified above 3.5 of the SD of a control region (lateral column of the cord). The integrated densitometry was calculated as the number of pixels and the average gray scales above the set background. Data are presented as mean ± SEM. $^*p < 0.05$, between groups indicated.

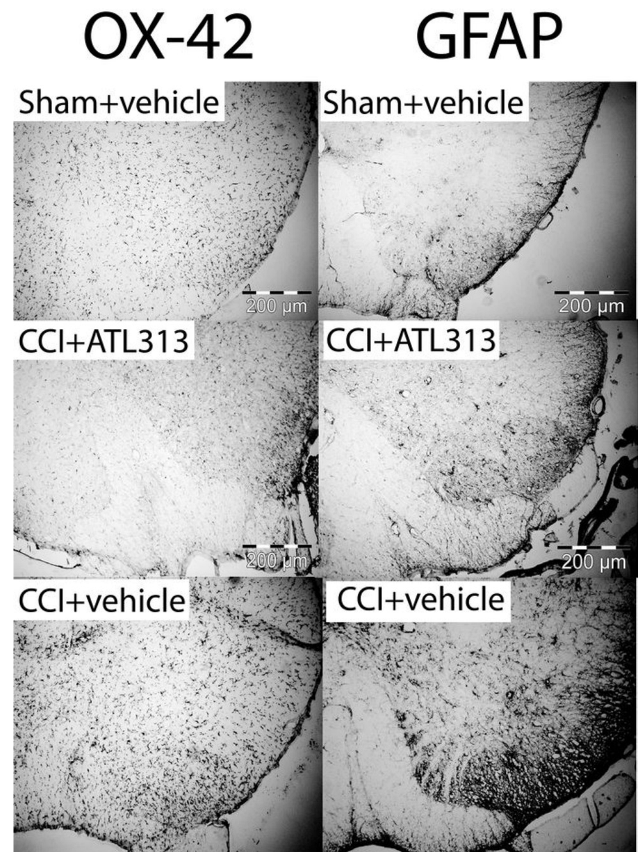


Figure 9. Representative photomicrographs of immunoreactivity of OX-42 and GFAP (10× magnification) are shown displaying the ipsilateral dorsal horn of the lumbar L4–L6 region at 1 week after intrathecal injection. The insets show the morphological changes of the microglia (OX-42) and astrocytes (GFAP) in the CCI rats. The activation of these glia is attenuated after intrathecal administration of an A_{2A} receptor agonist (ATL313) such that the morphology is closer to the sham-operated animals.

overlying meninges (drug effect: $p = 0.898$, $F_{(1,22)} = 0.017$), suggestive that the elevated *IL-10* gene expression is occurring in cells resident within the meninges or infiltrating from the periphery.

There was a significant decrease in *TNF-α* gene expression in the CSF cells (two-way ANOVA, drug effect, $p < 0.05$, $F_{(1,22)} = 4.591$) but no significant difference between CCI and sham ($p = 0.58$; $F_{(1,22)} = 0.316$) or interaction ($p = 0.58$; $F_{(1,21)} = 0.316$). The *TNF-α* mRNA attenuation by ATL313 does support previous *in vitro* experiments showing suppression of *TNF-α* production in peripheral immune cells (Hasko et al., 1996). In addition, like *IL-10* gene expression, there was no significant difference in *TNF-α* gene expression between any of the groups in the lumbar and meningeal tissue or the meningeal tissue alone (drug effect: lumbar and meninges: $p = 0.832$, $F_{(1,22)} = 0.046$; meninges: $p = 0.306$, $F_{(1,22)} = 1.094$).

