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Dissociation between morphine-induced spinal gliosis and analgesic tolerance by ultra-low-dose α_2-adrenergic and cannabinoid CB_1-receptor antagonists

Patrick Grenier[a], David Wiercigroch[b], Mary C. Olmstead[b] and Catherine M. Cahill[a,c]

Long-term use of opioid analgesics is limited by tolerance development and undesirable adverse effects. Paradoxically, spinal administration of ultra-low-dose (ULD) G-protein-coupled receptor antagonists attenuates analgesic tolerance. Here, we determined whether systemic ULD α_2-adrenergic receptor (AR) antagonists attenuate the development of morphine tolerance, whether these effects extend to the cannabinoid (CB_1) receptor system, and if behavioral effects are reflected in changes in opioid-induced spinal gliosis. Male rats were treated daily with morphine (5 mg/kg) alone or in combination with ULD α_2-AR (atipamezole or efaroxan; 17 ng/kg) or CB_1 (rimonabant; 5 ng/kg) antagonists; control groups received ULD injections only. Thermal tail flick latencies were assessed across 7 days, before and 30 min after the injection. On day 8, spinal cords were isolated, and changes in spinal gliosis were assessed through fluorescent immunohistochemistry. Both ULD α_2-AR antagonists attenuated morphine tolerance, whereas the ULD CB_1 antagonist did not. In contrast, both ULD atipamezole and ULD rimonabant attenuated morphine-induced microglial reactivity and astrogliosis in deep and superficial spinal dorsal horn. So, although paradoxical effects of ULD antagonists are common to several G-protein-coupled receptor systems, these may not involve similar mechanisms. Spinal glia alone may not be the main mechanism through which tolerance is modulated. *Behavioural Pharmacology* **29:241–254**

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Introduction

Opioids are highly efficacious in the treatment of moderate to severe acute postoperative and traumatic injury-induced pain. The therapeutic usefulness of these drugs in the treatment of many long-term and persistent pain states is limited by a decrease in efficacy (Ballantyne and Shin, 2008) and potency (Christie, 2008), as well as the development of opioid-induced hyperalgesia (Mao, 2002). One novel strategy to mitigate analgesic tolerance is through concomitant use of ultra-low-dose (ULD) G-protein-coupled receptor (GPCR) antagonists. ULD is defined as a concentration several log units below the levels that result in ligand-induced receptor activity. Preclinical studies have shown that long-term administration of ULD opioid antagonists, such as naloxone and naltrexone, does not inhibit opioid-induced pharmacological effects, but rather enhances the antinociceptive effects of morphine (Tsai *et al.*, 2008), suppresses the development of opioid analgesic tolerance (Shen and Crain, 1997; Powell *et al.*, 2002; Terner *et al.*, 2006; McNaull *et al.*, 2007; Tuerke *et al.*, 2011), and reduces opioid withdrawal symptoms (Mannelli *et al.*, 2004; Olmstead and Burns, 2005). This phenomenon has also been observed clinically, where ULD naltrexone enhances and prolongs oxycodone analgesia in patients experiencing osteoarthritis (Chindalore *et al.*, 2005) and reduces physical dependence compared with oxycodone alone in patients with long-term low back pain (Webster *et al.*, 2006).

This ULD phenomenon is not restricted to opioid receptor antagonists. Recent studies demonstrated that long-term intrathecal administration of an ULD α_2-adrenergic receptor (AR) antagonist, atipamezole, enhances clonidine analgesia in rats (Milne *et al.*, 2011). In addition, structurally diverse α_2-AR antagonists, including efaroxan (Milne *et al.*, 2013) and the α_{2A}-AR subtype selective BRL44408 (Milne *et al.*, 2014), are able to augment spinal morphine analgesia and attenuate development of short-term and long-term opioid tolerance (Milne *et al.*, 2008).

Given the close connections between opioid and cannabinoid (CB) systems, it is not surprising that ULD effects on analgesic tolerance extend to the CB receptor system. Opioid and CB receptor systems cross-modulate activity (Robledo *et al.*, 2008), are colocalized in spinal (Salio *et al.*, 2001) and supraspinal regions, and have similar signal

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transduction pathways; both are coupled to inhibitory G-protein pathways (Rodriguez *et al.*, 2001) and mediate analgesia by inhibiting release of pronociceptive neurochemicals. Short-term activation of either receptor type leads to analgesia, antinociceptive synergy is observed when both are activated (Cichewicz, 2004), and repeated stimulation leads to analgesic tolerance and receptor desensitization (Dewey, 1986; Olson *et al.*, 1998). ULD opioid antagonists attenuate the development of tolerance to (Paquette *et al.*, 2007) and enhance the antinociceptive effects of (Paquette and Olmstead, 2005) CB_1-receptor agonists. One goal of this study was to test the inverse relationship: whether ULD CB antagonists alter the development of tolerance to opioid agonists.

Although the paradoxical effect of ULD antagonists is more ubiquitous than once believed, the mechanism through which this occurs is not understood. One possibility is that modulation of spinal gliosis plays a role in ULD effects on analgesic tolerance. Repeated morphine administration leads to glial activation in the brain and spinal cord (Raghavendra *et al.*, 2002), causing a shift to a proinflammatory state (Watkins and Maier, 2004; Hutchinson *et al.*, 2007; Jo *et al.*, 2009). Importantly, pharmacological inhibition of glia attenuates the development of morphine tolerance (Song and Zhao, 2001; Mika *et al.*, 2009). Long-term coadministration of the ULD opioid antagonist, naltrexone, attenuates the development of morphine-induced spinal gliosis and opioid analgesic tolerance (Mattioli *et al.*, 2010). Because of similarities across receptor systems, we hypothesized that comparable effects would be observed with other GPCRs displaying paradoxical ULD antagonist activity, including the α_2-adrenergic and CB_1-receptor antagonists.

This study aimed to determine whether systemic administration of ULD α_2-AR antagonists enhances morphine analgesia and whether the mechanism of action was at least partially mediated through modulation of spinal glia. Thus, we investigated the long-term systemic effects of two chemically distinct ULD α_2-AR antagonists, atipamezole and efaroxan, and an ULD CB_1-receptor antagonist, rimonabant (RIM), on (i) the development of long-term morphine tolerance and (ii) morphine-induced spinal gliosis. To assess the latter, we used fluorescent immunohistochemistry and three-dimensional cellular reconstructions to quantify expression of CD11b and glial fibrillary acidic protein (GFAP), proteins that upregulate in activated microglia and astrocytes, respectively. This allowed us to assess changes in glial cell size associated with a proinflammatory phenotype that drives changes in receptor function and cell signaling pathways leading to the development of tolerance.

Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec, Canada) weighing 250–300 g were used for all experiments. Animals were pair-housed, kept on a reverse 12-h/12-h light/dark cycle with lights off at 07 : 00 h, and provided with free access to food and water in their home cage. Upon arrival, animals were allowed to habituate to their new surroundings for 3 days before handling and at least 7 days before the start of experimentation. All behavioral testing was performed blind to treatment during the animals' active phase, before 14 : 00 h. Animals were randomly assigned to treatment groups, and both animals within a cage received the same treatment.

All experiments were performed in accordance with guidelines set by the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee. Care was taken to minimize the number of animals needed for behavioral experiments that would still provide a robust enough effect to show statistical differences between treatment groups, if any. Although the nature of the study relies on exposure to a painful stimulus to assess changes in the antinociceptive effects of drugs over time, safety measures included the following: cutoff times to avoid tissue damage built into the experimental design to minimize distress, animals were only subjected to one test session per day, exposure to the nociceptive stimulus was terminated as soon as the rats withdrew from it, and the duration of the experiments was kept as short as possible while still allowing for robust development of opioid analgesic tolerance. Although perfusion for tissue collection needs to be done on live animals with a beating heart, the animals were deeply anesthetized during the procedure. To adequately model pain and reflex pathways, the species must be of sufficient complexity and exhibit structural and behavioral similarities to humans. Rats are a well-validated model for studies of this nature, and use of species lower on the phylogenetic tree would not provide useful behavioral data.

Thermal tail flick assay

The 5-cm distal portion of the rat tail was marked with a permanent black marker, and a thermal tail flick analgesiometer (IITC Life Science Inc., Woodland Hills, California, USA) was used to determine tail flick latencies, as described previously (D'Amour and Smith, 1941). Baseline thresholds were assessed before the start of each experiment on the same day, with the beam intensity adjusted to elicit tail flick latencies between 2 and 3 s, then kept constant throughout all trials. A cutoff was set at three times baseline (8 s) to avoid tissue damage.

