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Attenuation of morphine tolerance after antisense oligonucleotide knock-down of spinal mGluR1

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1 Chronic systemic treatment of rats with morphine leads to the development of opioid tolerance. This study was designed to examine the effects of intrathecal (i.t.) infusion of a metabotropic glutamate receptor 1 (mGluR1) antisense oligonucleotide, concomitant with chronic morphine treatment, on the development of tolerance to morphine's antinociceptive effects.

2 All rats received chronic (6 day) s.c. administration of morphine to induce opioid tolerance. Additionally, rats were treated with either mGluR1 antisense (AS), missense (MIS) or artificial cerebrospinal fluid (ACSF) by i.t. infusion *via* chronically implanted i.t. catheters connected to osmotic mini-pumps. The effects of acute i.t. or s.c. morphine on tail-flick latencies were assessed prior to and following chronic s.c. morphine treatment for all chronic i.t. infusion groups. mGluR1 protein level in the spinal cord was determined by Western blot analysis for all treatments, assessing the efficiency of knock-down with AS treatment.

3 Acute i.t. morphine dose-dependently produced antinociception in the tail-flick test in naïve rats. Systemic morphine-treated rats administered i.t. ACSF or MIS developed tolerance to i.t. morphine. Chronic i.t. infusion with mGluR1 AS significantly reduced the development of tolerance to i.t. morphine.

4 In contrast to i.t. morphine, tolerance developed to the antinociceptive effects of s.c. morphine, in all i.t. infusion groups, including the mGluR1 AS group.

5 The spinal mGluR1 protein level was dramatically decreased after mGluR1 AS infusion when compared to control animals (naïve and ACSF-treated animals).

6 These findings suggest that the spinal mGluR1 is involved in the development of tolerance to the antinociceptive effects of morphine. Selective blockade of mGluR1 may be beneficial in preventing the development of opioid analgesic tolerance.

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-2-amino-3 (hydroxy-5-methylisoxazol-4yl) propanoic acid; AS, antisense; AUC, area under the curve; i.t., intrathecal; LSD, least square difference; mGluR, metabotropic glutamate receptor; MIS, missense; %MPE per cent maximum possible effect; NMDA, N-methyl-D-aspartate; PI, phosphatidylinositol hydrolysis; PKC, protein kinase C; s.c., subcutaneous

Introduction

Although opioids are often used as analgesics, their therapeutic efficacy is limited by the development of tolerance (Martin, 1967). Both acute and chronic tolerance occur in animals (Cochin & Kornetsky, 1964; Yaksh, 1991) and humans (Houde *et al*., 1966; McQuay *et al*., 1981; 1992); although there is a great deal of variability of tolerance in humans, and often dose escalation may also depend on the worsening of the pathology causing pain (Foley, 1991; Portenoy, 1994). Throughout the years, many neurotransmitter systems have been implicated in the development of tolerance elicited by repeated morphine administration. One of the most prominent transmitters identified has been the excitatory amino acid glutamate. It has been found that concurrent treatment of rats with daily injections of morphine and either a non-selective EAA antagonist (kynurenic acid) or selective N-methyl-D-aspartate (NMDA) antagonists (MK-801 and ketamine) are effective in attenuating the development of tolerance to morphine's analgesic effect (Bilsky *et al*., 1996; Marek *et al*., 1991; Trujillo & Akil, 1991; 1994). NMDA antagonists have also been found to reduce opioid tolerance in humans with chronic pain (Bell, 1999; Clark & Kalan, 1995).

