

Intrathecal Delivery of a Mutant μ -Opioid Receptor Activated by Naloxone as a Possible Antinociceptive Paradigm

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ABSTRACT

Direct injection of double-stranded adeno-associated virus type 2 (dsAAV2) with a μ -opioid receptor (MOR) mutant [S4.45(196)A], and a reporter protein (enhanced green fluorescent protein) into the spinal cord (S2/S3) dorsal horn region of ICR mice resulted in antinociceptive responses to systemic injection of opioid antagonist naloxone without altering the acute agonist morphine responses and no measurable tolerance or dependence development during subchronic naloxone treatment. To develop further such mutant MORs into a therapeutic agent in pain management, a less invasive method for virus delivery is needed. Thus, in current studies, the dsAAV2 was locally injected into the subarachnoid space of the spinal cord by intrathecal administration. Instead of using the MORS196A mutant, we constructed the dsAAV2 vector with

the MORS196ACSTA mutant, a receptor mutant in which naloxone has been shown to exhibit full agonistic properties *in vitro*. After 2 weeks of virus injection, naloxone (10 mg/kg s.c.) elicited antinociceptive effect (determined by tail-flick test) without tolerance (10 mg/kg s.c., b.i.d. for 6 days) and significant withdrawal symptoms. On the other hand, subchronic treatment with morphine (10 mg/kg s.c., b.i.d.) for 6 days induced significant tolerance (4.8-fold) and withdrawal symptoms. Furthermore, we found that morphine, but not naloxone, induced the rewarding effects (determined by conditioned place preference test). These data suggest that local expression of MORS196ACSTA in spinal cord and systemic administration of naloxone has the potential to be developed into a new strategy in the management of pain without addiction liability.

Introduction

Although morphine is efficacious in the control of moderate and severe postoperative pain, it also induces adverse effects, such as respiratory depression, nausea, constipation, tolerance, physical dependence, and addiction. Frequently, non-opioid pharmacological strategies are used with opioid administration to alleviate the side effects while maintaining the analgesic potency of the opioids. For example, laxative or bulking agents are used to counteract opioid constipative effects. Dopaminergic or serotonergic (5-HT₃) antagonists are used for postoperative nausea and vomiting caused by opioid analgesics. In addition, peripheral opioid antagonists are used in the elimination of opioid side effects. Unlike centrally

acting opioid antagonists, such as naloxone or naltrexone, that could reverse the analgesic effects in postoperative patients (Abou-madi et al., 1976; Gairola et al., 1980; Lehmann et al., 1988), peripheral opioid antagonists such as methyl-naltrexone (the quaternary derivative of naltrexone) or alvimopan [a zwitterionic form of *trans*-3,4-dimethyl-4-(3-hydroxyphenyl) piperidine], because of their inability to penetrate the blood-brain barrier, have been used successfully to prevent the constipation, nausea, or vomiting effects in postoperative pain treatment (Taguchi et al., 2001; Bates et al., 2004). Unfortunately, tolerance and addiction to morphine remains with peripheral antagonists.

Other approaches were used to address the problem of opioid tolerance and addiction. With the cloning of the three opioid receptor types, MOR, δ -opioid receptor, and κ -opioid receptor (KOR) (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993, 1994; Meng et al., 1993), and the subsequent generation of respective receptor null mouse lines (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998; Zhu et al., 1999), MOR is shown to mediate the *in vivo* morphine effects.

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ABBREVIATIONS: dsAAV2, double-stranded adeno-associated virus type 2; MOR, μ -opioid receptor; KOR, κ -opioid receptor; CPP, conditioned place preference; EGFP, enhanced green fluorescent protein; ANOVA, analysis of variance; %MPE, percentage of maximum possible effect; AUC, area under the curve; DRG, dorsal root ganglion; CGRP, calcitonin gene-related peptide.

In the MOR(-/-) mice, the rewarding responses to morphine were not observed (Matthes et al., 1996). Clinical studies indicated that the mixed agonist-antagonists (agonist on KOR and antagonist on MOR), although having lower patient compliance because of their dysphoric properties from KOR, exhibit lower addictive liabilities (Rosow, 1987). Thus, the development of KOR agonists as pharmaceutical agents for pain control with low addiction liability has been the focus of many laboratories (Hasebe et al., 2004). This requires the development of ligands with specific binding to one opioid receptor type, which has been difficult even with the identification of putative receptor domains involved in MOR, δ -opioid receptor, and KOR ligand binding (Paterlini, 2005; Surratt and Adams, 2005).