Tolerance paradigm

Animals were randomly assigned to one of four groups: morphine (5 mg/kg, subcutaneous), morphine and ULD atipamezole (17 ng/kg, subcutaneous), ULD atipamezole alone (17 ng/kg, subcutaneous), or vehicle (0.9% saline, 0.1 ml/100 g, subcutaneous). Thermal tail flick latencies were assessed daily before and following drug injections (at 30 min, corresponding to analgesic peak effects produced by

morphine alone) for 7 days. Two-hour time courses were performed on the first and seventh day to compare changes in analgesic response profiles at the beginning and the end of the trial. This protocol was used to assess the effects of coadministration of ULD efaroxan (17 ng/kg, subcutaneous) compared with morphine alone in separate groups of animals. A third experiment was conducted in separate groups of animals to assess the effect of coadministration of ULD CB_1-receptor antagonist RIM (1, 5, 50 ng/kg) with morphine compared with morphine alone (5 mg/kg) or vehicle [5% dimethylsulfoxide (DMSO), 0.3% Tween-80 in 0.9% saline] over 7 days. Our previous studies reported that ULD α_2-adrenergic antagonists, atipamezole and efaroxan (Milne *et al.*, 2008, 2013, 2014), or CB_1-antagonist RIM (Paquette *et al.*, 2007) do not alter nociceptive thresholds, and therefore to minimize use of animals, these control groups were not repeated in all experiments.

Sacrifice, perfusion, and tissue isolation

On the eighth day, 24 h after the last drug treatment, rats were deeply anesthetized with sodium pentobarbital (75 mg/kg, intraperitoneal; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and perfused transaortically with 500 ml of cold 4% paraformaldehyde in 0.1 mol/l PBS. Rats were decapitated, and spinal cords were ejected and postfixed in ice-cold 4% paraformaldehyde for 30 min. Spinal cords were then cryoprotected by immersion in 30% sucrose (dissolved in 0.1 mol/l PBS) at 4°C for 48–72 h, and then snap-frozen in −45°C isopentane over dry ice and placed in a −80°C freezer for storage until the start of immunohistochemical labeling.

Immunohistochemistry

To assess whether the ULD α_2-adrenergic and CB_1-receptor antagonists modified spinal gliosis associated with tolerance from prolonged morphine treatment, immunohistochemistry was performed on lumbar spinal cord tissue to label and quantify markers expressed on astrocytes and microglia, GFAP and CD11b, respectively.

L4–L5 lumbar spinal cord sections were cut transversely (30 μm) using a freezing sledge microtome and collected in 0.1 mol/l Tris-buffered saline (TBS). Sections were washed 1 × 5 min in 0.1 mol/l TBS, and then 1 × 5 min in 0.1M TBS-Triton (TBS-T) to increase antibody penetration. Tissue was blocked for 2 h at room temperature with blocking buffer containing 10% normal goat serum (NGS) and 10% BSA in 0.1 mol/l TBS-T to reduce the likelihood of nonspecific labeling.

Sections were incubated with primary antibody solution containing 1% NGS and 1% BSA in 0.1 mol/l TBS-T overnight at 4°C to label microglia (anti-CD11b, raised in mouse, 1 : 1000 dilution, MLA257R, batch: 0404; anti-CD68, raised in mouse, 1:250 dilution, MCA341, AbD Serotec, Raleigh, North Carolina, USA) or astrocytes (anti-GFAP, raised in rabbit, 1 : 2500 dilution, Z0334, lot: 00045904; Dako, Glostrup, Denmark). Microglia and astrocyte labeling was performed on separate tissue sections.

Sections were then washed at room temperature 2 × 5 min, and then 2 × 10 min in 0.1 mol/l TBS-T before incubation at room temperature for 2 h (in the dark) with appropriate secondary antibodies conjugated to Alexa fluorophores (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 1 : 200 dilution in 5% NGS and 5% BSA in 0.1 mol/l TBS-T. GFAP-labeled tissue was incubated with a 488-nm anti-rabbit secondary, whereas the CD11b-labeled tissue sections were incubated with a 594-nm anti-mouse secondary. After incubation, sections were washed 3 × 10 min in 0.1 mol/l TBS-T, and then 1 × 10 min in 0.1 mol/l TBS before mounting on glass microscope slides in 0.1 mol/l TB, air-dried and coverslipped with Aquamount (Polysciences Inc., Warrington, Pennsylvania, USA). Slides were stored in the dark in the fridge at 4°C until imaging with a confocal microscope.

Confocal imaging

Imaging of CD11b and GFAP fluorescent labeling was performed on a confocal microscope (Leica TCS SP2 Multi Photon Laser Scanning confocal microscope; Leico Microsystems, Wetzlar, Germany). Images were captured, blind to treatment, within the deep dorsal horn (in lamina V) and the superficial dorsal horn (within laminae I,II). Images were captured in stacks through the z-plane (to allow for later three-dimensional reconstruction) at ×63 magnification under oil immersion. Settings such as gain and the number of passes (to clean up background noise) were optimized for each fluorophore at the beginning of the imaging and kept constant across all treatment groups for the entire duration, allowing comparison of fluorescent intensity across different animals and treatments.

Quantification of fluorescent labeling intensity

For quantification of average pixel intensity in two-dimensional images, all the layers of each three-dimensional stack were collapsed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA) by using its Z Project function (with Maximum Intensity set as the Projection Type). Collapsed images were 'measured' in ImageJ, and the mean pixel intensities were compared across treatment groups. Overall, three to six sections were imaged per animal in each area (deep and superficial dorsal horn) from at least three animals per group.

Three-dimensional reconstructions and assessment of cell size

Mean pixel intensity in a two-dimensional image gives a good assessment of the density of the cells in the image but does not provide information about cell size: another metric of gliosis. Thus, to compare cell size of astrocytes, three-dimensional reconstructions of the collected GFAP-labeled stacks were performed using ImagePro Plus 6.0 software (Media Cybernetics, Rockville, Maryland, USA). Stacks

were imported into ImagePro Plus, and Merge Images was used to create a video representation of the stack. Using the 3D Constructor, voxel and subsampling size was set at $x=4$, $y=4$, $z=1$ pixels and kept constant throughout. Global Transparency and Surface Value settings were optimized with the first stack and kept constant for all other stacks. No filters were used for volume measurements. Close edges was used, and Isosurface Simplification was set to medium as this gave the best and most consistent assessment of individual cells. The volume of all cells was determined throughout the entire stack. Cell volumes were sorted from largest to smallest, and the volumes of the three largest cells were recorded for each stack. The stacks were not adjusted or manipulated in any way, and the volumes were recorded exactly as the software detected them, with all settings kept constant. A total of four to six stacks were reconstructed in each area (deep and superficial dorsal horn) per animal from at least three animals per group.

Drug administration

All drugs were administered through once daily subcutaneous injection. For the ULD α_2-adrenergic antagonist studies, atipamezole hydrochloride (17 ng/kg; Orion Pharma, Esposo, Finland), efaroxan hydrochloride (17 ng/kg; Tocris Bioscience, Ellisville, Mississippi, USA), and morphine sulfate (MS, 5 mg/kg; Sandoz Canada Inc., Boucherville, Quebec, Canada) were dissolved in 0.9% sterile saline. The dose was selected on the basis of pilot studies. For the ULD CB_1-receptor antagonist study, MS (5 mg/kg) and ULD RIM (5 ng/kg) were dissolved in vehicle of 5% DMSO (Thermo Fisher Scientific) in 0.9% saline as used in previous experiments (Paquette *et al.*, 2007) owing to the poor solubility of the CB antagonist in aqueous solution. Because two different vehicles were used for the adrenergic and CB antagonists, more than one vehicle behavior group was included.

Data analysis

Behavioral responses were analyzed by two-way or three-way analysis of variance (ANOVA) using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, California, USA) with time as a within-subjects factor and morphine or ULD antagonist as between-subjects factors. P value less than 0.05 was considered statistically significant, and Bonferroni's or Tukey's post-hoc analysis was performed for multiple comparisons. Data are expressed as mean ± SEM. All data collected were included in the data analysis, and no data were omitted. The hypothesis tested was considered two tailed.

Statistical analyses for immunohistochemical experiments comparing mean pixel intensity of spinal CD11b and GFAP labeling and astrocyte hypertrophy were performed using two-way ANOVA with Tukey's post-hoc analysis. All GFAP cell volume measurements and 2D immunoreactivity in the ULD RIM experiment were analyzed by nonparametric two-tailed Mann–Whitney U-test.