In addition to NMDA receptors, glutamate acts post-synaptically on two other types of ionotropic receptors (α-2-amino-3(hydroxy-5-methylisoxazol-4yl) propanoid acid

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(AMPA) and kainate receptors, and a family of metabotropic receptors (mGluRs) (Schoepp & Conn, 1993). Most investigators have examined the involvement of NMDA receptors in morphine tolerance and dependence, but other glutamate receptors, particularly mGluRs, and related intracellular messenger systems have recently been implicated in morphine dependence (Fundytus & Coderre, 1994; 1996; 1997; Fundytus *et al*., 1997). Briefly, it was demonstrated that morphine withdrawal symptoms were attenuated with subtype-selective antagonists of mGluRs (Fundytus *et al*., 1997).

mGluRs are a family of receptors that are directly coupled, *via* guanine nucleotide regulatory (G) proteins to intracellular second messengers (Houamed *et al*., 1991; Martin *et al*., 1992; Masu *et al*., 1991). This family of mGluRs is classified into three groups based on sequence homology, signal transduction mechanisms and receptor pharmacology (Hayashi *et al*., 1994; Conn & Pin, 1997; Schoepp & Conn, 1993). Group I mGluRs, which include $mGluR_1$ and $mGluR_5$ produce an increase in phospholipase C (PLC) which stimulates phosphatidylinositol (PI) hydrolysis (Sladeczek *et al*., 1985; Sugiyama *et al*., 1987), and results in an increase in inositol-1,4,5-triphosphate (IP_3), intracellular Ca^{2+} (Berridge & Irvine, 1984) and protein kinase C (PKC) activity (Hug & Sarre, 1993; Nishizuka, 1986). Group II ($mGluR_2$ and $mGluR_3$) and group III ($mGluR_{4,6,7,8}$) mGluRs are negatively coupled to activation of adenylate cyclase and the production of cyclic adenosine 3′,5′-monophosphate (cAMP) (Schoepp & Conn, 1993; Conn & Pin, 1997).

Since $mGluR_1$ is found in laminae I and II of the dorsal spinal cord (Yung, 1998; Valerio *et al*., 1997), and has been implicated in pain processing (Fisher & Coderre, 1996; Fundytus *et al*., 2001; Neugebauer *et al*., 1999; Young *et al*., 1998), as well as opioid dependence (Fundytus & Coderre, 1994; 1996; Fundytus *et al*., 1997), we examined whether $mGluR_1$ in the spinal cord might contribute to the development of morphine tolerance. We tested this hypothesis by inducing a knock-down of $mGluR_1$ in the spinal cord with chronic i.t. infusion of an $mGluR_1$ antisense (AS) oligonucleotide. Here we show that chronic treatment of rats with an AS oligonucleotide targeting $mGluR_1$, concurrent with daily injections of morphine, attenuated the development of tolerance to the analgesic effects of morphine. Parts of this manuscript have been presented in abstract form (Sharif *et al*., 1999).

Methods

Subjects and surgery

Male Long Evans rats (Charles River), weighing 275–300 grams at the start of the experiment, were used in this study. Rats were housed in groups of 3–4, with food and water freely available. Rats were maintained on a 12 : 12 h light : dark cycle (lights on at 07:30 h). All experiments were approved by the animal care committee at the Clinical Research Institute of Montreal and were conducted in accordance with the Canadian guidelines on ethical treatment of animals in research.

Four days prior to morphine treatment, each rat was anaesthetized with sodium pentobarbitone (65 mg kg^{-1}, i.p.; MTC Pharmaceuticals) and a lumbar spinal catheter (PE-10 polyethylene tubing) was inserted in the i.t. space, according to the methods of either Yaksh & Rudy (1976) (rostral approach) or Storkson *et al*. (1996) (caudal approach). For the former, a small opening was made at the cisterna magna, and a catheter (PE 10 tubing attached to silicone tubing for attachment to an osmotic pump) was inserted into the subarachnoid space and caudally directed 8 cm to the lumbar enlargement of the spinal cord. After anchoring the catheter, an osmotic minipump (Alzet mini-osmotic pump, ALZA Corporation, model 2001) was attached to it and the pump was implanted subcutaneously. Only animals exhibiting no motor deficits as a result of the surgery were used for behavioural testing. Alternatively, the catheter was inserted through a 20 gauge needle which was used to perform a lumbar puncture between the L5 and L6 vertebrae in anaesthetized rats. After the catheter was pushed 3 cm beyond the needle tip, both the tubing and the needle were sutured to muscle by 3.0 silk sutures. The catheter was attached by its caudal end to a silicone tubing, which would later be connected to the infusion pump. The next day, rats were briefly anaesthetized with halothane to isolate the i.t. catheter, and following recovery, lidocaïne (2%, 50 μl) was injected through the catheter to test its position. Rats showing no hindlimb paralysis following lidocaïne injection were excluded from the study; osmotic pumps were attached and implanted, as above, in lidocaine-positive rats. For both rostral and caudal catheters, infusion pumps contained either artificial cerebral spinal fluid (ACSF), antisense (AS) or missense (MIS), and pumped at a rate of 1 μg h^{-1} for 7 days. Rats were then left to recover for 3 days before the chronic treatment with morphine.

