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Antisense oligonucleotide knockdown of $mGluR_1$ alleviates hyperalgesia and allodynia associated with chronic inflammation

Marian E. Fundytus[a,b,c,*], Michael G. Osborne[c], James L. Henry[a,d], Terence J. Coderre[c,e,f], Andy Dray[b]

[a]*Department of Physiology, McGill University, Montreal, Canada H3G 1Y6*
[b]*ASTRA Research Centre Montreal (now AstraZeneca R&D), St. Laurent, Canada H4S 1Z9*
[c]*Pain Mechanisms Laboratory, Clinical Research Institute of Montreal, Montreal, Canada H2W 1R7*
[d]*Department of Psychiatry, McGill University, Montreal, Canada H3A 1A1*
[e]*Anesthesia Research Unit, Department of Anesthesia, McGill University, Montreal, Canada H3G 1Y6*
[f]*McGill University Health Centre Research Institute, Montreal, Canada H3G 1A4*

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Abstract

Chronic inflammation induced by injection of complete Freund's adjuvant (CFA) into one hindpaw elicits thermal hyperalgesia and mechanical allodynia in the injected paw. Metabotropic glutamate receptors (mGluRs) have been implicated in dorsal horn neuronal nociceptive responses and pain associated with short-term inflammation. The goal of the present study was to assess the role of $mGluR_1$ in the hyperalgesia and allodynia associated with the CFA model of chronic inflammation. Here we show that antisense (AS) oligonucleotide knockdown of spinal $mGluR_1$ attenuates thermal hyperalgesia and mechanical allodynia in rats injected with CFA in one hindpaw. When intrathecal infusion of $mGluR_1$ AS oligonucleotide (50 μg/day) began prior to CFA injection, mechanical allodynia was attenuated from Days 1 to 8 following CFA injection, whereas heat hyperalgesia was attenuated on Day 1 and then from Days 4 to 8. When intrathecal infusion of $mGluR_1$ AS oligonucleotide was begun 2 days after CFA injection, both mechanical allodynia and heat hyperalgesia were attenuated at all time points following the oligonucleotide infusion. Thus, the present data suggest a role for $mGluR_1$ in persistent inflammatory nociception.

Keywords: Inflammation; Metabotropic glutamate receptor; Hyperalgesia; Allodynia; Antisense oligonucleotide

1. Introduction

The excitatory amino acid glutamate is a major contributor to inflammatory pain states. Glutamate release in the spinal cord dorsal horn is enhanced following inflammation (reviewed in Fundytus, 2001; Sasaki et al., 1998; Sorkin et al., 1992; Sluka and Westlund, 1992; Sluka and Willis, 1998; Omote et al., 1998). Several investigators have shown that antagonists at *N*-methyl-D-aspartate (NMDA) receptors alleviate hyperalgesia due to short-term inflammation induced by formalin or carrageenan (Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992; Ren et al., 1992; Vaccarino et al., 1993; Coderre and van Empel, 1994; Eisenberg et al., 1994; Hunter and Singh, 1994; Kristensen et al., 1994; Elliott et al., 1995; Lutfy and Weber, 1996; Chaplan et al., 1997; Davidson et al., 1997; Shimoyama et al., 1999; Sluka and Westlund, 1993a,b; Sluka et al., 1994; reviewed in Fundytus, 2001).

However, systemic or central administration of NMDA receptor antagonists is often associated with undesirable side-effects, such as motor incoordination and sedation, as well as cognitive deficits and psychotomimetic effects, in both rats (Cahusac et al., 1984; Hao and Xu, 1996) and humans (Arendt-Nielsen et al., 1996; Persson et al., 1995; Birch, 1995; Schugens et al., 1997; Muir et al., 1997; Murman et al., 1997; Max et al., 1995; Oye et al., 1992). Thus, perhaps NMDA receptors are not the optimal glutamate receptor target.

As well as NMDA receptors, glutamate acts at a family of receptors known as metabotropic glutamate receptors

* Corresponding author. Purdue Pharma L.P., 6 Cedar Brook Drive, Cranbury, NJ 08512, USA. Tel.: +1-609-409-5776; fax: +1-609-409-6922.
E-mail address: marian.fundytus@pharma.com (M.E. Fundytus).

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(mGluRs). These mGluRs are directly coupled to intracellular second messengers via guanine nucleotide regulatory (G) proteins, and are divided into three groups based on sequence homology, signal transduction mechanisms and receptor pharmacology (Hayashi et al., 1994; Conn and Pin, 1997). Group I mGluRs ($mGluR_1$ and $mGluR_5$) are positively coupled to phosphatidylinositol (PI) hydrolysis, and activation of these receptors leads to the release of intracellular Ca^{2+} and activation of protein kinase C (PKC) (Schoepp and Conn, 1993; Hayashi et al., 1994; Conn and Pin, 1997; Manzoni et al., 1990). Group II ($mGluR_2$ and $mGluR_3$) and Group III ($mGluR_{4,6,7,8}$) mGluRs are negatively coupled to the production of cyclic-3′,5′-monophosphate (cAMP) (Schoepp and Conn, 1993; Hayashi et al., 1994; Conn and Pin, 1997), but have differing receptor pharmacology (Saugstad et al., 1994). Activation of group I mGluRs enhances activity at NMDA receptors via a PKC-mediated mechanism (Raymond et al., 1994; Bleakman et al., 1992; Chen and Huang, 1992; Harvey and Collingridge, 1993; Kelso et al., 1992; Kitamura et al., 1993), while activation of Groups II and III mGluRs attenuates NMDA receptor activity (Martin et al., 1997).