Results

Analgesic tolerance

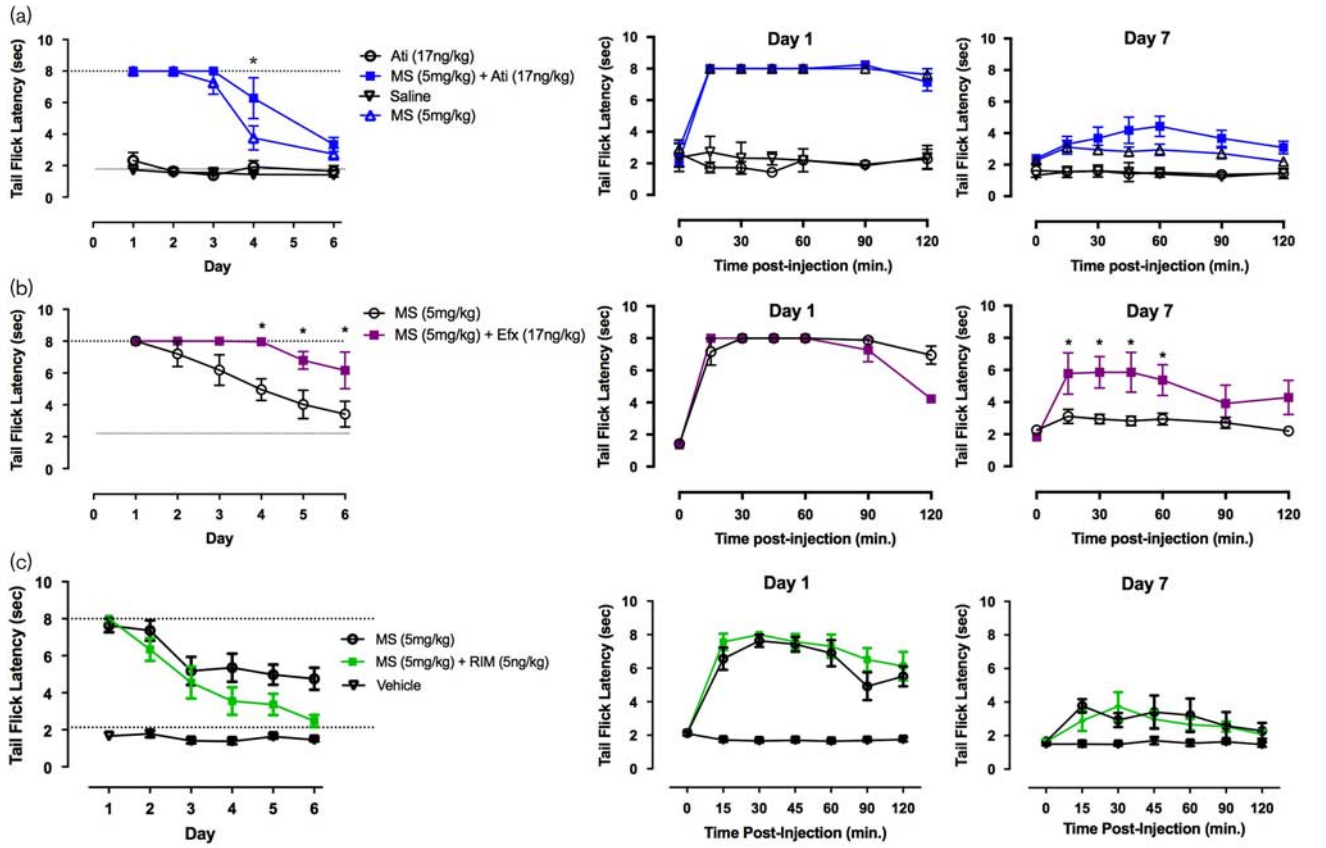
Long-term daily administration of morphine (5 mg/kg, subcutaneous, for 7 days) significantly reduced the ability of morphine to elicit thermal antinociception (Fig. 1a), as evidenced by a decrease in tail flick latencies measured 30 min after daily morphine injections (at peak effect). Concomitant administration of either ULD α_2-adrenergic antagonist (atipamezole or efaroxan) with morphine significantly attenuated the development of analgesic tolerance (Fig. 1a and b, left). Statistical analyses by two-way or three-way ANOVAs are presented in Table 1. Post-hoc analysis revealed a significant effect at day 4 in the atipamezole experiment and days 4–6 in the efaroxan experiment compared with animals receiving morphine alone. Long-term systemic administration of ULD atipamezole alone or saline vehicle did not change thermal withdrawal latencies over 7 days (Fig. 1a, left).

A 2-h time course of morphine or morphine + α_2-adrenergic antagonist was determined on days 1 and 7. On day 1, there was no difference in thermal tail flick latencies between animals receiving morphine and those receiving morphine coadministered with ULD atipamezole (17 ng/kg) (Fig. 1a, middle) or with ULD efaroxan (Fig. 1b, middle). For the atipamezole experiment, statistical analysis of the 2-h time course on day 7 revealed significant effects of morphine, atipamezole, and morphine × atipamezole (Table 1). Post-hoc analysis revealed no significant difference between the morphine and the morphine + atipamezole groups. However, a two-way ANOVA of morphine and morphine + atipamezole groups revealed a significant effect of treatment [$F(1,42)=19.03$, $P<0.001$] but no difference was identified at any specific time point. For the efaroxan experiment, two-way ANOVA of the 2-h time course on day 7 for the morphine and morphine + efaroxan groups revealed significant effects of treatment and time (Table 1). Animals that had been treated with morphine showed tail flick latencies not significantly different from their baseline responses before any drug treatment, whereas the animals that had been chronically treated with morphine + efaroxan (Fig. 1b, right) had significantly higher tail flick latencies compared with the morphine only groups at 15–60 min after injection.

To determine whether short-term synergistic or additive effects exist between morphine and ULD atipamezole that would not be detected at maximal analgesic doses of morphine, we coadministered ULD atipamezole with submaximal doses of morphine. No additive effects of the combination were evident (data not shown), suggesting that any potential additive or synergistic effects do not account for the ability of ULD atipamezole to attenuate the development of tolerance.

Over the 2-h time course following the first injection, both morphine alone and morphine + ULD RIM (5 ng/kg) significantly increased tail flick latencies over the entire 2 h, compared with vehicle at all time points (Fig. 1c). However,

Fig. 1



Long-term coadministration of ULD α_2-adrenergic receptor antagonists Ati or Efx, but not ULD CB_1-receptor antagonist RIM, attenuates the development of MS tolerance. Left: thermal tail flick latencies at 30 min after injection (peak effect) across 6 days of testing. Middle: 2-h time course following the first injection. Right: 2-h time courses following the final injection on day 7. MS was administered with ULD Ati (a), ULD Efx (b), or ULD RIM (c). The lower dotted boundary lines indicate mean baselines before the start of the trial within each cohort. The upper dotted boundary lines indicate the cutoff. Data displayed as mean ± SEM. Group sizes: $n = 4$–5 per group for the ULD atipamezole and efaroxan studies; $n = 8$/group for the ULD RIM study. Significant difference $^*P < 0.05$, compared with MS. Ati, atipamezole; Efx, efaroxan; MS, morphine; RIM, rimonabant; ULD, ultra-low dose.

there was no significant effect of treatment, indicating that RIM had no effect on the development of morphine analgesic tolerance (see Table 1 for statistical analyses). A two-way ANOVA of day 1 of morphine and morphine + RIM revealed no significant difference between groups: tail flick latencies of animals treated with morphine alone did no differ significantly over the 2 h of testing from those treated with morphine + ULD RIM (Fig. 1c, middle). By day 7, however, the antinociceptive effects of morphine were absent, and coadministration of RIM with morphine had no effect on the development of analgesic tolerance (see Table 1 for statistics). Two other doses of ULD RIM were also assessed (1 ng/kg, 50 ng/kg), but neither of those two doses had any effect on morphine antinociception or tolerance development either (data not shown).