Discussion

This study documents that a single intrathecal bolus injection of A_{2A}R agonists reverse neuropathic pain for 4+ weeks. This unprecedented, enduring reversal occurred for both mechanical allodynia and thermal hyperalgesia. The A_{2A}R agonist effects likely involve suppression of astrocyte and microglial activation based on activation marker analysis plus involvement of the anti-inflammatory cytokine IL-10 in the pain suppression as IL-10 receptors are expressed by spinal glia, but not spinal neurons (Ledeboer et al., 2003). The effects are not unique to one A_{2A}R-selective compound, as comparable results were obtained using two structurally distinct A_{2A}R-selective agonists (ATL313 and CGS21680). Whether the increased potency of ATL313, relative to CGS21680, reflects differences in receptor affinity, tissue penetration, relative rates of degradation/clearance, or other differences is unknown. The A_{2A}R agonists are not analgesic, but rather antiallodynic/antihyperalgesic, as they have no effect on nonneuropathic animals. Furthermore, the long-duration A_{2A}R agonist effect in neuropathic rats is not opioid mediated, another positive feature for considering targeting A_{2A}R for pain control. The attenuation of neuropathic pain is mediated by A_{2A}R activation initially, but the sustained reversal of allodynia is likely mediated by IL-10 release, possibly from resident or recruited cells in the intrathecal space. The efficacy of A_{2A}R-selective agonists in producing sustained reversal of neuropathic pain is not limited to the therapy being administered shortly (10 d) after neuropathic pain onset as equally potent, sustained reversal of neuropathic pain is observed when treatment is administered 6 weeks after CCI as well (Loram et al., 2009).

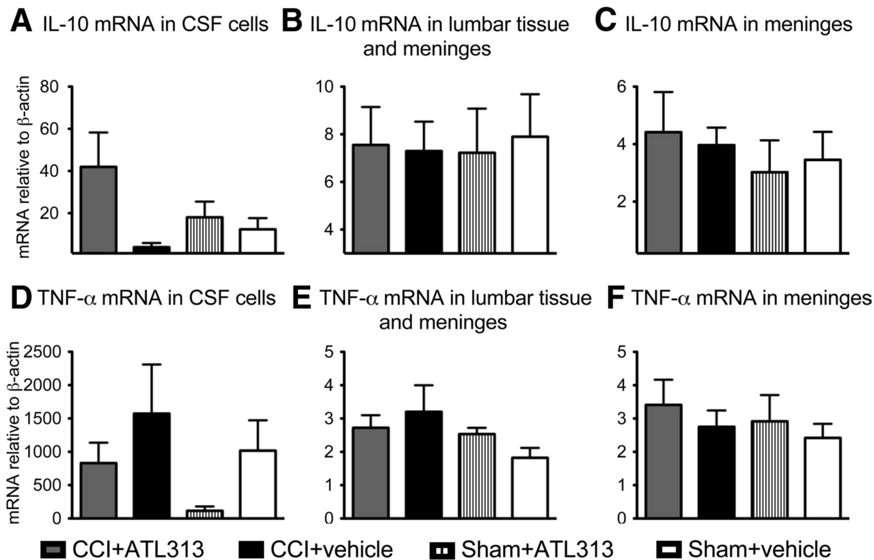


Figure 10. Gene expression mRNA expression in tissue collected 1 week after administration of ATL313 (1 μM) or vehicle in CCI or sham-operated rats. Intrathecal drug administration was 10 d after CCI/sham surgery ($n = 7$–10/group). There was a significant elevation in *IL-10* mRNA in CSF cells after ATL313 administration compared with vehicle controls (two-way ANOVA, drug effect, $p < 0.05$, $F_{(1,21)} = 4.604$; surgery effect, $p = 0.46$, $F_{(1,21)} = 0.57$) (***A***). There was no significant change in *IL-10* mRNA in lumbar and meningial tissue (two-way ANOVA, drug effect, $p = 0.898$, $F_{(1,21)} = 0.017$; surgery effect, $p = 0.933$, $F_{(1,21)} = 0.007$) (***B***) or meninges alone (drug effect, $p = 0.332$, $F_{(1,21)} = 0.976$) (***C***). There was a significant increase in *TNF-α* mRNA in CSF cells after CCI plus vehicle compared with sham plus vehicle (CSF cells, $p < 0.05$, $F_{(1,21)} = 3.792$) (***D***) but not in CCI plus ATL313 versus CCI plus vehicle. There was no significant difference in *TNF-α* gene expression in the lumbar and meninges tissue between any of the groups (drug effect, $p = 0.832$, $F_{(1,21)} = 0.046$) (***E***) or in the meninges alone (drug effect, $p = 0.306$, $F_{(1,21)} = 1.094$) (***F***). Data are presented as mean ± SEM and analyzed using ANOVA. Gray bar, CCI plus ATL313 (1 μM); solid bar, CCI plus vehicle; vertical striped bar, sham plus ATL313 (1 μM); white bar, sham plus vehicle. *$p < 0.05$.