In addition to NMDA receptors, mGluRs have been shown to be involved in the mediation of nociception associated with models of short-term inflammation. Administration of nonselective and selective mGluR antagonists reduces dorsal horn neuronal responses and pain behaviors following inflammation induced by mustard oil, carageenan or formalin (Young et al., 1994, 1995, 1997; Neugebauer et al., 1994; Fisher and Coderre, 1996).

Current technology allows for direct targeting of specific proteins, including receptor proteins, with antisense (AS) oligonucleotides. AS oligonucleotides bind to mRNA via Watson–Crick base pairing. Formation of an AS–mRNA complex leads to the inhibition of protein translation. This technology is termed AS oligonucleotide knockdown of a protein. Young et al. (1998) recently showed that AS oligonucleotide knockdown of spinal $mGluR_1$ reduces dorsal horn neuronal responses to mustard oil administration to the skin, a model of short-term inflammation.

Recently, we have shown that AS oligonucleotide knockdown of spinal $mGluR_1$ significantly reduces hyperalgesia and allodynia associated with a chronic constriction injury of the sciatic nerve in rats, without affecting thermal or mechanical sensitivity in the uninjured paw or in sham-operated animals (Fundytus et al., 2000). We also showed that $mGluR_1$ AS oligonucleotide treatment restores opioid efficacy, and reduces NMDA receptor hypersensitivity, in neuropathic rats (Fundytus et al., 2001). Concomitant with these behavioural effects, we demonstrated that intrathecal administration of $mGluR_1$ AS oligonucleotide significantly reduces the relative amount of $mGluR_1$ protein in lumbar spinal cord, and reduces PKC activation in lumbar spinal cord dorsal horn (Fundytus et al., 2001). In the present study, we examine further the role of $mGluR_1$ in chronic pain states, using a model of persistent inflammatory hyperalgesia. AS treatment was begun either prior to injection of complete Freund's adjuvant (CFA), to assess whether $mGluR_1$ AS could prevent the development of inflammatory hyperalgesia, or after CFA injection to determine whether $mGluR_1$ AS could reverse established inflammatory hyperalgesia. Here, we show that AS oligonucleotide knockdown of spinal $mGluR_1$ significantly reduces heat hyperalgesia and mechanical allodynia associated with CFA-induced chronic inflammation of one hindpaw. Parts of these data have previously been presented in abstract form (Fundytus et al., 1998b).

2. Methods

2.1. Subjects, surgery and induction of inflammation

Subjects were male Wistar rats weighing 325–375 g at the beginning of the experiment. Rats were housed three to four per cage with food and water freely available, and a 12:12-h light–dark cycle (lights on at 06:00 h).

All surgical and testing procedures conformed to the ethical guidelines stipulated by the Canadian Council on Animal Care, and were approved by the animal care committee at the Clinical Research Institute of Montreal. Intrathecal catheters were inserted using a lumbar catheterization method (Storkson et al., 1996). We chose this method because it is associated with minimal trauma, as the catheter is inserted from the back, and thus does not slide the length of the spinal cord, and therefore does not cause spinal cord damage. Because of the minimal trauma associated with this catheterization method, we were able to attach mini osmotic pumps at the same time, eliminating the need for a second surgery. During intrathecal surgery, rats were anesthetized with 60 mg/kg sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Quebec). The catheter was attached to an Alzet mini osmotic pump (Alzet Model 2001; ALZA, CA; infusion rate of 1 μl/h for 7 days) containing either artificial cerebrospinal fluid (ACSF), AS oligonucleotide solution or missense (MS) oligonucleotide solution. Mini-osmotic pumps were placed subcutaneously on the back. Rats were infused intrathecally for 7 days.

Chronic unilateral inflammation was induced by transdermally injecting 25 μl of CFA (1 mg/ml heat-killed and dried *Mycobacterium tuberculosis*; each milliliter of vehicle contains 0.85 ml paraffin oil + 0.15 ml mannide monooleate) (Sigma, Oakville, Ontario, Canada) in each of the dorsal and ventral surfaces of one hindpaw. Rats were anesthetized with 2.5% halothane in carbogen (95% O_2, 5% CO_2) for injection of CFA.