Effects of ultra-low-dose antagonists on morphine-induced gliosis

To quantify changes in microglial and astrocyte activation, CD11b and GFAP were labeled correspondingly through fluorescent immunohistochemistry, and stacks were captured on a confocal microscope within the deep and superficial dorsal horn of the spinal cord, in animals treated for 7 consecutive days with morphine (5 mg/kg), morphine + ULD atipamezole (17 ng/kg), ULD atipamezole alone (17 ng/kg), or saline (vehicle). In separate groups of animals, morphine, morphine + ULD RIM, or vehicle (5% DMSO, 0.3% Tween-80 in saline) were also assessed. Representative micrographs of microglial (CD11b) and astrocyte (GFAP) cell surface markers are presented (Fig. 2). As previously reported, morphine significantly increased expression of microglial protein expression, indicative of opioid-induced neuroinflammation. This was evident in both the superficial (Fig. 3b) and deep (Fig. 3a) dorsal horn of the spinal cord (see Table 2 for statistics). Post-hoc analyses revealed a significant difference between saline and morphine in vehicle-treated groups in the deep ($P < 0.01$) and superficial spinal cord ($P < 0.001$). However, there was no significant difference between saline and morphine treatment in the atipamezole-treated groups in either the superficial or deep

Table 1 **Summary of statistical analysis of data presented in Fig. 1**

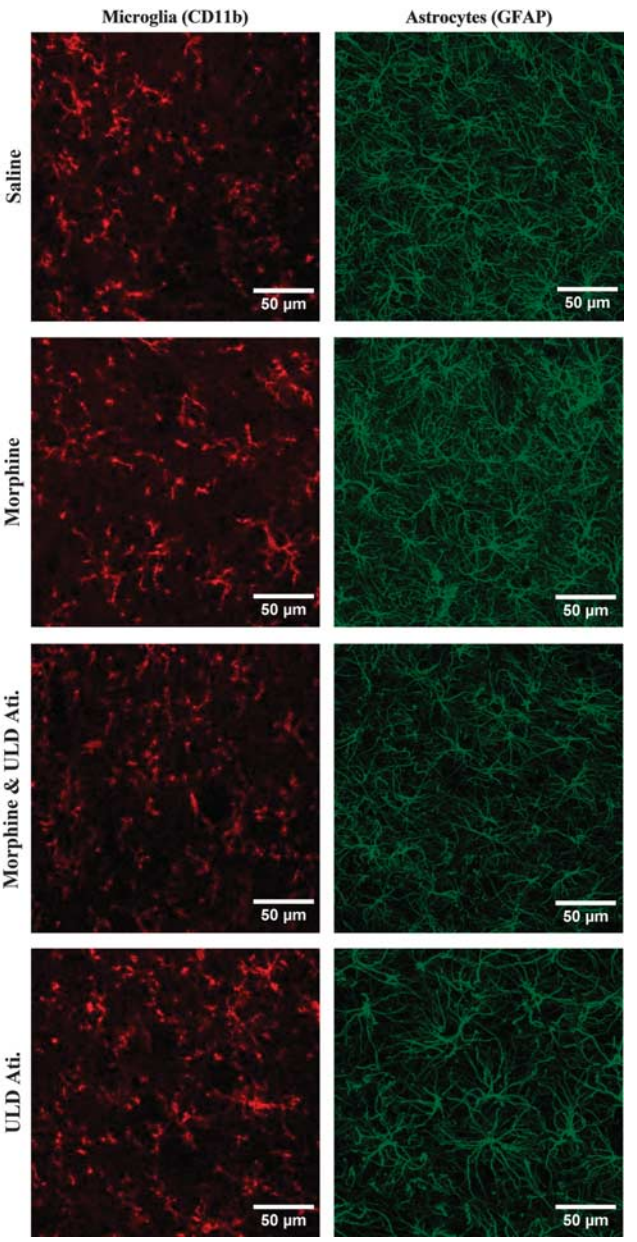
Three-way ANOVA	Fig. 1a – time course	*F*	*P* value	Summary
	Time	$F(4,60) = 27.63$	< 0.0001	***
	Morphine	$F(1,60) = 565$	< 0.0001	***
	Atipamezole	$F(1,60) = 1.824$	0.1819	NS
	Morphine × atipamezole	$F(1,60) = 6.659$	0.0123	*
Three-way ANOVA	Fig. 1a – day 1	*F*	*P* value	Summary
	Time	$F(6,84) = 46.34$	< 0.0001	***
	Morphine	$F(1,84) = 2270$	< 0.0001	***
	Atipamezole	$F(1,84) = 0.680$	0.4119	NS
	Morphine × atipamezole	$F(1,84) = 4.774$	0.0317	*
Three-way ANOVA	Fig. 1a – day 7	*F*	*P* value	Summary
	Time	$F(6,84) = 2.053$	< 0.0675	NS
	Morphine	$F(1,84) = 132.1$	< 0.0001	***
	Atipamezole	$F(1,84) = 15.34$	0.0002	***
	Morphine × atipamezole	$F(1,84) = 19.09$	< 0.0001	***
Two-way ANOVA	Fig. 1b – time course	*F*	*P* value	Summary
	Time	$F(5,30) = 11.67$	< 0.0001	***
	Treatment	$F(1,6) = 8.615$	0.0261	*
	Interaction	$F(5,30) = 2.776$	0.0355	*
Two-way ANOVA	Fig. 1b – day 1	*F*	*P* value	Summary
	Time	$F(6,36) = 104.7$	< 0.0001	***
	Treatment	$F(1,6) = 2.982$	0.2488	NS
	Interaction	$F(6,36) = 5.532$	0.0004	***
Two-way ANOVA	Fig. 1b – day 7	*F*	*P* value	Summary
	Time	$F(6,60) = 11.22$	< 0.0001	***
	Treatment	$F(1,10) = 8.715$	0.0145	*
	Interaction	$F(6,60) = 5.324$	0.0002	***
Two-way ANOVA	Fig. 1c –time course	*F*	*P* value	Summary
	Time	$F(5,70) = 23.47$	< 0.0001	***
	Treatment	$F(1,14) = 4.005$	0.0637	NS
	Interaction	$F(5,70) = 1.914$	0.103	NS
Two-way ANOVA	Fig. 1c – day 1	*F*	*P* value	Summary
	Time	$F(5,110) = 5.93$	< 0.0001	***
	Treatment	$F(1,22) = 0.005$	0.9458	NS
	Interaction	$F(5,110) = 0.268$	0.9297	NS
Two-way ANOVA	Fig. 1c – day 7	*F*	*P* value	Summary
	Time	$F(6,84) = 4.23$	0.0009	***
	Treatment	$F(1,14) = 0.098$	0.7589	NS
	Interaction	$F(6,84) = 0.731$	0.6256	NS

ANOVA, analysis of variance.
Significance is denoted as $^{*}P < 0.05$ and $^{***}P < 0.001$.

dorsal spinal cord (Fig. 3a and b). Data collected from multiple trials are represented in the figure, and no data points were excluded.

Similar to the effects of long-term morphine treatment on microglial reactivity, morphine treatment also increased expression of the astrocytic cytoskeletal protein GFAP.