Instead of developing more specific ligands for the opioid receptors, or opioid ligand "cocktails" to enhance the recycling and resensitization of MOR, leading to lowering of the tolerance and dependence responses (He and Whistler, 2005), we decided to focus on developing a therapeutic agent using a mutant MOR that could be activated by the opioid alkaloid antagonists naloxone and naltrexone. The initial identification of this mutant in which the conserved Ser in the fourth transmembrane domain (TM4) was mutated to Leu [S4.45(196)L] suggested that the mutation did not affect agonist receptor affinity or potency (Claude-Geppert et al., 2005). Rather, the S4.45(196)L mutation resulted in opioid antagonists activating Kir3 or inhibiting adenylyl cyclase activity (Claude-Geppert et al., 2005). The ability of antagonists to activate the mutant receptor in vivo was demonstrated by generating a S4.45(196)A knock-in mouse line (Yang et al., 2003). In addition to morphine, naloxone and naltrexone could elicit antinociceptive responses in the MORS196A mice. Chronic morphine treatment resulted in tolerance and dependence development in these mice. However, long-term treatment of these mice with naloxone or naltrexone did not produce tolerance, and the withdrawal signs were less severe (Yang et al., 2003). Thus antagonist-mediated activation of MORS196A in vivo could be a probable pain management paradigm.

The utility of this approach was demonstrated by delivering the MORS196A mutant to the dorsal horn area of the spinal cord (S2/S3) region by using double-stranded adeno-associated virus type 2 (dsAAV2). Mice injected with dsAAV2 exhibited a naloxone-mediated antinociceptive response that was not accompanied by tolerance or dependence development during subchronic antagonist treatment (Chen et al., 2007). These results suggest that opioid antagonist is activating the mutant but not the endogenous MOR. However, the exposure of spinal cord segment after partial dorsal laminectomy during dsAAV2 injection greatly reduces the utility of this approach.

To develop the mutant MOR as a therapeutic agent further, we decided to explore the possibility of introducing the virus at the subarachnoid space of the spinal cord by intrathecal injection. Instead of delivering MORS196A, a MOR with additional mutations at transmembrane 7 [T7.44(327)A] and C7.47(330)S in which opioid antagonists exhibit full agonistic properties (Claude-Geppert et al., 2005), was delivered by dsAAV2. We found that naloxone showed antinociceptive effects without the development of tolerance, physical dependence, and rewarding effect [de-

termined by conditioned place preference (CPP)] in these mice after the expression of MORS196ACSTA by intrathecal injection of dsAAV2-MORS196ACSTA-EGFP. Therefore, naloxone has great potential to be developed into a new strategy in the management of pain by delivering the mutated MOR gene intrathecally.

Materials and Methods

Construction of the dsAAV2 Virus

The original dsAAV-cytomegalovirus-EGFP shuttle vector was obtained from Xiao Xiao (University of North Carolina, Chapel Hill, NC). The cloned MOR or the mutant MORS4.45AT7.44AC7.47A (MORS196ACSTA) with EGFP as reporter was cloned into the dsAAV2 expression cassette pV4.1c containing the cytomegalovirus promoter as described previously (Chen et al., 2007). The dsAAV2 was produced in human embryonic kidney 293 cells by using the adenovirus-free, triple-plasmid cotransfection method (Wang et al., 2003) and purified by heparin affinity column chromatography following previously published methods (Clark et al., 1999; Wang et al., 2003). The virus titer of the dsAAV2 was determined to be 10^{13} viral particles/ml.

Animals

Male ICR mice (30–35 g) were used in this study. All mice were kept in an animal room with a 12-h light/dark cycle at a temperature of $25 \pm 2^\circ\text{C}$ and humidity of 55%. A standard diet and water were provided ad libitum. The care of animals was carried out in accordance with institutional and international standards (*Principles of Laboratory Animal Care*, National Institutes of Health), and the protocol had the approval of the Institutional Animal Care and Use Committee of the National Defense Medical Center (Taiwan, Republic of China). There were at least eight mice in each group in the beginning of the experiments.

Injection of the dsAAV2 into the Subarachnoid Space of the Spinal Cord

The direct lumbar puncture method (Wigdor and Wilcox, 1987; Fairbanks, 2003) was applied in awake, conscious mice. Mice were covered with a soft cloth over the head and upper body and gripped firmly by the pelvic girdle (iliac crest). Five microliters of the virus was injected with a 50- μl Hamilton syringe (Hamilton Co., Reno, NV) attached to a 30-gauge, 0.5-inch sterile disposable needle, which was inserted into the intrathecal space at the cauda equine region according to the method described by Wigdor and Wilcox (1987). Puncture of the dura was indicated by a flick of the tail.

Determination of the Antinociceptive Effect of Drugs

Drug-induced antinociception was evaluated by using the tail-flick test (D'Amour and Smith, 1941). Using a tail-flick apparatus (Ugo Basile, Comerio, Italy), the intensity of the heat source was set at 40, which resulted in the basal tail-flick latency to occur between 2.5 and 3.5 s for most of the animals. The tail-flick latency was recorded at 30, 60, 90, 120, and 180 min after drug administration. The percentage of maximum possible effect (%MPE) was calculated for each mouse at each time point according to the following formula: %MPE = [(postdrug latency - predrug latency)/(cutoff latency - predrug latency)] \times 100. The predrug latency was determined from an average of three predrug determinations, and cutoff latency was selected at 10 s. The trapezoidal rule, without extrapolation to infinite time, was used to calculate area under the %MPE versus time curves (AUCs) for each individual animal as shown in Fig. 2. The ED₅₀ value was measured by an up and down method (Dixon, 1965). In brief, the tail-flick response was tested at the peak effect of morphine or naloxone, which occurs approximately 20 to 30 min after drug administration. A series of trials ($n = 6$) was carried out