2.2. Oligonucleotides

As described in earlier studies (Fundytus et al., 2000, 2001), we designed an AS (5′-GAG CCG GAC CAT TGT GGC-3′) oligonucleotide complementary to base pairs 371–

388 of the rat mGluR$_1$ gene RATGPCR. A MS (5′-GAG CCG *AG*C *AC*T *GTG T*GC-3′) oligonucleotide was designed by taking the AS sequence and mismatching 4 base pair couples (creating eight mismatches between the AS and MS sequences). Oligonucleotides were purchased from Medicorp (Montreal, PQ). We used unmodified, phosphodiester-bonded (PO) oligonucleotides because this formulation has been shown to be both stable and nontoxic in the central nervous system (Akhtar and Agrawal, 1997; Wahlestedt, 1994; Whitesell et al., 1993; Yaida and Nowak, 1995). The vehicle used to dissolve the oligonucleotides, and as the vehicle treatment, was artificial cerebrospinal fluid (ACSF) (128.6 mM NaCl, 2.6 mM KCl, 1.0 mM $MgCl_2$, 1.4 mM $CaCl_2$, phosphate buffered to pH 7.4). Vehicle, AS and MS were continuously infused intrathecally, via the catheter, in a volume of 1 μl/h. The daily dose of AS and MS was 50 μg/day. We chose this dose of oligonucleotide based on previous experiments utilizing AS technology. Effective knockdown of receptors has been achieved with doses as low as 1 μg/day, up to doses as high as 720 μg/day (Wahlestedt, 1994). This dose of AS and MS oligonucleotides was not found to produce any motoric or sedative side-effects, as examined using placing, righting and grasping reflexes.

2.3. Assessment of heat hyperalgesia and mechanical allodynia

Heat sensitivity was measured by applying focussed radiant heat to the plantar surface of each hindpaw and measuring the latency for the rat to withdraw its paw (Hargreaves et al., 1988). Heat hyperalgesia was assessed by calculating the percent decrease in latency (from baseline) on Days 1 to 8 after CFA injection. Data were analyzed by repeated measures ANOVA with intrathecal treatment and paw (injected vs. contralateral) as the independent groups factors and days post CFA injection as the repeated measures factor. Significant results were further analyzed with post-hoc Fisher's LSD *t*-tests.

Mechanical sensitivity was measured by applying thin filaments (von Frey hairs) to the plantar surface of the hindpaw and determining the 50% response threshold (in grams) for paw withdrawal using the up–down method of filament presentation exactly as described in Chaplan et al. (1994). Mechanical allodynia was assessed by calculating the percent decrease in 50% response threshold (from baseline) on Days 1–8 after CFA injection. Data were analyzed by repeated measures ANOVA with intrathecal treatment and paw (injected vs. contralateral) as the independent groups factors and days post CFA injection as the repeated measures factor. Significant results were further analyzed with post-hoc Fisher's LSD *t*-tests. Heat and mechanical sensitivity were measured in the same group of rats, with a minimum interval of 1 h between the tests. Because oligonleotides were administered intrathecally, and therefore would be unlikely to affect the periphery, paw volume was not measured in this study.

2.4. Treatment schedule

2.4.1. Pre-CFA treatment group

Three days before injection of CFA, rats were implanted with intrathecal catheters attached to mini osmotic pumps containing either ACSF ($n=7$), AS ($n=6$) or MS ($n=6$) solution. Heat and mechanical sensitivity were measured prior to surgery or CFA injection (baseline), and again 1, 2, 4, 6 and 8 days after CFA injection. Mean baseline withdrawal latencies from radiant heat for the ipsilateral paw were: ACSF-treated = 13.43 ± 1.52 s, MS-treated = 11.48 ± 1.94 s, AS-treated = 12.75 ± 0.98 s. Mean baseline 50% withdrawal thresholds for the ipsilateral paw were: ACSF-treated = 10.41±1.48 g, MS-treated=12.95±0.95 g, AS-treated= 9.67± 2.30 g. This treatment schedule was employed to see if mGluR$_1$ AS could prevent the *development* of inflammatory pain. Western blot analysis was carried out on lumbar spinal cords from rats in this treatment group.

2.5. Post-CFA treatment group

A separate group of rats was injected with CFA, followed by implantation of intrathecal catheters attached to mini osmotic pumps containing either ACSF ($n=7$), AS ($n=6$) or MS ($n=5$) solution 2 days later. Heat and mechanical sensitivity were measured prior to surgery or CFA injection (baseline), and again 1, 2, 4, 6 and 8 days after CFA injection. Mean baseline withdrawal latencies from radiant heat for the ipsilateral paw were: ACSF-treated = 13.43 ± 1.52 s, MS-treated = 14.33 ± 1.95 s, AS-treated = 17.12 ± 0.74 s. Mean baseline 50% withdrawal thresholds for the ipsilateral paw were: ACSF-treated = 10.41 ± 1.48 g, MS-treated = 9.71 ± 1.88 g, AS-treated = 12.48 ± 1.27 g. This treatment was employed to determine whether AS oligonucleotide knockdown of spinal mGluR$_1$ could *reverse* hyperalgesia due to an established inflammatory injury.