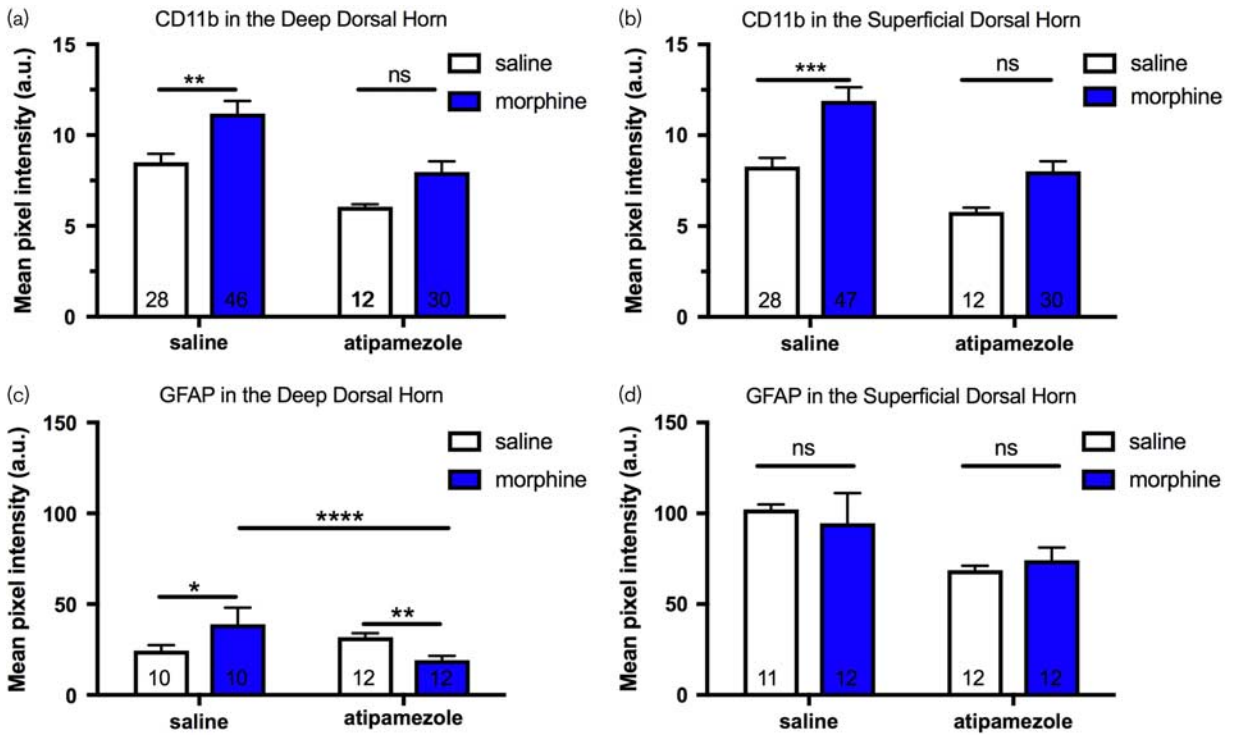
Fig. 2



Representative micrographs demonstrating microglia (CD11b, left column in red) and astrocytes (GFAP, right column in green) by fluorescent immunohistochemistry in the superficial dorsal horn of rats treated chronically for 7 days with saline vehicle, morphine (5 mg/kg), morphine + ULD Ati (17 ng/kg), or ULD Ati alone. × 63 magnification under oil immersion. Ati, atipamezole; GFAP, glial fibrillary acidic protein; ULD, ultra-low dose.

However, this was only evident in the deep dorsal horn (Fig. 3c and d, see Table 2 for statistics). Post-hoc analysis revealed that vehicle-treated animals that received long-term treatment with morphine had significantly higher labeling in the deep dorsal horn (Fig. 3c) than the saline-treated controls ($P < 0.001$). This effect was not blocked by atipamezole, as there remained a significant difference between morphine-treated and saline-treated groups. However, there was a

Fig. 3



Coadministration of ULD atipamezole reduces chronic morphine-induced spinal gliosis in the deep and superficial dorsal horn of the spinal cord. Graphs show mean pixel intensity of CD11b (a, b) and GFAP (c, d) labeling in collapsed stacks of the deep (a, c) and superficial (b, d) dorsal horn of the L4–L5 spinal cord following 7 days of injections with saline, morphine alone (5 mg/kg), morphine + ULD atipamezole (17 ng/kg), or ULD atipamezole alone. Three to six images were captured per region per animal from at least three animals per group. For the CD11b study, data were pooled from three separate trials, and all collected data from those trials are shown, giving a total of 12–46 images per group for the CD11b study. For the GFAP study, 10–12 collapsed stacks were quantified per group. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. a.u., arbitrary units; GFAP, glial fibrillary acidic protein; ULD, ultra-low dose.

significant decrease in morphine-induced GFAP expression between vehicle and atipamezole treatment ($P < 0.001$).

To assess changes in the size of individual astrocytes, three-dimensional models were quantified within the deep and superficial dorsal horn of morphine + vehicle-treated and morphine + atipamezole-treated groups. Representative cells are shown as wireframe models drawn to scale to allow for visual comparison (Fig. 4a–d). Quantification of reconstructed GFAP-labeled stacks showed that coadministration of ULD atipamezole significantly decreased astrocyte cell volume in both the deep ($U = 240$, $P < 0.001$) and superficial ($U = 78$, $P < 0.001$) dorsal horn (Fig. 4e) compared with animals receiving morphine and vehicle.

Similar to the aforementioned atipamezole experiment, in the ULD RIM experiment, morphine significantly increased CD11b expression in vehicle-treated animals in the superficial ($U = 1$, $P < 0.001$) and deep ($U = 39$, $P < 0.001$) dorsal spinal cord compared with saline-treated animals (Figs 5 and 6). In contrast, animals coadministered ULD RIM + morphine showed significantly lower levels of CD11b immunoreactivity compared with the morphine + vehicle group for both the deep ($U = 32$, $P < 0.001$) and superficial ($U = 4$, $P < 0.001$) dorsal spinal cord. Moreover, no significant difference was noted in CD11b immunoreactivity of saline + vehicle and ULD RIM + morphine groups in either the deep ($U = 112$, $P =$ NS) or superficial ($U = 121$, $P =$ NS) spinal cord. For the RIM experiment, we also determined the effects of treatment on another marker of microglial reactivity (CD68). Similar to the effects of morphine on CD11b, in the vehicle-treated groups, morphine significantly increased CD68 expression in the deep ($U = 30$, $P < 0.001$) and superficial ($U = 43$, $P < 0.001$) spinal cord compared with saline treatment (Fig. 6). The concomitant administration of RIM with morphine significantly blocked the morphine-induced increase in CD68 in the spinal cord, as there was no significant difference between the RIM + morphine-treated and saline-vehicle-treated groups and a significant difference between the morphine + vehicle and RIM + morphine groups (Fig. 6).

In vehicle-treated animals, morphine significantly increased GFAP expression in the deep ($U = 29$, $P < 0.001$) but not superficial ($U = 88$, $P =$ NS) dorsal spinal cord (Fig. 7). Coadministration of ULD RIM with morphine significantly decreased GFAP immunoreactivity compared with the

Table 2 Summary of statistical analysis of data presented in Fig. 3

Two-way ANOVA	Fig. 3a	*F*	*P* value	Summary
	Morphine	$F(1,124) = 20.14$	< 0.0001	***
	Atipamezole	$F(1,124) = 13.3$	0.0004	***
	Interaction	$F(1,124) = 0.357$	0.5511	NS
Two-way ANOVA	Fig. 3b	*F*	*P* value	Summary
	Morphine	$F(1,113) = 15.6$	0.0001	***
	Atipamezole	$F(1,113) = 13.12$	0.004	***
	Interaction	$F(1,113) = 0.7235$	0.3968	NS
Two-way ANOVA	Fig. 3c	*F*	*P* value	Summary
	Morphine	$F(1,39) = 0.1529$	0.6980	NS
	Atipamezole	$F(1,39) = 7.647$	0.0086	**
	Interaction	$F(1,39) = 36.38$	< 0.0001	***
Two-way ANOVA	Fig. 3d	*F*	*P* value	Summary
	Morphine	$F(1,43) = 0.092$	0.7633	NS
	Atipamezole	$F(1,43) = 70.03$	< 0.0001	***
	Interaction	$F(1,43) = 0.0918$	0.7633	NS

ANOVA, analysis of variance.
Significance is denoted as $^{**}P < 0.01$ and $^{***}P < 0.001$.

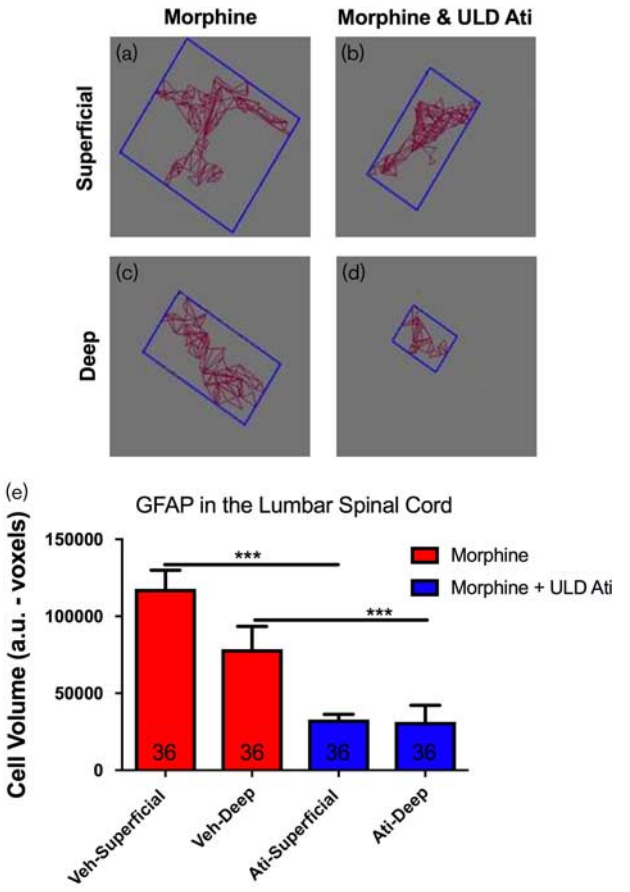
morphine-treated and vehicle-treated group ($U = 47$, $P < 0.01$). No significant difference was observed between the MS + RIM and vehicle-saline group ($U = 118$, $P =$ NS).