following the rule of a decrease in drug dose after inhibition of the tail-flick response (%MPE \geq 50%) and an increase in drug dose after no inhibition of the tail-flick response (%MPE <50%). Each rat was tested in only one trial. The ED₅₀ value was determined from the relationship $ED_{50} = X_r + k \times d$ (Dixon, 1965), where X_r was the last dose administered, k was the tabular value outlined by Dixon, and d was the interval between doses.

Detection of the Expression of Mutated μ -Opioid Receptors

Mice were anesthetized with chlorohydrate (350 mg/kg i.p.) and perfused transcardially with Tyrode calcium-free buffer (116 mM NaCl, 5.36 mM KCl, 1.57 mM MgCl₂ · 6H₂O, 0.405 mM MgSO₄, 1.23 mM NaH₂PO₄, 5.55 mM glucose, 26.2 mM NaHCO₃, pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (187.5 mM KH₂PO₄, 212.5 mM Na₂HPO₄ · 12H₂O). The lumbar spinal cord and L1–L4 dorsal root ganglion (DRG) were dissected and then put in 20% sucrose solution (in 0.1 M phosphate buffer) overnight at 4°C. The samples were then embedded in OCT compound and frozen immediately in a –80°C freezer. Serial transverse spinal cord slices and DRG (10 μ m) were sectioned with a cryostat at –20°C. The slices were mounted on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany), and the EGFP expression of the mutated μ -opioid receptors was visualized with a fluorescence microscope. To investigate whether these mutated μ -opioid receptors were colocalized with calcitonin gene-related peptide (CGRP), a well characterized marker of the DRG sensory neurons and related afferent fiber terminals, immunohistochemistry was done in DRG at the L1–L4 level. Antibodies were diluted in blocking buffer (0.01 M phosphate-buffered saline containing 1% goat serum and 0.1% Triton X-100). Anti-CGRP antibody (AB5920; Millipore Bioscience Research Reagents, Temecula, CA) was used at a dilution of 1:2000. Secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was diluted 1:200 to against rabbit or mouse IgG that was coupled with rhodamine for immunofluorescence detection. Sections were then washed with phosphate-buffered saline, cleared, and mounted onto coverslips using mounting medium (HIS002B; Serotec, Oxford, UK). The processed sections were examined with an Olympus (Tokyo, Japan) fluorescence instrument (model BH2-RFL-T3) on an upright microscope and an Olympus BX50 camera with SPOT software (version 4.6; Diagnostic Instruments, Inc., Sterling Heights, MI).

Experiment 1. To determine whether naloxone would induce antinociceptive effect in mice intrathecally injected with dsAAV2-MORS196ACSTA and whether this effect would last for the period (8 weeks) when we would carry out the acute and subchronic drug treatments, the tail-flick tests of naloxone (10 mg/kg s.c.) were done before gene transfer and at 1, 2, 3, and 8 weeks after gene transfer.

Experiment 2. To determine subchronic drug treatment-induced tolerance and physical dependence, mice were separated into three groups. Mice in the control group were injected with saline intrathecally and tested with saline (intrathecal saline–saline); mice in the morphine or naloxone group were injected with dsAAV-MORS196ACSTA intrathecally and tested with morphine or naloxone. Before intrathecal injection of saline or virus, the antinociceptive effects of saline (10 ml/kg s.c.), naloxone (10 mg/kg s.c.), or morphine (10 mg/kg s.c.) were tested as pretest data (before intrathecal acute). The acute antinociceptive effects (including ED₅₀ and AUC) of each drug were determined 2 weeks after intrathecal injection of saline or virus (after intrathecal acute). And then the mice were subchronically treated with saline (10 ml/kg s.c.), naloxone (10 mg/kg s.c.), or morphine (10 mg/kg s.c.) twice a day for 6 days. The AUC values (after intrathecal chronic) of saline or drug in each group were evaluated again in the morning of the sixth day. On the seventh day, the ED₅₀ values were measured by the up and down method (Dixon, 1965) in the morning, and the required dose to make up to 10 mg/kg for each animal was injected right after the test. On the

eighth day, mice were first injected with morphine or naloxone for 30 min and then injected with naltrexone (10 mg/kg i.p.) and placed into test chambers consisting of transparent round plastic boxes (20 cm diameter, 33 cm height). Naltrexone-induced precipitated withdrawal symptoms, such as the number of jumping and rearing moves, were counted for 15 min. The amount of feces and urine were weighed after the determination of withdrawal signs. The body weight losses were determined after 4 h of naltrexone injection. The natural withdrawal symptoms (jump, paw tremor, wet dog shaking, teeth chattering, diarrhea, ptosis, piloerection) were also counted at 14 and 38 h after the last drug treatment. Jump and paw tremor frequencies were recorded during the test time, and a score of 1 was assigned to every three jumps or five paw tremors. Diarrhea and ptosis events were recorded for every 5-min interval in which they occurred (maximal score = 6). The presence or absence of piloerection was noted. A global opiate withdrawal score was calculated by summing the values for each sign (Papaleo and Contarino, 2006).