2.6. Western blot analysis (protein determination)

On the 7th day of infusion in rats pretreated with oligonucleotides, rats were decapitated, and their spinal cords were pressure ejected and rapidly frozen. Spinal cords were stored at −70 °C until analysis. The lumbar enlargement of each spinal cord was homogenized in Tris buffer containing protease inhibitors (leupeptin, aprotinin, pepstatin, 4-amidinophenylmethanesulfonyl fluoride hydrochloride). Group I mGluRs are most likely found in the dorsal horn in lumbar spinal cord (Berthele et al., 1999; Alvarez et al., 2000). Concentration of protein in each sample was determined using the method of Bradford (Bradford, 1976). The concentration of protein in each sample fell on the linear portion of the curve. For separation, 20 μg of total protein was loaded onto the gel for separation by electrophoresis (SDS-PAGE, 5% polyacrylamide gel). After separation, proteins were electrotransferred to PVDF membrane. The membrane was probed with an anti-rat mGluR$_1$ or anti-rat mGluR$_5$ antibody

(primary antibody) raised in rabbits (Upstate Biotechnology, Lake Placid, NY). These antibodies are raised against the C termini of the receptors, a region that is *unique* to each of these receptors, and specificity was verified with immunoblotting (Martin et al., 1992; Abe et al., 1992; Upstate Biotechnology). The primary antibody was later labeled with a peroxidase-conjugated anti-rabbit antibody (secondary antibody; Jackson Immunoresearch Laboratories, Westgrove, PA). The secondary antibody was detected by chemiluminescence (Boehringer-Mannheim Roche Diagnostics, Laval, QC) and the membranes were apposed to Kodak Biomax MR film for 1 min. Band density was measured using Alpha Imager software. The $mGluR_1$ is a protein of approximately 133–142 kDa (Houamed et al., 1991; Martin et al., 1992; Masu et al., 1991) and the $mGluR_5$ protein is approximately 128 kDa (Abe et al., 1992). Mean density was calculated from transblots of separate animals ($n=2$–4 per group). Data were analyzed using two-tailed Student's *t*-tests on binding density scores.

3. Results

3.1. Heat hyperalgesia

In the pre-CFA treatment group, ACSF- and MS-treated rats displayed significant heat hyperalgesia from Days 1 to 8 after CFA injection, as indicated by a large decrease from baseline in response latency (Fig. 1A) when radiant heat was applied to the ventral surface of the injected paw. Although there was no significant effect of oligonucleotide treatment on Day 2 after CFA injection, heat hyperalgesia of the injected hindpaw was attenuated in AS-treated rats on Day 1 and Days 4–8 after CFA injection, as indicated by longer response latencies (Fig. 1A). There were no differences in heat sensitivity between groups on the contralateral hindpaw.

In the post-CFA treatment group, all rats initially displayed heat hyperalgesia, as indicated by a large decrease from baseline in response latency of the injected hindpaw (Fig. 1B) on Day 1 after CFA injection, prior to the infusion of oligonucleotides. Oligonucleotide infusion began on Day 2 after CFA injection. ACSF- and MS-treated rats remained hyperalgesic from Days 4 to 8 after CFA injection, as indicated by a continued reduction in response latency in the injected paw. In contrast, heat hyperalgesia was attenuated in the injected hindpaw of AS-treated rats, as indicated by an increase in response latency following drug infusion on Days 4–8 after CFA injection (Fig. 1B). There were no differences in heat sensitivity between groups in the contralateral hindpaw.