Antinociceptive testing

The tail-flick test was used for antinociceptive testing. This test involved measuring the latency (s) for the rat to withdraw its tail from a hot water bath (55°C). The rat was hand-held during testing to minimize stress associated with prolonged immobilization. Next the rat's tail was placed in the hot water up to 5 cm from the tip, and the latency to flick or curl the tail from the water was recorded. Baseline responses were typically 2–3 s and a cut-off was imposed at 10 s to prevent tissue damage.

Drugs

Rats were continuously infused i.t. with ACSF, $mGluR_1$ AS, or $mGluR_1$ MIS oligonucleotides for 7 days. We used an AS oligonucleotide targeting $mGluR_1$ (AS: 5′-GAG CCG GAC CAT TGT GGC-3′), previously described in Fundytus *et al*. (2001), (whose sequence is complementary to base pairs 371–388 of the mRNA of rat $mGluR_1$ gene), an $mGluR_1$ MIS oligonucleotide (whose sequence is comparable to the $mGluR_1$ AS), but in which some nucleotides have been changed as indicated by the underlining (5′-GAG CCG AGC ACT GTG TGC-3′), or the vehicle ACSF (aqueous solution of (in mM): NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; phosphate buffered, pH 7.33). Oligonucleotides were purchased from Medicorp Inc (Montreal, PQ, Canada). We used unmodified, phosphodiester-bonded oligonucleotides because this formulation has been shown to be both stable and non-

toxic in the central nervous system (Whitesell *et al*., 1993; Yaida & Nowak, 1995). Vehicle, AS and MS were continuously infused i.t. through the catheter at a rate of 1 μl h^{-1}. The daily dose of AS and MS was 50 μg day^{-1}. 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^{-1}, up to doses as high as 720 μg day^{-1} (Wahlestedt, 1994). This dose of AS and MS oligonucleotide was not found to produce any motor or sedative side-effects, as examined using placing, righting and grasping reflexes.

Pre-treatment testing (Naïve rats)

First, all rats were tested prior to any treatment for their baseline tail-flick latency. Next, separate groups of rats were injected with i.t. morphine (3, 10 or 30 μg in a 20 μl volume) *via* acute lumbar puncture, while briefly anaesthetized with halothane. Three additional groups of rats were injected with s.c. morphine (1, 3, 10 mg kg^{-1}). Tail-flick latencies were recorded every 15 min for 60 min post-morphine administration to determine morphine's dose-dependent antinociceptive effects in naïve rats.

Chronic morphine administration

Three days after the i.t. ACSF, $mGluR_1$ MIS or $mGluR_1$ AS infusion started, rats were injected with escalating doses of morphine (Sabex, Mississauga, ONT, Canada) every 12 h for 5 days (8, 10, 10, 15 and 15 mg kg^{-1}, s.c.) to induce tolerance to morphine's antinociceptive effects.