Experiment 3. To determine the rewarding effects induced by morphine or naloxone, the CPP test was carried out as reported previously (Chen et al., 2007).

In this study, a distinctive environment was paired repeatedly with administration of a drug, and a different environment was associated with the nondrugged state. The CPP test apparatus, made from an acrylic plastic box (33 × 15 × 15 cm), was divided into three compartments. Two identically sized compartments (15 × 15 × 15 cm) were constructed at both sides, separated by a narrower compartment (3 × 15 × 15 cm). The compartments were connected by guillotine doors (7 × 3.5 cm) in the central unit. One of the large compartments was covered by mosaic-type paper (3 × 3 cm black and white squares) on the three walls and as a visual cue; the other large compartment was covered by purely white paper. To give more visual cues, blue and red light bulbs were hung separately above the two large compartments. During the experiments, the CPP apparatus was kept in an isolated dark room, which was free from noise. After each behavioral test or place conditioning, the whole box was cleaned thoroughly to prevent the interference from the smell of feces and urine. For CPP conditioning, the mice were given saline in the morning and morphine (5 mg/kg i.p.) or naloxone (10 mg/kg i.p.) in the afternoon for 6 days. A distinctive environment was paired repeatedly with administration of saline, and a different environment was associated with drug injection. The animals were kept for 40 min in the corresponding compartment with the guillotine doors closed. CPP tests were performed on the day before conditioning and the day after conditioning (day 7). Place preference was determined by placing the mice into the central compartment of the apparatus with the guillotine doors opened for 15 min. The time that the mice stayed in each compartment was recorded to determine the place preference. The measurement of drug rewarding effect was determined by an increase in the time spent in the compartment previously paired with drug injection than the time spent in the saline-paired compartment.

Statistical Analysis

The data were expressed as means \pm S.E.M. Student's *t* test, one-way ANOVA, repeated-measures ANOVA, and Newman-Keuls test were used to analyze the data. A difference was considered to be significant at $P < 0.05$.

Chemicals

Morphine hydrochloride was purchased from the National Bureau of Controlled Drugs, National Health Administration (Taipei, Taiwan, Republic of China). Naloxone, naltrexone, paraformaldehyde, and glucose were purchased from Sigma-Aldrich (St. Louis, MO). Chlorohydrate, MgSO₄, H₂PO₄, and Na₂HPO₄ · 12H₂O were purchased from Nacalai Tesque (Kyoto, Japan). KCl, KH₂PO₄, MgCl₂ · 6H₂O, NaCl, and NaHCO₃ were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

Results

Expression of the Mutated MOR in Mice after Intrathecal Injection of dsAAV2-MORS196ACSTA-EGFP. Our previous studies with the spinal cord dorsal horn injection of virus has validated the feasibility of using dsAAV2 as the delivery vehicle for the mutant opioid receptor. To develop a less invasive method for the transgene delivery, intrathecal injections of the dsAAV2 were attempted. Expression of the transgene was monitored by the EGFP fluorescence. Similar to previous studies (Chen et al., 2007), the intrathecal injection of the dsAAV2 resulted in the expression of the MORS196ACSTA at the L3–L5 spinal cord starting from 2 weeks after the virus injection. A representative picture of EGFP fluorescence in mice 8 weeks after the intrathecal injection is shown in Fig. 1B. Furthermore, the DRG located at the L3–L4 also exhibited EGFP fluorescence in all sizes of cells 8 weeks after the intrathecal injections (Fig. 1, C–F). When the DRGs were stained for CGRP (red; Fig. 1D), a marker for sensory neurons, colocalization of CGRP with the EGFP was observed (Fig. 1, E and F). The colocalization of the mutant MOR with CGRP suggests probable expression of the mutant opioid receptor within the sensory neurons associated with nociception.

The antinociceptive effects of saline, morphine, or naloxone 2 weeks after intrathecal injection of dsAAV2-MORS196ACSTA-EGFP are shown in Fig. 2A. The AUC values were calculated from the area under the time-response curve of Fig. 2A and are shown in Fig. 2B.