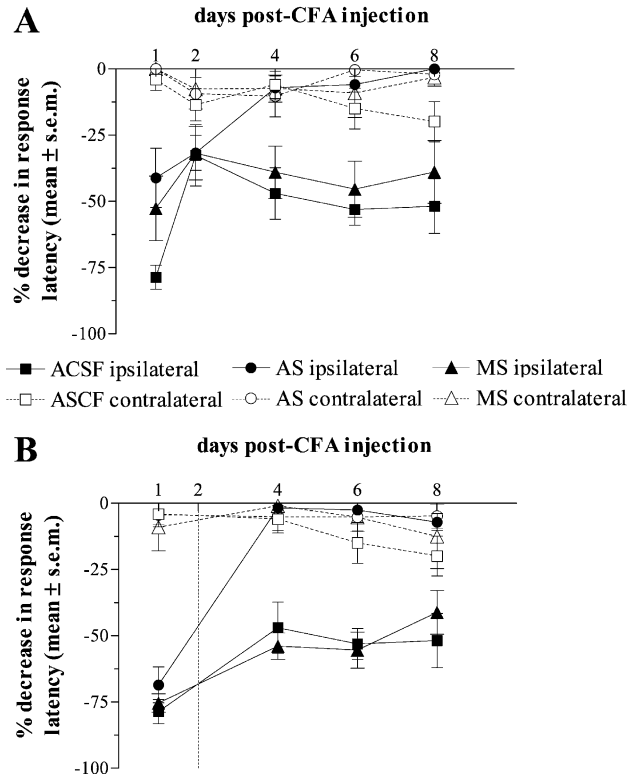

Fig. 1. Heat hyperalgesia. Percent change from baseline in the response (paw withdrawal) latency to radiant heat applied to the ventral surface of the inflamed and contralateral hindpaws of rats injected with CFA on Days 1–8 after CFA injection. (A) Pre-CFA treatment group: mean percent decrease in response latency in ACSF-, AS- and MS-treated rats. ANOVA indicated a significant intrathecal treatment by hindpaw interaction [$F(2,32)=5.93$, $P<.01$] and a significant intrathecal treatment by day interaction [$F(8,128)=2.52$, $P<.05$]. Post-hoc Fisher's LSD *t*-tests indicated that the percent decrease in withdrawal latency of the injected paw was significantly attenuated in AS-treated rats compared to ACSF- and MS-treated rats, while there was no difference between groups in the contralateral hindpaw. Post-hoc Fisher's LSD *t*-tests showed that AS-treatment attenuated heat hyperalgesia on Days 1, 4, 6 and 8, but not on Day 2 following CFA injection. (B) Post-CFA treatment group: mean percent decrease in response latency in ACSF-, AS- and MS-treated rats. Oligonucleotide infusion began on Day 2 after CFA injection (indicated by the dotted line). ANOVA indicated a significant intrathecal treatment by hindpaw × Day interaction [$F(6,90)=2.93$, $P<.05$]. Post-hoc Fisher's LSD *t*-tests showed that the decrease in withdrawal latency was significantly greater in the injected paw for all intrathecal treatment groups on Day 1 following CFA injection, prior to oligonucleotide infusion. On Days 4–8 after CFA injection, the decrease in withdrawal latency of the injected paw was significantly attenuated in AS-treated rats compared to ACSF- and MS-treated rats, and in AS-treated rats the paw withdrawal latency was not different between injected and contralateral paws.

3.2. Mechanical allodynia

In the pre-CFA treatment group, ACSF- and MS-treated rats displayed significant mechanical allodynia on Days 1–8 after CFA injection, as indicated by a large decrease from baseline in 50% response threshold (grams) in response to von Frey hair stimulation of the plantar surface of the injected paw (Fig. 2A). Mechanical allodynia of the injected hindpaw was attenuated in AS-treated rats on all test days as indicated by the significantly higher 50% response thresholds (Fig. 2A). There were no differences in mechanical sensitivity between groups in the contralateral hindpaw.

In the post-CFA treatment group all rats initially displayed mechanical allodynia of the injected hindpaw on Day

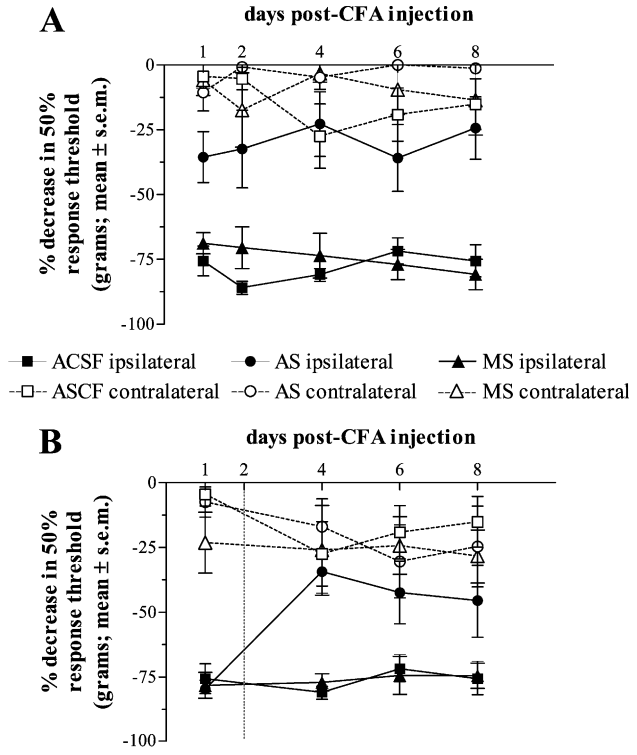

Fig. 2. Mechanical allodynia. Percent change in 50% response threshold to von Frey hair stimulation of the ventral surface of the injected and contralateral hindpaws of rats injected with CFA on Days 1–8 after CFA injection. (A) Pre-CFA treatment group: mean percent decrease in 50% response threshold in ACSF-, AS- and MS-treated rats. ANOVA indicated a significant intrathecal treatment by hindpaw interaction [$F(2,30)=4.63$, $P<.05$]. Post-hoc Fisher's LSD *t*-tests indicated that the decrease in 50% response threshold in the injected hindpaw was attenuated in AS-treated rats, compared to ACSF- and MS-treated rats on all test days. (B) Post-CFA treatment group: mean percent decrease in 50% response threshold in ACSF-, AS- and MS-treated rats. Oligonucleotide infusion began on Day 2 after CFA injection (indicated by dotted line). ANOVA indicated a significant intrathecal treatment by hindpaw interaction [$F(2,32)=8.03$, $P<.01$]. Post-hoc Fisher's LSD *t*-tests showed that the decrease in 50% response threshold in the injected paw was attenuated in AS-treated rats, compared to ACSF- and MS-treated rats (from Days 4 to 8 after CFA injection).

1 after CFA injection, prior to oligonucleotide infusion, as indicated by a large decrease in 50% response threshold compared to baseline (Fig. 2B). Following oligonucleotide infusion, ACSF- and MS-treated rats remained allodynic from Days 4 to 8 after CFA injection, as indicated by a continued large decrease from baseline 50% response threshold (Fig. 2B). In contrast, mechanical allodynia was attenuated in the injected hindpaw of AS- treated rats, as indicated by significantly higher 50% response thresholds on Days 4–8 after CFA injection (Fig. 2B). There were no differences in mechanical sensitivity between groups in the contralateral hindpaw.