To date, only gene therapy produces longer than ~1 d of pain relief from single injections. Even gene therapy is challenging in the intrathecal space, with single-dose viral vectors failing in efficacy by ~2 weeks (Milligan et al., 2005a,b) and repeated injections of optimized gene therapies required for more extended pain resolution. Given the difficulties of bringing intrathecal gene therapy to clinical trials, identifying novel drug approaches for extended pain relief may be ideal. Also, once-monthly intrathecal drugs that induce endogenous spinal IL-10 avoid potential peripheral immunosuppression inherent in daily systemic administration of glial activation inhibitors, as these may compromise the responses of peripheral immune cells as well (Watkins and Maier, 2003).

IL-10 gene therapy studies provide insights for the potential of single dose intrathecal $A_{2A}R$ agonists for long-term pain control. Foremost is that no tolerance develops with extended IL-10 exposure for resolving thermal hyperalgesia and allodynia. Optimized IL-10 gene therapy provides 3+ months of pain suppression, and when neuropathic pain does reoccur, an additional IL-10 gene therapy treatment again reverses neuropathic pain (Sloane et al., 2009b). Uncompromised pain reduction over extended time periods is recapitulated here, in which single intrathecal doses of ATL313 delivered once each month provided sustained, potent suppression of neuropathic pain. In addition, IL-10 gene therapy operates by driving IL-10 production by cells within the CSF space, with IL-10 induced in this manner acting as a protracted intrathecal delivery of IL-10 to spinal cord glia, thereby suppressing pain (Sloane et al., 2009b). Upregulation of IL-10 in CSF cells appears likely to be recapitulated by single-dose intrathecal $A_{2A}R$ agonists, suggestive that this may well contribute to both suppression of spinally mediated neuropathic pain and glial activation.

Previous studies report that $A_{2A}R$ activation reduces neuroinflammatory symptoms in spinal cord injury, including motor deficits and markers of neuronal injury (Cassada et al., 2002; Reece et al., 2006). We have recently extended this to show that the $A_{2A}R$ agonist, CGS21680, also potently suppresses paralysis in a rat model of multiple sclerosis (Loram et al., 2009). Thus, $A_{2A}R$ agonism has far broader potential clinical utility than neuropathic pain.

No previous reports identified that $A_{2A}R$ agonists elevate IL-10 *in vivo*, either peripherally or centrally, or that blocking IL-10 relieves symptoms, such as neuropathic pain. Unlike *in vitro* studies in which IL-10 is measured within 24 h after 24 h of constant $A_{2A}R$ agonist exposure (Hasko et al., 1996; Kreckler et al., 2006; Csoka et al., 2007b), we have identified that the increase in IL-10 production is sustained *in vivo* after $A_{2A}R$ agonist administration in neuropathic animals for 1+ week but most likely 4 weeks given the duration of effect on neuropathic pain. Comparable with *in vitro* studies, it appears that an inflammatory stimulus is required, such as that seen after CCI surgery and subsequent neuropathy, in order for the $A_{2A}R$ agonist to potentiate the IL-10 production. Although we did not identify a significant interaction between surgery and drug effect in the IL-10 mRNA, higher values were obtained in $A_{2A}R$ agonist-treated neuropathic rats compared with vehicle-treated neuropathic animals.