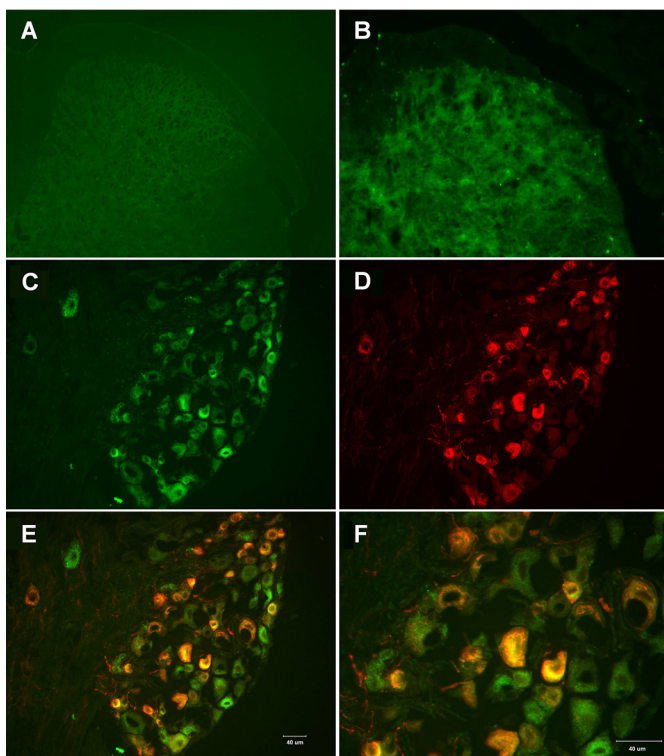


Fig. 1. A and B, fluorescence micrographs of the lumbar spinal cord 8 weeks after intrathecal administration of saline (A) or dsAAV2-MORS196ACSTA-EGFP (B) are shown with the 20 \times objective. C to F, fluorescence micrographs of the L3 dorsal root ganglion 8 weeks after intrathecal administration of dsAAV2-MORS196ACSTA-EGFP are shown with the 20 \times objective (C–E) or the 40 \times objective (F). EGFP fluorescence (C), CGRP immunofluorescence (D), and the merging of EGFP (green) with CGRP immunofluorescence (red) (E and F) revealed colocalization (yellow) of MORS196ACSTA and CGRP.

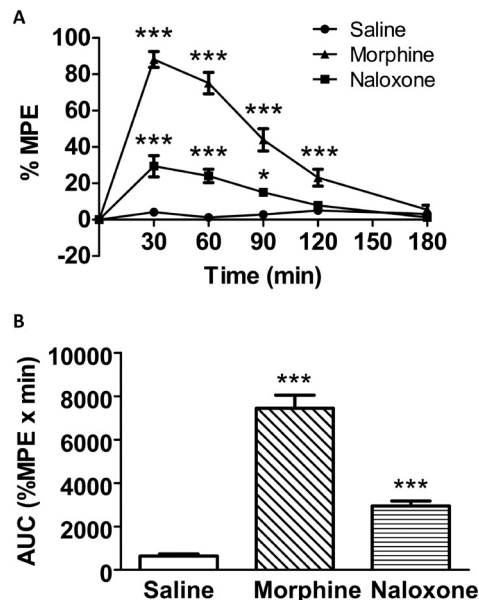


Fig. 2. A, time-response curves of saline, naloxone (10 mg/kg, s.c.), and morphine (10 mg/kg s.c.) 2 weeks after intrathecal injection of dsAAV2-MORS196ACSTA. B, the area under curve values derived from A. Data are presented as mean %MPE \pm S.E.M. ($n = \sim 7$ –10). One-way ANOVA and Newman-Keuls test were used to analyze the data. *, $P < 0.05$; ***, $P < 0.001$ compared with saline group at each time point.

Naloxone Elicited Antinociceptive Responses in Mice Injected with dsAAV-MORS196ACSTA Intrathecally. As shown in Fig. 3, naloxone (10 mg/kg s.c.) did not elicit any antinociceptive effect before gene transfer. The

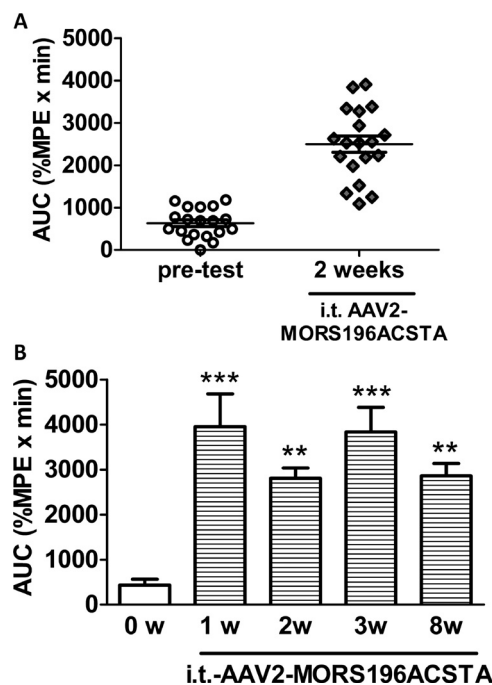


Fig. 3. The antinociceptive effects of naloxone in ICR mice after intrathecal administration of dsAAV2-MORS196ACSTA. A, the AUC values for naloxone (10 mg/kg s.c.) before and 2 weeks after MORS196ACSTA gene transfer ($n = 19$). B, the antinociceptive effects of naloxone (10 mg/kg s.c.) before (0w) and 1 week (1w), 2 weeks (2w), 3 weeks (3w), and 8 weeks (8w) after MORS196ACSTA gene transfer. Data are presented as mean \pm S.E.M. ($n = 6$). Repeated-measures ANOVA and Newman-Keuls test were used to analyze the data. **, $P < 0.01$; ***, $P < 0.001$ compared with week 0.