Post-treatment testing

The day following the 5 days of chronic s.c. morphine treatment, rats were tested after either i.t. (3, 10 or 30 μg in a 20 μl volume) or s.c. (10 mg kg^{-1}) morphine injection, according to the same testing schedule described above for pre-treatment testing. The rats that received s.c. or i.t. morphine for the pre-treatment test also received s.c. or i.t. morphine, respectively, for the post-treatment test. The s.c. post-treatment trials were performed first in rats given chronic i.t. infusion through catheters implanted rostrally. The i.t. post-treatment trials were performed in rats given chronic i.t. infusion through caudal catheters. In i.t.-ACSF-infused rats, the chronic s.c. morphine treatment normally produces tolerance to the analgesic effect of acute s.c. or i.t. morphine. A dose response curve for i.t. morphine-induced antinociception was obtained. However, rats injected with s.c. morphine were tested only with the highest dose (10 mg kg^{-1}) of morphine. A dose response curve was not completed after it was determined that the i.t. treatments did not influence morphine tolerance observed with this high dose of s.c. morphine.

Western blot analysis

After assessing the behavioural data, we performed a Western blot analysis to determine the $mGluR_1$ protein level in the lumbar spinal cord of i.t. infused animals. The groups compared included ACSF, $mGluR_1$ MIS, $mGluR_1$ AS or naïve rats. Lumbar spinal cords were taken from naïve rats and rats in each treatment group 5 days after the beginning of the morphine treatment. Rats were decapitated, and spinal cords quickly removed (pressure ejection) and frozen at $-70^{\circ}C$. Samples were prepared for Western blot analysis by homogenizing lumbar spinal cords in buffer containing protease inhibitors (leupeptin, aprotinin, pepstatin, 4-amidinophenylmethanesulphonyl fluoride hydrochloride). The spinal cords from three rats in each group were collected and a triplicate analysis was performed. Concentration of protein in each sample was determined using the method of Bradford (1976). For separation, 20 μg of total protein was loaded onto the gel for electrophoresis. The concentration of protein in each sample fell on the linear portion of the curve. Proteins were separated by gel electrophoresis (SDS–PAGE) on a 5% polyacrylamide gel, and electrotransferred to PVDF membrane. The membrane was probed with a primary antibody, anti-rat $mGluR_1$ IgG (raised in rabbits, Upstate Biotechnology, NY, USA), and later tagged with a peroxidase-conjugated donkey anti-rabbit antibody (secondary antibody, Jackson Immunoresearch). The primary antibody is raised against the C termini of the receptors, a region that is *unique* to this receptor, and specificity has been verified with immunoblotting (Abe *et al*., 1992; Martin *et al*., 1992; Upstate Biotechnology). After incubation with secondary antibody, the membrane was treated with chemiluminescent substrate (Boehringer Mannheim, Germany), and apposed to Kodak Biomax MR film. Density of binding was measured using Alpha Imager Software and Scion Imaging Software (NIH). The $mGluR_1$ is a protein of approximately 133–142 kD (Houamed *et al*., 1991; Martin *et al*., 1992; Masu *et al*., 1991).

Data analysis

Tail-flick latencies were converted to per cent maximum possible effect (%MPE) scores (%MPE = (test latency – baseline)/(cut-off – baseline)) * 100. From the %MPE scores we calculated an area under the curve (AUC) for 15 to 60 min after i.t. morphine injection to indicate degree of analgesia. The AUC scores for each treatment group were compared to each other and to scores in naïve rats using two-way ANOVA with treatment group and dose as independent factors. The analgesic effects of the highest doses of i.t. and s.c. morphine were also plotted as a time-effect curve for each dose of morphine tested, and the %MPE scores were subjected to two-way repeated measures ANOVA with a treatment (independent group) factor and time (repeated) factor. Significant main effects from the ANOVA analysis were further assessed using Fisher's LSD *post-hoc* test.

Differences between treatment groups in density of binding obtained in the Western blot analysis were compared by one-way ANOVA followed by Fisher LSD *post-hoc* test.