Adenosine modulation may reduce neuropathic pain via activation of adenosine receptors either/or within spinal cord or resident or recruited immunocompetent cells within meninges or CSF (Ribeiro et al., 2002; Hasko and Cronstein, 2004; Dare et al., 2007). We have previously shown that meningeal cells can produce proinflammatory cytokines after activation both *in vivo* and *in vitro* (Wieseler-Frank et al., 2007). Also, CSF cells significantly increase after peripheral neuropathy and intrathecal IL-10 gene therapy, and IL-10 gene therapy shifts the cells from a predominantly ED1, or classically activated macrophage phenotype, to that of ED2, the alternatively activated or anti-inflammatory phenotype (Sloane et al., 2009b). Potentially, given increased IL-10 mRNA in CSF cells in response to $A_{2A}R$ agonists, such drugs may alter the status of the cells recruited to, or resident within, the CSF and/or meninges into alternatively activated macrophages producing anti-inflammatory IL-10, especially under neuroinflammatory circumstances. The observed glial suppression may reflect $A_{2A}R$ agonist diffusing into spinal tissue altering glial function. Alternatively, it is possible that downstream mediators produced within CSF after $A_{2A}R$ agonists can penetrate spinal tissue and affect glia. In both $A_{2A}R$ knock-out mice, and in our study of $A_{2A}R$ agonists, neuropathic pain was attenuated, correlated with reduction in glial activation markers within the spinal cord (Bura et al., 2008). These seemingly contradictory findings may reflect that glial activation in knock-out

mice was attenuated by a reduction in peripheral A_{2A}R activation, known to be pronociceptive (Taiwo and Levine, 1990; Doak and Sawynok, 1995; Khasar et al., 1995), thereby affecting inputs to the spinal cord. In contrast, in our study the glial activation was attenuated by direct A_{2A}R action within the spinal cord or, more likely, diffusion of CSF-derived IL-10, suppressing glial activation.

Our data are unique in that a single intrathecal A_{2A}R agonist injection produces remarkably enduring reversal of allodynia compared with previous reports in which A_{2A}R effects were only measured within the first 4 h (Lee and Yaksh, 1996; Poon and Sawynok, 1998; Yoon et al., 2005; Zahn et al., 2007), a time when our results show suboptimal impact on allodynia. Also, all previous A_{2A}R agonist studies have used nanomolar doses, below our lowest dose of ATL313 (0.1 μM), a dose at which we did not detect an effect. Given that the effects from A_{2A}R activation take a few hours to achieve, timing of behavioral observations is critical. In a spinal nerve ligation model of neuropathic pain, intrathecal adenosine (nonselective adenosine receptor agonist) and an A_1R agonist each attenuated allodynia for up to 24 h, but no observations were reported after that (Lavand'homme and Eisenach, 1999; Zahn et al., 2007). Studies using the nonselective agonist NECA (5-*N*-ethylcarboxamidoadenosine) suppressed thermal and tactile nociception in rats, dissipating within 1 h (DeLander and Hopkins, 1987). Systemic or intrathecal A_1R agonists produce antihyperalgesia and antiallodynia, resolving within 2 h (Reeve and Dickenson, 1995; Lee and Yaksh, 1996; Sawynok, 1998; Curros-Criado and Herrero, 2005). Therefore, it is possible that activating neuronal adenosine receptors is short-lived versus the prolonged resetting of glial and/or CSF/meningeal immune cells after activation of A_{2A}Rs. Reversal of allodynia, observed here, show that unlike IL-10 protein or IL-1 receptor antagonist (Milligan et al., 2005b; Ledeboer et al., 2007), which produce immediate effects, at least 4 h and possibly even 24 h is required to optimize the reversal of allodynia. This slow onset of reversal, and given that IL-10 neutralizing antibodies failed with coadministration with the A_{2A}R agonist, suggests that downstream mediators, such as IL-10, require time to develop and/or that immune cells need to be recruited and then phenotypically shifted to an anti-inflammatory profile before a full benefit can occur for neuropathic pain.

The potential impact of A_{2A}R agonists as therapeutic agents has not yet been realized, at least for neuroinflammatory diseases (Yan et al., 2003), since too short a time course has been included or the *in vivo* models have limited the ability to identify the astounding duration of drug effect. We are currently investigating the effect of A_{2A}R agonists on a model of multiple sclerosis (experimental autoimmune encephalitis) and revealing dramatic improvement of motor function after single intrathecal A_{2A} agonist administration (Loram et al., 2009), comparable with that seen after pDNA-IL-10 (Sloane et al., 2009a). If such observations are any indication, A_{2A}R agonists are potentially exciting candidates for the treatment of chronic pain and possibly other neuroinflammatory diseases.

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