3.3. Western blot analysis

Western blot analysis showed that although there appeared to be a slight increase in binding density of $mGluR_1$ IgG in lumbar spinal cord from ACSF- and MS-treated rats compared to naive rats, there were no statistically significant differences in $mGluR_1$ binding density between either ACSF- or MS-treated rats and naive rats (Student's *t*-test, $P>.05$; Fig. 3). There was also no difference in $mGluR_1$ protein between ACSF- and MS- treated rats ($P>.05$; Fig. 3). Although peak binding density of $mGluR_1$ IgG was lower in lumbar spinal cord from AS-treated rats versus naive rats, the results failed to reach statistical significance ($P=.08$; Fig. 3). However, binding density of $mGluR_1$ IgG was significantly less in AS-treated rats versus either ACSF- ($P<.05$) or MS-treated rats ($P<.05$). These results suggest that intrathecal administration of $mGluR_1$ AS induced a reduction in $mGluR_1$ protein in lumbar spinal cord. Western blot analysis of $mGluR_5$ showed that $mGluR_1$ AS treatment did not

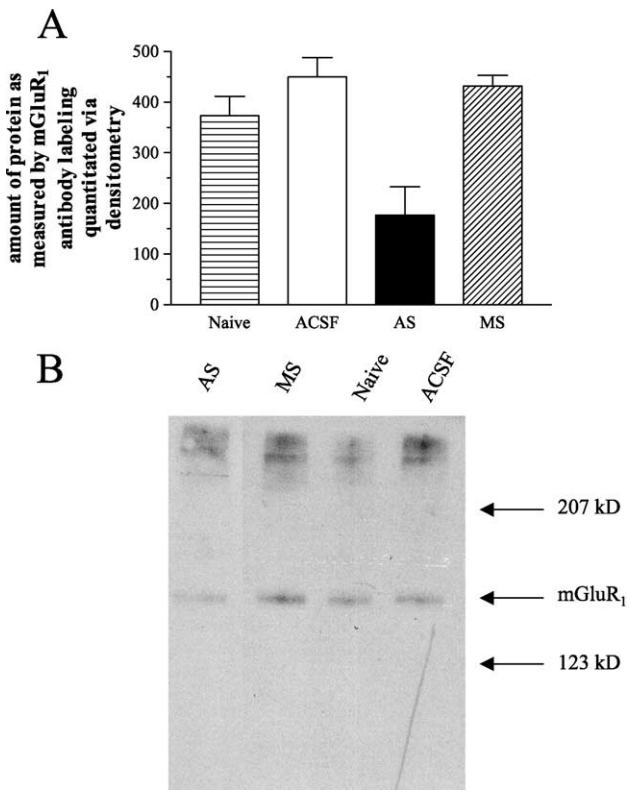

Fig. 3. Representative Western blots and histogram summary results ($n=2–4$ per group) from Western blot analysis of lumbar spinal cords taken from naive rats (no treatment), and ACSF-, AS- and MS-treated rats injected with CFA after 7 days of oligonucleotide infusion. (A) Peak binding density of $mGluR_1$ IgG in lumbar spinal cords taken from naive, and ACSF-, AS- and MS-treated rats injected with CFA. Although there appeared to be a slight increase in binding density of $mGluR_1$ IgG in lumbar spinal cord from ACSF- and MS-treated rats compared to naive rats, there were no statistically significant differences in $mGluR_1$ binding density between either ACSF- or MS-treated rats and naive rats (Student's *t*-test, $P>.05$). There was no difference in $mGluR_1$ protein between ACSF- and MS-treated rats ($P>.05$). Although peak binding density of $mGluR_1$ IgG was lower in lumbar spinal cord from AS-treated rats versus naive rats, the results failed to reach statistical significance ($P=.08$). However, binding density of $mGluR_1$ IgG was significantly less in AS-treated rats versus either ACSF-treated rats ($P<.05$) or MS-treated rats ($P<.05$). (B) Sample Western blot showing binding of $mGluR_1$ IgG to the $mGluR_1$ protein.

decrease the amount of $mGluR_5$ protein in lumbar spinal cord ($P>.05$ versus either ACSF- or MS-treated rats). These Western blot results are similar to what we have previously seen (Fundytus et al., 2001).