Results

Dose-response curve for the effect of acute i.t. morphine injection on the tail-flick latencies

Figure 1 illustrates the dose-dependent antinociceptive effects produced by i.t. administration of morphine in naïve rats and chronic s.c. morphine-treated rats that had i.t. infusions. The

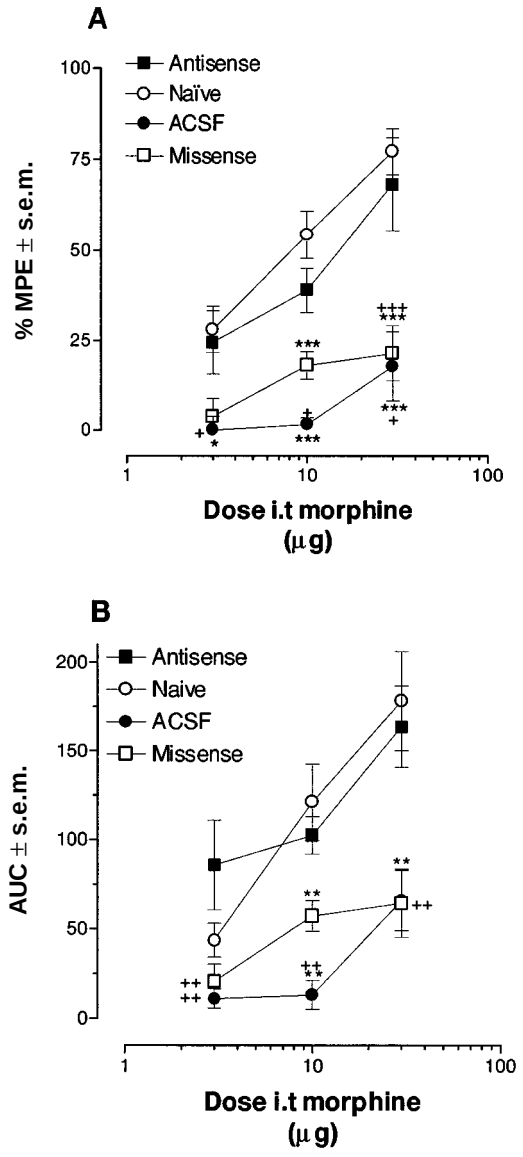

Figure 1 Antinociceptive effects of acute intrathecal (i.t.) injections of morphine on tail-flick latencies in naïve rats and chronic morphine-treated rats given i.t. infusions. Shown are (A) the per cent maximum possible effect (%MPE) obtained for the period of peak morphine effect, and (B) the area under the curves (AUCs) for over the entire 60 min testing session, obtained after acute i.t. injection of 3, 10 or 30 μg morphine in various groups of rats. Groups include naïve rats, or in rats that received chronic s.c. morphine treatment concomitantly with chronic i.t. $mGluR_1$ AS, mGluR MIS or ACSF. $^{**}P<0.01$, $^{***}P<0.001$ represent values that are significantly different from the pre-treatment naïve group, and $\dagger P<0.05$; $\dagger\dagger P<0.01$; $\dagger\dagger\dagger P<0.001$ represent values that are significantly different from the $mGluR_1$ AS group.

figure shows that rats treated with i.t. ACSF or $mGluR_1$ MIS developed tolerance to the analgesic effects of morphine after the 5-day period of chronic s.c. morphine treatment, since the peak analgesic effect (Figure 1A) and the areas under the curve (AUC; Figure 1B) for doses over 3 μg of i.t. morphine were significantly decreased compared to those in naïve rats. This was confirmed by a significant main effect of treatment group in the ANOVA ($F(3,60)=34.0$, $P<0.001$). *Post-hoc* analysis revealed that for the 10 and 30 μg doses of morphine, the ACSF- and $mGluR_1$ MIS-treated rats were significantly different from the naïve group. Figure 1 also shows that rats treated with the $mGluR_1$ AS exhibit a dose-dependent antinociception that is not significantly different from the effects obtained naïve rats (as confirmed by *post-hoc* analysis, Fisher's LSD). Furthermore, this opioid-induced antinociception is significantly higher than the effects induced in rats treated with ACSF or $mGluR_1$ MIS. *Post-hoc* analysis (Fisher's LSD) revealed a significant decrease in the morphine-induced antinociception in ACSF-treated and $mGluR_1$ MIS-treated animals when compared to naïve or $mGluR_1$ AS-treated animals.