AUC value of naloxone was 434.6 ± 128.6 (%MPE \times min), which was similar to that of saline [472.2 ± 81.3 (%MPE \times min)]. In contrast, starting from 1 week after intrathecal administration of dsAAV2-MORS196ACSTA, 79% (15/19) of the mice responded to naloxone (10 mg/kg s.c.) with an average AUC value equal to 3951.2 ± 731.9 (%MPE \times min) (Fig. 3). The naloxone-induced antinociceptive response remained constant when we measured 2, 3, or 8 weeks after the intrathecal injection of the virus. This effect of naloxone paralleled the observed EGFP immunofluorescence (Fig. 1).

Subchronic Drug Treatment-Induced Tolerance and Physical Dependence. Two weeks after gene transfer, the naloxone-induced antinociceptive effect was determined. Only the mice that had AUC values more than 2000

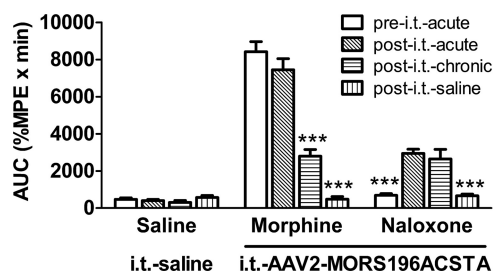


Fig. 4. Tolerance induced by morphine but not naloxone in mutated MOR gene-transferred mice. The acute antinociceptive effects of morphine (10 mg/kg s.c.) or naloxone (10 mg/kg s.c.) were determined before and after gene transfer. The subchronic effects of morphine or naloxone were determined again after 6 days of treatment (10 mg/kg s.c., b.i.d.). After subchronic drug treatment, the effect of saline (10 ml/kg) was tested (after intrathecal saline). The effects of saline injection acutely or subchronically in the intrathecal saline group (no gene transferred) were also determined as the control. Data are presented as mean \pm S.E.M. ($n = \sim 7-10$). Repeated-measures ANOVA and Newman-Keuls test were used to analyze the data. ***, $P < 0.001$ compared with after intrathecal acute effect of each group.

TABLE 1

Tolerance induced by morphine or naloxone in mice injected with dsAAV2-MORS196ACSTA-EGFP intrathecally

The ED₅₀ values of morphine or naloxone to inhibit the tail-flick responses were determined by the up-down method. After 2 weeks of gene transferred, animals were tested for the ED₅₀ values of morphine or naloxone and then chronically treated with morphine (10 mg/kg s.c., b.i.d. for 6 days) or naloxone (10 mg/kg s.c., b.i.d. for 6 days). The ED₅₀ values were then redetermined. The values represent the mean \pm S.E.M. ($n = \sim 6-8$).

Treatment	AAV2-MORS196ACSTA Intrathecally	
	ED ₅₀ (mg/kg) of Morphine	ED ₅₀ (mg/kg) of Naloxone
Before chronic treatment	1.3 ± 0.2	8.4 ± 1.2
After chronic treatment	6.0 ± 0.9 (4.6-fold)	8.4 ± 1.2 (1-fold)

TABLE 2

Physical dependence induced by chronic morphine or naloxone in mutated MOR gene transferred mice by intrathecal injection of dsAAV-MORS196ACSTA

After chronic treatment with saline (10 ml/kg s.c., b.i.d.), morphine (10 mg/kg s.c., b.i.d.), or naloxone (10 mg/kg s.c., b.i.d.) for 7 days, withdrawal symptoms were precipitated by injection of naltrexone (10 mg/kg i.p.) 30 min after morphine (10 mg/kg s.c.) or naloxone (10 mg/kg s.c.) on day 8. Withdrawal signs (jumping, rearing, feces, and urine) were counted for 15 min. Body weight changes were recorded and calculated before and after 1 and 4 h of naltrexone injection. Data are presented as mean \pm S.E.M. ($n = \sim 7-10$). One-way ANOVA and Newman-Keuls test were used to analyze the data.