4. Discussion

In the present study, we showed that AS oligonucleotide knockdown of spinal $mGluR_1$ alleviates thermal hyperalgesia and mechanical allodynia in a rat model of chronic inflammation. When rats were infused intrathecal with $mGluR_1$ AS oligonucleotide prior to CFA injection, mechanical allodynia was attenuated from Days 1 to 8 after CFA injection, whereas heat hyperalgesia was attenuated on Day 1 and then from Days 4 to 8 after CFA injection. The lack of effect of AS treatment on heat hyperalgesia in Day 2 after CFA injection in the pretreatment group may be due to one of two things. First, ACSF- and MS- treated rats had longer response latencies on Day 2 than on either Day 1 or Days 4–8 after CFA injection. Second, it appears that the effects of AS treatment were not maximal until 4 days after CFA injection (7 days of oligonucleotide infusion). It is also interesting to note that when rats were treated with AS prior to the induction of inflammation, the beneficial effects outlasted the treatment. This effect has previously been observed with both NMDA receptor antagonists (Mao et al., 1992a,b) or $mGluR_1$ AS (Fundytus et al., 2001). When infusion of $mGluR_1$ AS oligonucleotide began after injection of CFA, when the inflammatory pain syndrome was established, AS treatment attenuated heat hyperalgesia and mechanical allodynia at all time points after infusion of AS. These results suggest that knockdown of spinal $mGluR_1$ can *reverse* hyperalgesia and allodynia associated with chronic inflammatory pain. It is especially interesting to note that, whereas the maximal effect of AS treatment occurred after 7 days of oligonucleotide infusion in the pretreatment group, AS was maximally effectively after only 2 days of infusion in the posttreatment group. Both of these time points correspond to Day 4 after CFA injection, when the inflammatory hyperalgesia is well established. It can therefore be hypothesized that AS knockdown of $mGluR_1$ produces the best therapeutic effect when the inflammatory pain is well established. There is, however, an interesting mechanistic hypothesis, discussed in a paper by Hua et al. (1998). Hua and colleagues showed that 3 days of intrathecal NK1 AS was ineffective in reducing flinching in the formalin test in otherwise naive animals. However, when testing was preceded by an intrathecal dose of substance P (SP) to activate and induce internalization of the NK1 receptor, NK1 AS treatment attenuated the flinching response. AS treatment inhibits the synthesis of new receptor protein, but does not affect the activity of protein already present in the membrane. Thus, if the receptor is not previously activated and internalized (with at least some degradation) there may be enough functional receptor protein available to elicit a full physiological response. If the receptor protein has been depleted by activation, then AS inhibition of new protein synthesis would be sufficient to reduce the physiological response. In addition, it has been shown that Group I mGluRs generally stay away from the postsynaptic specialization, and rather show a peri-synaptic or extra-synaptic expression, occurring most predominantly (50% of staining) within a 60 nm annulus surrounding the edge of the postsynaptic specialization, with the rest being found at more distant positions (Ottersen and Landsend, 1997; Luján et al., 1997). This suggests that Group I mGluRs may only be significantly activated under conditions of excess glutamate release. In our present experiments, it is possible that an excess of chronic glutamate release in animals with established inflammation has led to a more dynamic state of $mGluR_1$ than is present in animals that received initiation of AS treatment prior to CFA injection. Thus, there may be a higher turnover rate of the receptor as a result of ongoing stimulation, allowing, in turn, the AS effect to become apparent at earlier time points in previously inflamed animals. Western blot analysis showed that $mGluR_1$ AS oligonucleotide infusion reduced the amount of $mGluR_1$ protein in the lumbar enlargement of spinal cord of CFA-injected rats. However, the reduction in $mGluR_1$ failed to reach significance when CFA-injected AS-treated rats were compared to naive rats. Perhaps, this is because CFA injection may cause a slight up-regulation of $mGluR_1$ (as suggested by the slightly higher $mGluR_1$ IgG binding density seen in ACSF- and MS-treated rats compared to naive rats), making it difficult to reach statistical significance when comparing with naive rats. Although the present study did not include Western blot analyses on rats given CFA injections prior to AS infusion, a future study could be done to determine whether the degree of knockdown after 2 days of oligonucleotide infusion in preinflamed animals is equivalent to the degree of knockdown after 7 days of oligonucleotide infusion in postinfusion inflamed animals.

Because of the robust behavioral effect, it would be interesting to determine more precisely the location of the $mGluR_1$ knockdown. There are some discrepant results as to where in the spinal cord $mGluR_{1\alpha}$ is found. One of the first studies showed that $mGluR_{1\alpha}$ mRNA is expressed at high levels in rat spinal cord (Valerio et al., 1997). Using immunocytochemistry, some studies have shown high levels of $mGluR_{1\alpha}$ immunoreactivity in laminae I–III of rat spinal cord dorsal horn (Yung, 1998). Laminae I and II contain glomerular afferents that most likely arise from nociceptive afferents. Other investigators have shown weak labeling of $mGluR_{1\alpha}$ in lamina I, with strong labeling in lamina II (Tang and Sim, 1999), while others yet see weak labeling in lamina I, with no labeling in lamina II (Alvarez et al., 2000). Alvarez et al. (2000) observed $mGluR_{1\alpha}$-like immunoreactivity generally distributed throughout laminae III–X. Glomerular terminals in lamina III arise largely from low-threshold mechanoreceptive afferents. In the present

study, we saw a robust effect of knockdown of $mGluR_{1\alpha}$ at reducing mechanical allodynia. If $mGluR_{1\alpha}$ is largely found in the deeper laminae, as seen by Alvarez et al (2000), this would explain the behavioral effect we observed.