Time-course for the effect of acute s.c. or i.t. morphine injection on the tail-flick test

Figure 2A shows the time course for antinociception induced by an acute s.c. injection of morphine (10 mg kg^{-1}). After the 5 days of chronic s.c. morphine treatment, all rats developed tolerance to morphine's antinociceptive effects, as shown in Figure 2A. The opioid-induced antinociception is, in all groups, significantly lower than that obtained in naïve rats not exposed to chronic morphine treatment; (i.e., there was a significant main effect of group in the ANOVA: $F(3,80)=27.7$, $P<0.001$).

Figure 2B illustrates the time course of acute i.t. morphine-induced antinociception (30 μg) in all treatment groups following chronic s.c. morphine treatment. Statistical analysis demonstrated a significant difference between groups (ANOVA: $F(3,80)=68.0$, $P<0.001$). *Post-hoc* analysis revealed that i.t. morphine-induced antinociception is significantly attenuated in ACSF and $mGluR_1$ MIS-treated rats compared to naïve and $mGluR_1$ AS-treated rats. Furthermore, there was no significant difference between i.t. morphine-induced antinociception in $mGluR_1$ AS-treated compared to naïve rats at all time points with the exception of the first time interval (15 min).

Quantification of $mGluR_1$ protein on day 8 of the treatment

Figure 3A shows a decrease in the density of binding of the $mGluR_1$ protein from $mGluR_1$ AS-treated rats that is significantly different when compared to naïve and ACSF-treated rats. ANOVA revealed a significant effect of treatment group ($F(3,8)=4.1$, $P<0.05$). *Post-hoc* analysis (Dunnett's *t*-test) revealed a significant decrease ($42\pm8\%$) in the protein level of $mGluR_1$ in the $mGluR_1$ AS group, but not the $mGluR_1$ MIS ($18\pm17\%$) or ACSF ($3\pm3\%$) group, when compared to the naïve group. Figure 3B shows a representative immunoblot of the $mGluR_1$ protein levels from the four different treatment groups.

Discussion

This study demonstrates that the knockdown of spinal $mGluR_1$ receptors is effective in attenuating the development of morphine tolerance when the test dose of morphine was given as a spinal injection, but not as a systemic injection. We speculate that the lack of effect of the AS in preventing morphine tolerance when the test morphine was given s.c. is