Precipitated Withdrawal Symptoms	Control (Saline)	Morphine	Naloxone
Weight of feces and urine (g)	0.12 ± 0.05	0.54 ± 0.09 ***	0
Jumping	0	15.3 ± 2.9 **	0.1 ± 0.1
Rearing	64.1 ± 4.7	256.6 ± 13.2 ***	79.1 ± 5.0
Body weight loss (g)			
1 h after naltrexone injection	0.66 ± 0.18	0.62 ± 0.25	0.60 ± 0.12
4 h after naltrexone injection	1.74 ± 0.46	1.42 ± 0.49	2.00 ± 0.23

** $, P < 0.01$; *** $, P < 0.001$ compared with control group.

(%MPE \times min) were considered as responsible mice and were used in further studies. By calculating the subsequent values of AUC (Fig. 4) or ED₅₀ (Table 1), it can be demonstrated that the ability of morphine (10 mg/kg s.c.) to elicit antinociceptive effect before or 2 weeks after intrathecal injection of dsAAV2 was not altered. The AUC values of morphine were 8417.1 ± 546.7 (%MPE \times min) (before) and 7447.9 ± 600.6 (%MPE \times min) (after), respectively. These values indicate that injection of the virus did not alter the endogenous μ -opioid receptor responses. Furthermore, intrathecal injection of dsAAV2 did not alter the subchronic morphine effect either. After the mice were subchronically treated with morphine (10 mg/kg s.c., b.i.d.) for 6 days, the AUC value was reduced to 2791.9 ± 364.3 (%MPE \times min) ($P < 0.001$) and the ED₅₀ value of morphine was increased from 1.3 ± 0.2 to 6.0 ± 0.9 mg/kg after 6 days of morphine treatment, reflecting a 5-fold tolerance development. In contrast to morphine-induced antinociceptive effect, subchronic naloxone activation of the mutated MOR (MORS196ACSTA) in the spinal cord did not result in tolerance. The ED₅₀ of naloxone was 8.4 ± 1.2 mg/kg (Table 1) and the AUC was 2318.8 ± 436.7 (%MPE \times min) (Fig. 4), which was not significantly different from the ED₅₀ (8.4 ± 1.2 mg/kg) and AUC [2947.0 ± 225.1 (%MPE \times min)] measured before subchronic drug treatment. To ensure that the antinociception effect was induced by morphine or naloxone but not nonspecific effect, the effect of saline was tested after subchronic drug treatment. As shown in Fig. 4, saline did not elicit any antinociception effect in each group.

Another undesirable side effect of chronic opioid agonist is physical dependence. This is reflected in the appearance of withdrawal symptoms such as rearing, jumping, and weight loss upon the withdrawal of the agonist. In this study, a selective MOR antagonist, naltrexone (10 mg/kg i.p.), was administered to precipitate the withdrawal symptoms. Predictably, obvious rearing and jumping behaviors were seen in mutated MOR gene-transferred mice subchronically treated with morphine (10 mg/kg s.c., b.i.d. for 7 days). However, naloxone (10 mg/kg s.c., b.i.d. for 7 days) did not induce significant withdrawal signs as shown in Table 2. Although measurable loss in body weight was observed, there was no significant difference in these two groups of mice compared with the saline group.

The natural withdrawal symptoms were also assessed in this study (see Fig. 6). At 14 and 38 h after the last morphine treatment, significant withdrawal signs were shown, and the global opiate withdrawal scores were 6.49 ± 1.34 ($P < 0.001$, vs. 1.40 ± 0.41 in control group) and 5.40 ± 1.54 ($P < 0.01$, vs. 0.80 ± 0.32 in control group), respectively. However, the

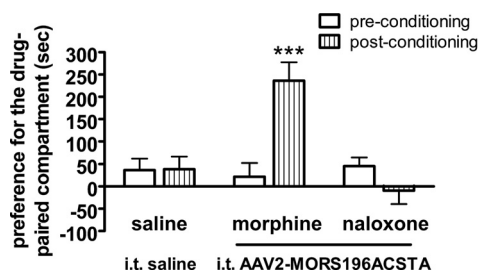


Fig. 5. Morphine, but not naloxone, induced the rewarding effects in mice that had been gene-transferred by intrathecal administration of dsAAV2-MORS196ACSTA. Data are expressed as preference for the drug-paired compartment as determined by time spent in the drug-paired compartment minus time spent in the saline-paired compartment. Data are presented as mean \pm S.E.M. ($n = \sim 7-10$). Student's paired t test was used to analyze the data. ***, $P < 0.001$ compared with preconditioning.

withdrawal of naloxone after subchronic treatment did not induce significant withdrawal signs. The global opiate withdrawal scores in the naloxone groups were 0.47 ± 0.24 and 1.32 ± 0.53 at 14 and 38 h after the last naloxone treatment, respectively.

Rewarding Effects Induced by Morphine but Not Naloxone. The rewarding effects of morphine or naloxone in mutated MOR gene-transferred mice were determined by CPP test (Chen et al., 2007). Our results showed that morphine (10 mg/kg s.c.) induced the drug rewarding effect as anticipated (Fig. 5). The time spent in the morphine-paired compartment was significantly increased after conditioning ($P < 0.001$). However, saline or naloxone (10 mg/kg s.c.) treatment did not induce the drug rewarding effects as shown in Fig. 5.

Discussion

The results of our present study indicated that the intrathecal administration of dsAAV2-MORS196A can accomplish similar antinociceptive effects of naloxone as direct injection of the virus at the dorsal horn area of the spinal cord as reported previously (Chen et al., 2007). The expression of the transgene at the lumbar region of the spinal cord, with the retrograde transport of the transgene into the DRG neurons, resulted in the ability of opioid receptor antagonist to elicit antinociceptive activities after intrathecal injection of the virus.