Astrocyte cell volumes from the ULD RIM experiment were quantified from three-dimensional reconstructions of GFAP-labeled stacks (Fig. 8). An increase in astrocyte hypertrophy was observed in the morphine group, compared with vehicle, in both the superficial ($U = 667$, $P < 0.001$) and deep ($U = 628$, $P < 0.001$) dorsal horn. In the ULD RIM-treated groups, RIM + morphine was not significantly different from the saline-vehicle treatment group (superficial: $U = 1079$, $P =$ NS) but was significantly different from the morphine + vehicle group (deep: $U = 623$, $P < 0.001$, superficial: $U = 745$, $P < 0.01$), demonstrating that RIM treatment significantly blocked morphine-induced astrogliosis.

Discussion

The main objective of this study was to determine whether previous reports of intrathecal administration of ULD α_2-AR antagonists attenuating opioid tolerance could be replicated using a more clinical translational dose regimen (systemic administration). A secondary goal was to test the hypothesis that these paradoxical ULD effects are common to another class of GPCR involved in pain modulation, the CB_1 receptor. Finally, we examined whether observed behavioral changes correlated with temporal changes in spinal gliosis induced by long-term morphine treatment. Although both α_2-AR antagonists, atipamezole and efaroxan, attenuated morphine analgesic tolerance, the CB_1-antagonist RIM did not. In contrast, both atipamezole and RIM attenuated the development of long-term morphine-induced spinal gliosis. These data suggest that the ULD effects are not ubiquitous among GPCRs involved in modulation of nociception, and that

Fig. 4



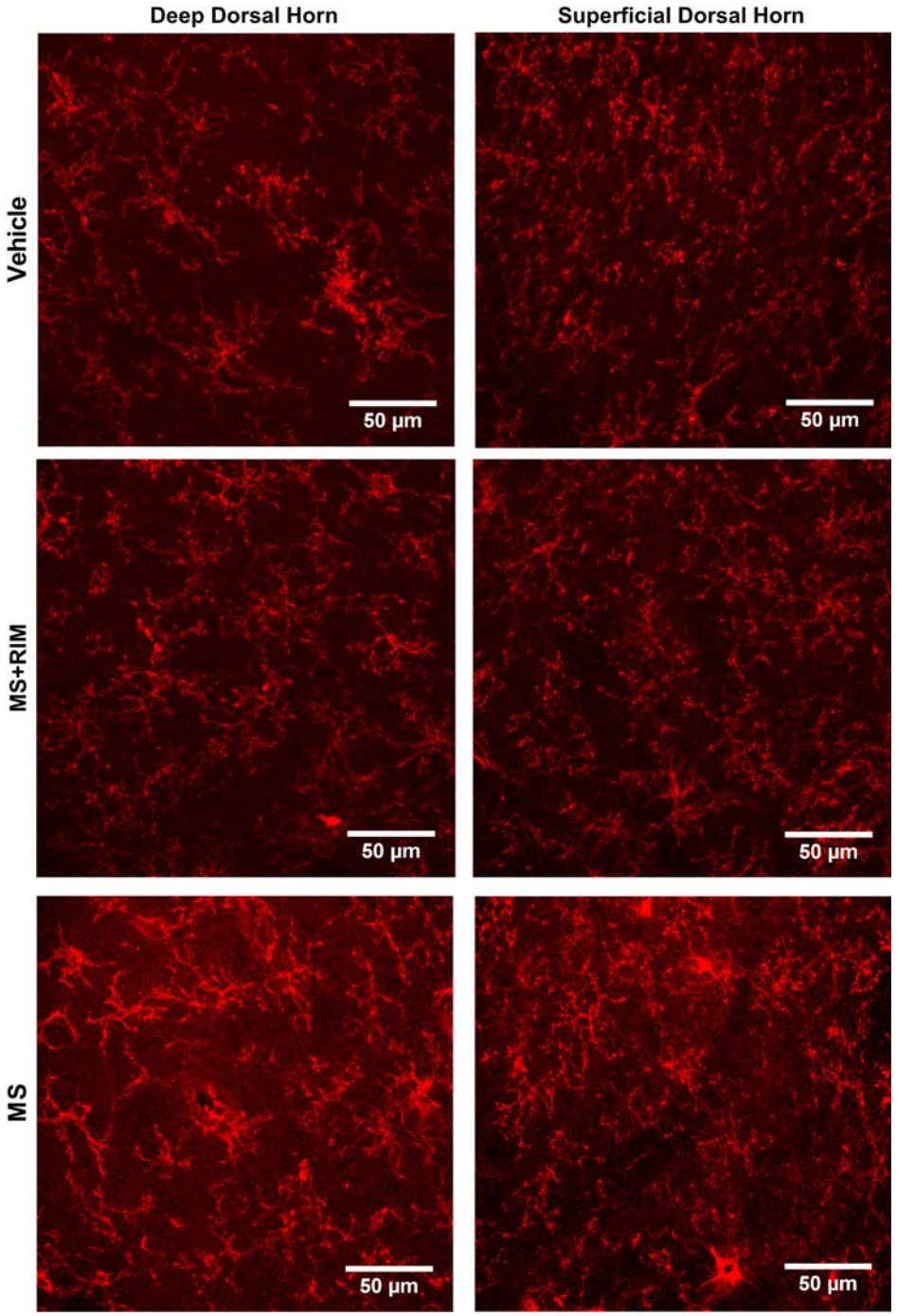
Coadministration of ULD atipamezole reduces long-term morphine-induced astrocyte hypertrophy in the deep and superficial dorsal horn of the lumbar spinal cord. The figure shows representative three-dimensional reconstructions of astrocytes in the superficial (a, b) and deep (c, d) dorsal horn of the spinal cord in animals treated over long term with morphine (5 mg/kg) or morphine plus ULD atipamezole (17 ng/kg) daily for 7 days. (e) Quantification of astrocyte cell volume. Wireframes of the cells are represented to scale. Gray box dimensions are 250 × 250 pixels. The three largest cells were quantified in each area of each stack, with four stacks per animal from three animals per group; total $n = 36$/group. Bars represent mean ± SEM. $^{***}P < 0.001$. Ati, atipamezole; a.u., arbitrary units; GFAP, glial fibrillary acidic protein; ULD, ultra-low dose.

the mechanism of action may be distinct, given both adrenergic and CB receptor antagonists prevented opioid-induced spinal gliosis, but not the development of analgesic tolerance.

These results are consistent with previous studies demonstrating that intrathecal administration of α_2-AR antagonists attenuated the development of morphine analgesic tolerance in both short-term and long-term opioid tolerance models (Milne *et al.*, 2008, 2013).

It seems likely, therefore, that α_2-AR antagonists are exerting effects in the present study, at least partially, through an action within the spinal cord. Lilius *et al.*