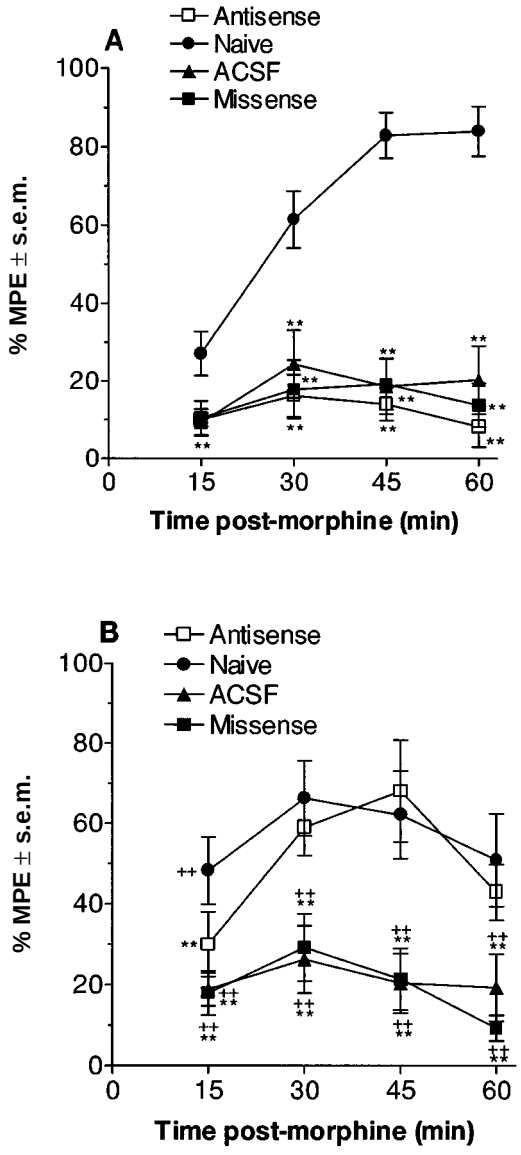

Figure 2 Time course of the antinociceptive effects of (A) acute s.c. (10 mg kg^{-1}) and (B) acute i.t. (30 μg) injection of morphine on tail-flick latencies in rats. Shown are the % maximum possible effect (%MPE) scores every 15 min over the 60 min testing session obtained after acute s.c. or i.t. morphine injection in naïve rats, or in rats that received chronic s.c. morphine treatment concomitantly with chronic i.t. infusion of $mGluR_1$ AS, $mGluR_1$ or ACSF. $^{**}P<0.01$ represent values that are significantly different from the pre-treatment group. $\dagger\dagger P<0.01$ represents values significantly different from that of the $mGluR_1$ AS-treated group.

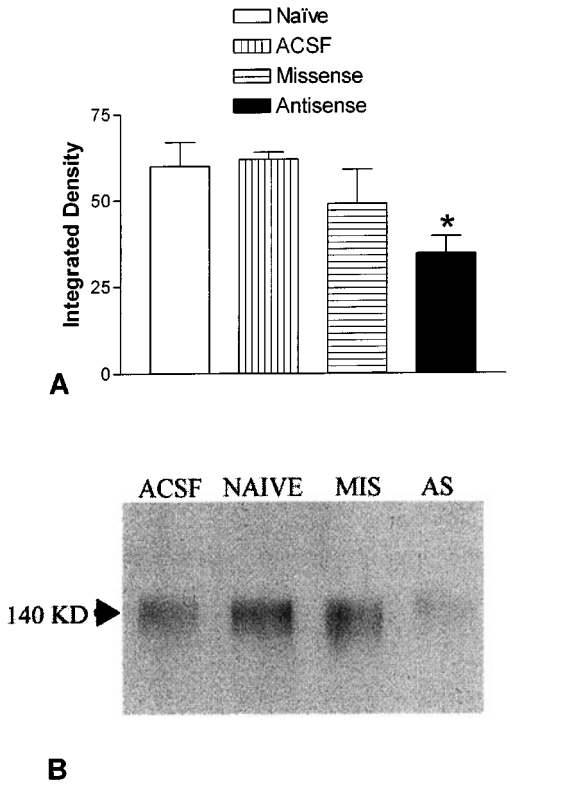

Figure 3 (A) The effects of $mGluR_1$ AS, $mGluR_1$ MIS, and ACSF infusion at the lumbar level of the spinal cord on the quantification of binding density of the $mGluR_1$ receptor proteins in spinal cord tissues. The quantification of binding density of $mGluR_1$ receptor proteins is obtained after incubating the spinal cord tissue with an $mGluR_1$ antibody. The decrease in protein binding intensity as compared to naïve animals is $18\pm17\%$ for the MIS-treated group, $3\pm3\%$ for the ACSF-treated group, and 42 ± 8 for the AS-treated group ($^{*}P<0.05$ represents values that are significantly different from ACSF treated group). (B) Representative immunoblots of the $mGluR_1$ protein levels from the four different treatment groups. Shown are immunoblots using $mGluR_1$ antibodies after gel electrophoresis of lysates from spinal cord segments L3-L6 of naïve, ACSF-, AS- or MIS-treated animals. Bands at (140 kD) represent $mGluR_1$ protein.

due to the fact that the AS treatment was spinal, and has limited effects in the brain. Thus, chronic systemic morphine treatment will produce tolerance at supraspinal sites that are not as greatly affected by the i.t. AS. The tolerance in supraspinal, and potentially peripheral, sites would then affect the analgesic effects of s.c., but not i.t. morphine. Indeed, our previous study (Fundytus *et al*., 2001) indicated that while i.t. $mGluR_1$ AS treatment produces a 57% decrease in mGluR protein in spinal cord, it produces considerably lower effects in tissue taken from the thalamus and periaqueductal grey (18–25%). It is possible, however, that the differential effects of the spinal AS treatment for the s.c. and i.t. morphine post-test conditions may depend on the different catheter placement methods used (i.e. rostral vs caudal, respectively). Minor damage or irritation associated with the rostral catheter placement method may have influenced the effectiveness of the AS treatment, since the osmotic pump was implanted immediately after the catheter; unlike following caudal catheter placement, where the pump implantation was delayed 24 h.