Previous data suggest that Group I mGluRs play a role in mediating short-term inflammatory pain. It has previously been shown that the nonselective mGluR antagonist L-AP3, as well as the relatively selective Group I mGluR antagonist (*S*)-4CPG, attenuate dorsal horn neuronal responses to repeated mustard oil application to the skin (Young et al., 1994, 1995, 1997). Antagonism of Group I mGluRs also reduces neuronal hypersensitivity in rats with an inflamed knee joint (Neugebauer et al., 1994), while having no effect in normal animals. In subsequent studies of the role of Group I mGluRs in pain processing, Neugebauer et al. (1999) showed that both L-AP3 and (*S*)-4CPG, as well as the more selective antagonists AIDA (selective for $mGluR_1$) and CPCCOEt (more potent at $mGluR_1$ than $mGluR_5$), inhibit dorsal horn neuronal responses to brief high intensity cutaneous stimulation and reduce capsaicin-induced central sensitization of dorsal horn neurons (Neugebauer et al., 1999). Recently, it has been shown that the relatively selective Group I mGluR antagonist (*S*)-4CPG slightly decreases formalin-induced nociception (Fisher and Coderre, 1996). However, the specific role of mGluRs cannot be determined using these antagonists. In addition to its antagonism of mGluRs, L-AP3 also has antagonistic actions at NMDA receptors (Birse et al., 1993). Moreover, (*S*)-4CPG has a dual action whereby it is an antagonist at Group I mGluRs, but an agonist at Group II mGluRs (Hayashi et al., 1994; Eaton et al., 1993; Watkins and Collingridge, 1994). Therefore, it is unclear whether the beneficial actions of (*S*)-4CPG can be attributed solely to antagonism of Group I mGluRs, or whether activation of Group II mGluRs also plays a role. Moreover, CPCCOEt does not differentiate entirely between $mGluR_1$ and $mGluR_5$. The antagonist AIDA has been purported to be selective for $mGluR_1$, therefore, results obtained by Neugebauer et al. (1999) suggest that perhaps $mGluR_1$ in particular is involved in pain processing.

Other data suggest that group I mGluRs may be only minimally involved in acute pain transmission. Zahn and Brennan (1998) showed that antagonism of mGluRs does not attenuate hyperalgesia associated with an incision injury of the hindpaw in rats that mimics acute postoperative pain. The fact that Neugebauer et al. (1994) saw no reduction in dorsal horn neuronal responses in normal animals (while observing a robust effect in animals with an inflamed knee joint) also suggests that mGluRs are more important for pain transmission when a longer-term injury is present. Recent data from our laboratory also suggest that Group I mGluRs may not play a significant role in acute or short-term pain, but play a pivotal role in chronic pain. Thus, although intrathecal administration of either (*S*)-4CPG (Fisher et al., 1998) or antibodies selective for either $mGluR_1$ or $mGluR_5$ (Fundytus et al., 1998a,b) significantly reduced chronic neuropathic pain, intrathecal antibodies had no effect on heat sensitivity or formalin-induced nociception in naive rats (Fundytus et al., 1998a,b). We also showed that although knockdown of spinal $mGluR_1$ reduced hyperalgesia and allodynia in the hindpaw ipsilateral to a sciatic nerve constriction, there was no significant effect in either the contralateral paw or in sham-operated rats (Fundytus et al., 2001). However, Young et al. (1998) found that in addition to reducing dorsal horn neuronal responses to repeated mustard oil application, spinal knockdown of $mGluR_1$ increased response latencies in the tail-flick test in naive rats. The discrepancy in results between the two knockdown studies may be due to differences in the oligonucleotide formulation, or in the placement of the spinal catheter. Two other studies suggest a role for group I mGluRs in acute pain transmission. In adult female sheep with no injury or tissue damage, intrathecal administration of low doses (50 nmol) of (*S*)-3,5-DHPG induced a decrease in mechanical withdrawal threshold, while a mid-range dose (500 nmol) failed to alter mechanical threshold, and the highest dose used (5 μmol) induced a significant increase in mechanical withdrawal threshold (Dolan and Nolan, 2000). In this study, the selective Group I mGluR antagonist, AIDA, alone had no effect, and the authors suggested that the Group I mGluR pathway is not tonically active in non-injured animals (Dolan and Nolan, 2000). However, in another study, intracerebroventricular administration of AIDA in mice induced an increase in pain threshold in the hotplate and acetic acid writhing tests, with a bell-shaped dose–response curve (Moroni et al., 1997). Yet, another group of investigators found that the nonselective mGluR antagonist AP3 did not affect the flexion withdrawal reflex in either uninjured or inflamed (intra-articular mustard oil) spinalized rats (Silva et al., 1997), in agreement with our observations. Thus, the role of Group I mGluRs in acute pain transmission remains controversial, and complicated. Further research is necessary to clarify the degree of involvement of Group I mGluRs in acute or short-term pain versus chronic pain.

In summary, these and previous data suggest that Group I mGluRs, and particularly $mGluR_1$, play a vital role in the mediation of inflammatory pain. The degree of involvement of $mGluR_1$ in acute versus longer-term (chronic) pain remains to be definitively determined. However, data from our laboratory and others suggest that $mGluR_1$ may be a viable target for the development of new analgesics. Moreover, our data suggest that AS oligonucleotide knockdown of $mGluR_1$ may be particularly efficacious.

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