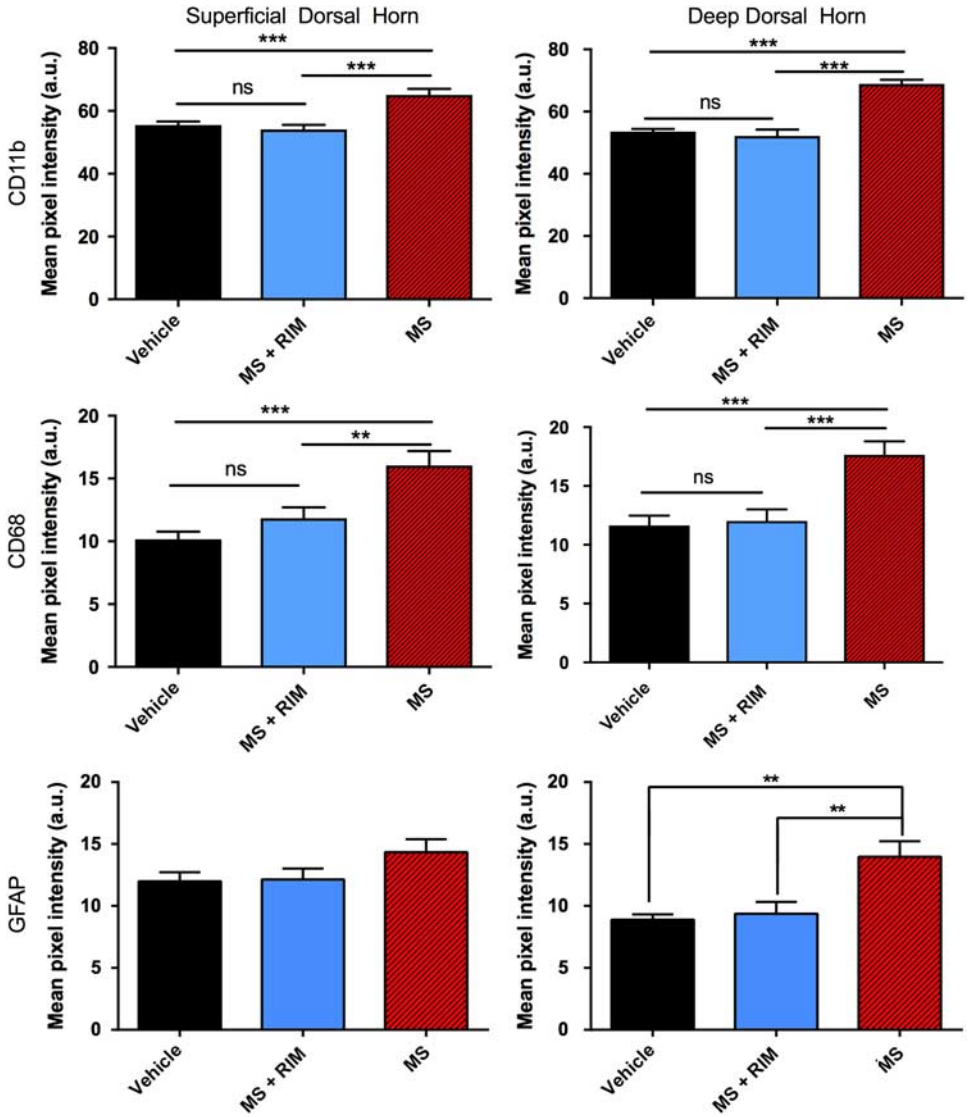
Fig. 5



Representative micrographs showing intensity of CD11b labeling of microglia within the deep (left column) and superficial lumbar dorsal horn in animals chronically treated for 7 days with vehicle (top), MS plus ultra-low-dose CB_1-receptor antagonist RIM (5 ng/kg), or MS alone (5 mg/kg). × 63 magnification under oil immersion. MS, morphine; RIM, rimonabant.

(2012) also reported that spinal administration of ULD atipamezole attenuated the development of morphine tolerance, but their finding that systemic administration had no effect on this measure contradicts our current results. This discrepancy can be explained by methodological differences between the two studies: subcutaneous doses of atipamezole in the study of Lilius *et al.* (2012) were several-fold higher than ours, and their dosing paradigm to induce morphine tolerance was more intense. Specifically, we used daily injection of 5 mg/kg morphine for 7 days, whereas Lilius

Fig. 6

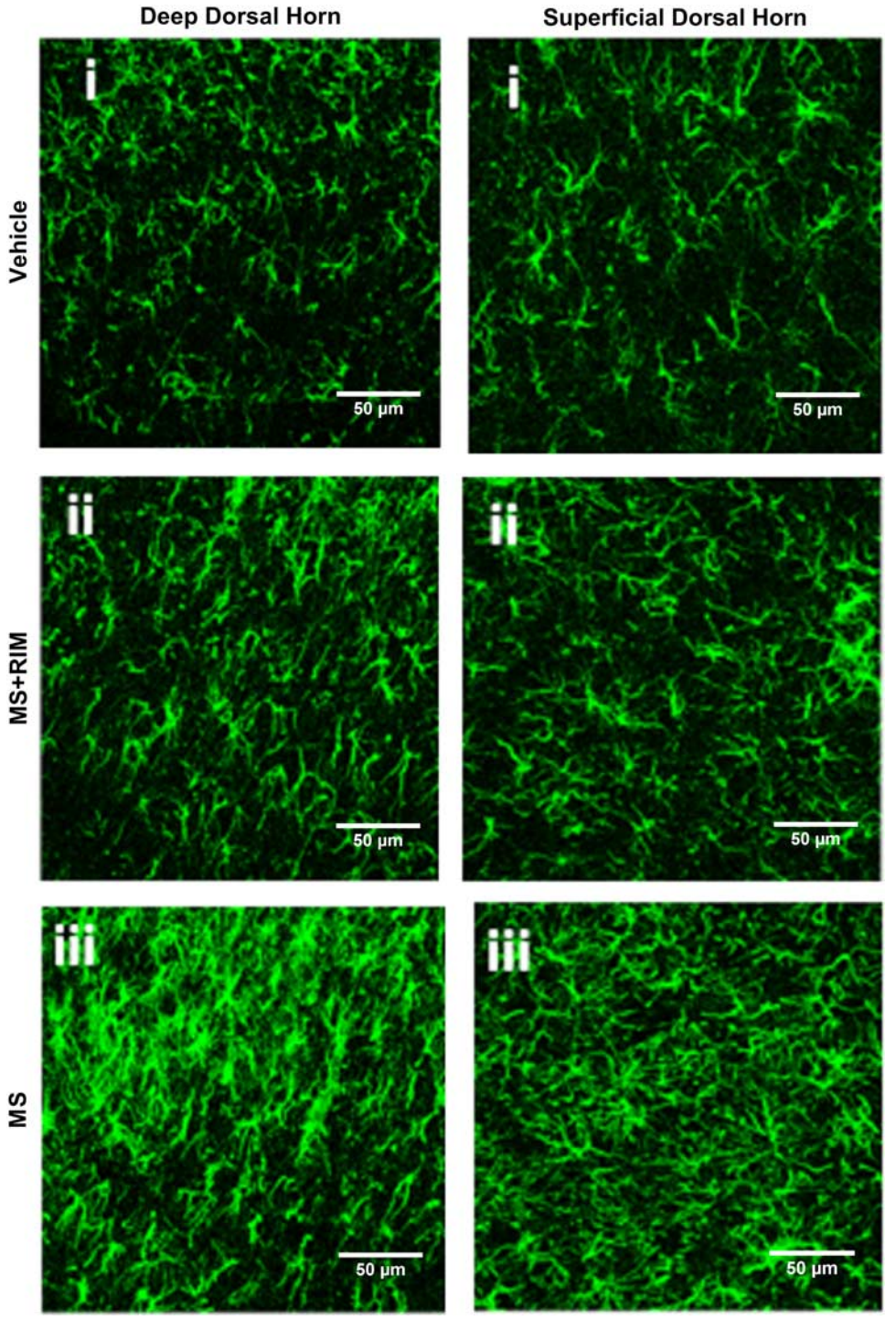


Quantification of mean pixel intensity of CD11b, CD68, and GFAP superficial is on the left and the deep is on the right. Morphine treatment significantly increased fluorescence intensity of microglial (CD11b and CD68) in both deep and superficial dorsal spinal cord. Morphine treatment also increased GFAP fluorescence intensity in the deep but not superficial dorsal horn. Animals cotreated with ultra-low-dose RIM showed an attenuation of morphine-induced spinal gliosis in both areas commonly associated with the development of analgesic tolerance. Five to six sections were imaged per animal per area, with at least three animals from each group, giving a total $n = 16$/group. Bars represent mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. GFAP, glial fibrillary acidic protein; MS, morphine; RIM, rimonabant.

et al. (2012) administered morphine twice daily for 4 days in an escalating dosage paradigm starting at 10 mg/kg on day 1 and rising to 30 mg/kg on day 4. We also treated animals chronically with the ULD α_2-AR antagonists daily with or without morphine; Lilius *et al.* (2012) administered atipamezole as a single injection in naive or morphine-tolerant animals at the end of the dosing paradigm.

Two α_2-AR antagonists were used in the present study. The dose of atipamezole was selected from a pilot study that identified an optimal dose for eliciting behavioral responses. Efaroxan has a similar molecular weight to atipamezole and was therefore administered at the same dose. In competitive binding studies using RX821002 [a selective antagonist at α_2-ARs that does not bind to imidazoline receptors (Clarke and Harris, 2002)], atipamezole was shown to have the highest binding affinity for α_2-ARs in the rat cortex (Ki = 0.2 nmol/l) compared with other selective ligands, and several-fold higher than efaroxan (Renouard *et al.*, 1994). Different potencies may explain some variations in behavioral responses to the two drugs. Because more robust effects were observed with atipamezole compared with efaroxan, cellular effects of atipamezole were assessed in the immunohistochemical studies.