This is the first study demonstrating a role for spinal $mGluR_1$ in the development of tolerance to the antinociceptive effect of i.t. morphine; although we have previously demonstrated that $mGluR_1$ AS reversed the decreased opioid sensitivity observed in neuropathic rats (Fundytus *et al*., 2001). It is possible that in the present study the chronic AS treatment may have enhanced the analgesic effect of morphine (rather than reducing tolerance); however, we have also previously shown that the efficacy of morphine in naïve rats is not altered by $mGluR_1$ AS (Fundytus *et al*., 2001). Other mGluRs were not examined in this study of morphine tolerance; however, we have previously examined the

involvement of other mGluRs in morphine dependence (Fundytus & Coderre, 1994; 1997; Fundytus *et al*., 1997). The involvement of group I mGluRs, and particularly $mGluR_1$, in nociception (Fisher & Coderre, 1996; Fundytus, 2001; Neugebauer *et al*., 1999; Young *et al*., 1998) suggested that this receptor subtype would be a good starting point.

A role of mGluRs in opioid tolerance is not unexpected, since previous studies have demonstrated that opioids influence both glutamate transmission and glutamate-linked second messengers, and vice-versa (Fundytus & Coderre, 1999a,b). Thus, opioids have been found to activate PLC (Okajima *et al*., 1993; Smart *et al*., 1995; Tsu *et al*., 1995), stimulate PI hydrolysis (Leach *et al*., 1986; Periyasamy & Hoss, 1990; Smart *et al*., 1994, and increase intracellular Ca^{2+} release (Jin *et al*., 1992), as well as increasing PKC (Kramer & Simon, 1999; Narita *et al*., 1994b). Furthermore, PKC stimulates the secretion of β-endorphin in pituitary and hypothelamic neurons (Abou-Samira *et al*., 1987; Kapcala *et al*., 1992). Evidence also indicates that inhibitors of intracellular Ca^{2+} release and PKC reduce either opioid tolerance or dependence (Fundytus & Coderre, 1996; Mao *et al*., 1995; Mayer *et al*., 1995; Narita *et al*., 1994a). Importantly, PKC has been found to produce both a desensitization of μ-opioid receptors (Fan *et al*., 1998; Kramer & Simon, 1999; Mestek *et al*., 1995; Ueda *et al*., 1995), and a sensitization of NMDA receptors (Chen & Huang, 1992; Gerber *et al*., 1989), both effects that could contribute to the development of opioid tolerance (see Fundytus & Coderre, 1999a, b; Mao *et al*., 1995; Mayer *et al*., 1995). A recent study by Xie *et al*. (1999) showed that PLCβ deficient mice are more sensitive to the analgesic effects of morphine. By reducing the potential for $mGluR_1$-stimulated increases in PLC, mGluR knockdown should produce a reduction in each of these intracellular messengers (Ca^{2+}, IP_3, PKC), and may subsequently reduce morphine tolerance.

In conclusion, we showed that the knockdown of spinal $mGluR_1$ receptors prevents the development of tolerance to the antinociceptive effects of i.t. morphine. We speculate that this inhibition of morphine tolerance is due to pre-empting an increase in PI hydrolysis, thus preventing an increase in PKC activation that causes desensitization of the μ-opioid receptors, as previously hypothesized by Fundytus & Coderre (1999a, b) for opioid dependence. Additional effects could depend on reductions in nitric oxide, intracellular Ca^{2+}, phospholipases, and other messengers stimulated by group I mGluRs. Clearly, the results suggest a role for spinal $mGluR_1$ in the development of morphine tolerance.

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