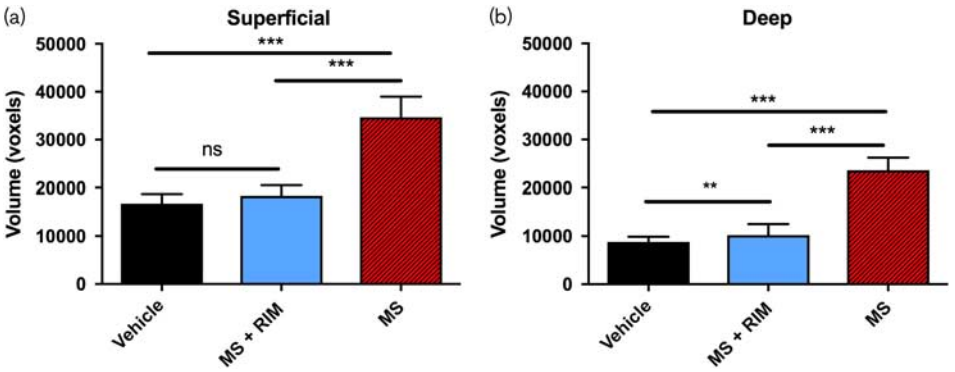
Fig. 7



Representative micrographs showing intensity of GFAP labeling of astrocytes within the deep (left column) and superficial (right column) lumbar dorsal horn in animals chronically treated for 7 days with vehicle (top), MS plus ultra-low-dose CB_1-receptor antagonist RIM (5 ng/kg), or morphine alone (5 mg/kg). ×63 magnification under oil immersion. GFAP, glial fibrillary acidic protein; MS, morphine; RIM, rimonabant.

Although the mechanism is currently unknown, ULD α_2-AR antagonists may be attenuating the development of opioid tolerance by modulating neuronal–glial interactions. Long-term morphine administration (Hutchinson *et al.*, 2007) causes activation of microglia and astrocytes in the peripheral and central nervous system, leading to the enhanced production of proinflammatory cytokines and other inflammatory mediators that can also contribute to the development

Fig. 8



Long-term coadministration of ultra-low-dose RIM attenuates chronic morphine-induced increases in astrocyte cell size associated with a shift to a proinflammatory state that is believed to contribute to tolerance development. Glial fibrillary acidic protein-labeled stacks were reconstructed in three-dimensions using ImagePro software and their size was quantified. Attenuation of spinal astrogliosis was observed in both the superficial (a) and deep (b) dorsal horn. Five to six stacks were reconstructed per animal per area, with at least three animals from each group, giving a total $n = 16$/group. Bars represent mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. GFAP, glial fibrillary acidic protein; MS, morphine; RIM, rimonabant.

of opioid analgesic tolerance (Raghavendra *et al.*, 2002). Previously, we reported that administration of a ULD opioid antagonist, naltrexone, inhibits long-term morphine-induced glial activation (Mattioli *et al.*, 2010). Here, we extend the findings to another system, reporting that long-term coadministration of ULD atipamezole with morphine decreased microglial reactivity and astrogliosis (inferred by CD11b and GFAP immunolabeling, respectively) compared with animals treated with morphine alone. The change in morphine-induced gliosis is small but significant, and it should not be expected that drug administration would have dramatic effects on glia cell size, because cell size is measured by changes in cytoskeletal proteins and even the effects of morphine are not more than a 25% increase in glial proteins. The magnitude of the responses observed here is consistent with past literature studies assessing ULD opioid antagonists on morphine-induced gliosis. It is unknown if these ULD antagonists are acting directly or indirectly on α_2-ARs on the glial cells to alter the development of tolerance. Studies using RT-PCR and radioligand binding, mRNA expression, and protein expression have shown that all α-ARs are represented in astrocytes (Aoki, 1992; Porter and McCarthy, 1997; Chen and Hertz, 1999; Hertz *et al.*, 2004; Hutchinson *et al.*, 2011), therefore the potential of these effects being direct is plausible.

Given the extent of cross-modulation between opioid and CB systems and previous evidence that ULD opioid antagonists modulate tolerance to a CB_1-receptor agonist (Paquette and Olmstead, 2005; Paquette *et al.*, 2007), it was surprising that we did not observe an effect of a ULD CB_1-receptor antagonist on morphine-induced tolerance. At the same time, this supports the contention that cross-talk between opioid and CB systems is asymmetrical (Valverde *et al.*, 2004). Although only one dose of the CB_1-receptor antagonist is shown in this study, two other doses were assessed (1 and 50 ng/kg). None of the three doses of RIM was able to modulate the development of opioid tolerance, despite the 5 ng/kg dose being able to attenuate astrocyte and microglial activation in the spinal dorsal horn associated with tolerance development. The expression of both CD11b and GFAP was reduced with coadministration of ULD RIM with morphine, and three-dimensional reconstructions of astrocytes showed that the morphine-induced increase in cell size associated with the phenotypic shift to a proinflammatory state was prevented. The only region where a difference was not observed was in the GFAP labeling in the superficial dorsal horn. In this region, morphine did not cause a significant upregulation in GFAP expression; thus, despite the same general trend in all immunohistochemical graphs, any effect of coadministration of ULD RIM might have been masked. This fits with the attenuation of astrocyte hypertrophy in the same region in the three-dimensional reconstructions. The expression of CD68, a marker of reactive microglia, was also attenuated by coadministration of ULD RIM. CD68 is predominately expressed on 'activated' microglia following the proinflammatory phenotypic shift and not on quiescent microglia (Bachstetter *et al.*, 2013; Kaser-Eichberger *et al.*, 2016).

Although there is a temporal correlation between spinal glial activation and loss of opioid analgesia, attenuation of gliosis by ULD antagonists alone does not appear to be sufficient to prevent tolerance development. Thus, we identify a dissociation between markers of neuroinflammation and gliosis with interventions that were previously reported to attenuate the development of analgesic tolerance. Although the paradoxical effects of ULD GPCR antagonists are more ubiquitous than once believed, the exact mechanisms of action may be distinct, depending on the class of receptor, its localization, and what changes in expression or function are induced by the model. Alternate mechanisms that could explain the

ability of the α_2-AR antagonists to attenuate opioid tolerance include changes in G-protein coupling and signaling, which have been investigated for ULD opioid antagonists (Chakrabarti *et al.*, 2005; Wang *et al.*, 2005, Wang and Burns, 2006). It is unknown if a similar coupling shift occurs with ULD α_2-adrenergic antagonists, and mechanistically, data interpretation become much more complicated when investigating cross-modulation of two different GPCR systems. In addition, a better understanding of the functional interaction between classes of GPCRs, including the possibility of heterodimer formation, additivity, and synergy when multiple receptors are activated simultaneously, and differential changes in receptor expression in various cell types, will lead to a better understanding of the paradoxical effects of antagonists at ULD.

Ultimately, although the behavioral changes we observed are small, we argue that this may indeed be clinically relevant. In a clinical study where pain ratios assessed on a visual analog scale score (100 mm scale) was 34 or lower, a change of 13 was considered meaningful (see Bird and Dickson, 2001), although with greater pain scores, larger differences were needed to be considered clinically relevant. These data show that a 13% change was needed to be clinically meaningful, so although our effect is small, it may still be clinically relevant. The magnitude of the results observed here is comparable with previous studies that investigated the use of ULD opioid antagonists such as naloxone and naltrexone in preventing the loss of morphine potency over time in animal models. The use of ULD opioid antagonists has actually translated well in terms of clinical effects in human patients (Chindalore *et al.*, 2005; Webster *et al.*, 2006), and it remains to be seen whether the effects of ULD α_2-adrenergic antagonists also translate from animal models to humans.

The next step is to investigate the use of ULD α_2-AR antagonists in more clinically meaningful models, including models of nerve injury and opioid reward. Management of long-term pain and the overuse of opioids are complex and controversial issues that place a significant burden on patients and the health care system as a whole. Use of ULD α_2-AR antagonists could reduce opioid tolerance and thus the total amount of opioids needed to manage pain, diminishing adverse effects and the dangers of escalating doses over time.

Acknowledgements

Conflicts of interest

Patrick Grenier was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) postgraduate scholarship (PGS-D). Catherine M. Cahill and Mary C. Olmstead received funding from Canadian Institutes of Health Research (CIHR). For David Wiercigroch there are no conflicts of interest.

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