There are obvious advantages when dsAAV2 is administered intrathecally rather than directly injected into dorsal horn area, such as ease, safety, and high percentage in the success rate (79%). These factors are critical if the mutant opioid receptor is to be developed further as a therapeutic agent. Although naloxone was shown to exhibit full agonistic properties in activating the MORS196ACSTA mutant in vitro (Claude-Geppert et al., 2005), our current AUC analyses of the tail-flick responses reflected that naloxone had less effect than morphine at the same dose (10 mg/kg s.c.). The reduced effect observed with naloxone could reflect the relatively low amount of mutant MOR being expressed in the spinal antinociceptive neurons compared with the normal MOR at the spinal or supraspinal level on which morphine could act.

The choice of dsAAV2 for current gene delivery vector is based mainly on its unique features such as safety, high titers, broad host range, transduction of quiescent cells, and

vector integration (Xiao et al., 1997). Intrathecal gene transfer is an attractive approach for targeting spinal mechanisms of nociception. Several studies have shown that recombinant adeno-associated virus could successfully transfer genes into rats' spinal cord by intrathecal administration and the transgenes could be expressed in spinal cord and DRG (Milligan et al., 2005; Wang et al., 2005; Storek et al., 2006). The disadvantage of using dsAAV2 is the relative small size of the transgene that it could accommodate, i.e. < 2 kb. To restrict the transgene expression within the nociceptive neurons, especially those normally expressing endogenous MOR, the 5' untranslated region and elements that could control the expression of MOR must be included in the dsAAV expression cassette. With the minimal promoter of MOR that could regulate the spatial expression of the receptor to be ~ 1.3 kb (Liang et al., 1995), dsAAV might not be the optimal gene transfer vehicle. Other viral vectors that could accommodate larger sizes of transgene and could infect neurons, such as lentivirus, or other methods for gene delivery, such as nanoparticles, must be developed for the eventual therapeutic delivery of the MOR mutant.

In this study, we assessed both the antagonist-precipitated withdrawal signs and natural withdrawal symptoms to determine the development of physical dependence. Because naltrexone also acts as an agonist when interacted with the mutated opioid receptors (Claude-Geppert et al., 2005), to assess precipitated withdrawal signs by injecting naltrexone seems not to be an ideal method to determine the development of physical dependence induced by naloxone. To rule out the effects resulted from naltrexone-induced activation of mutated opioid receptor, the natural withdrawal symptoms were also assessed in the present study. Our data indicated subchronic morphine, but not naloxone, induced significant natural withdrawal symptoms (Fig. 6).

Nausea, vomiting, and constipation are very common, irritating side effects of opiates in clinic patients. Opiate-induced nausea and vomiting are caused by activation of the μ -opioid receptors in the brainstem chemoreceptor trigger zone, and the constipating effects of opioids are mediated through an action on the μ -opioid receptors in the enteric nervous system (Schumacher and Basbaum, 2009). Because

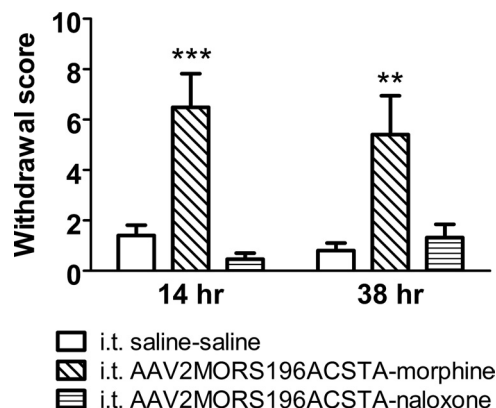


Fig. 6. The global natural withdrawal scores after chronic saline (10 ml/kg s.c., b.i.d., 6 days), morphine (10 mg/kg s.c., b.i.d., 6 days), or naloxone (10 mg/kg s.c., b.i.d., 6 days) treatment in control (intrathecal saline) or gene-transferred mice (intrathecal AAV2MORS196ACSTA). Data are presented as mean \pm S.E.M. ($n = \sim 7-10$). One-way ANOVA and Newman-Keuls test were used to analyze the data. **, $P < 0.01$; ***, $P < 0.001$ compared with intrathecal saline-saline group.

naloxone acts only on the mutated MOR expressed in the spinal cord, as an agonist if we transfer the mutated MOR by intrathecal injection, we can expect that naloxone will not cause these side effects of nausea, vomiting, and constipation. Furthermore, long-term use of opioid agonists to treat chronic pain may induce tolerance, physical dependence, and addiction, and our current intrathecal injection of dsAAV2-MORS196ACSTA and systemical administration of naloxone clearly demonstrate an alternative method for pain management without the side effects of tolerance and dependence development. Especially, the absence of place preference by naloxone indicates that this mutant receptor activated by naloxone could be a future therapeutic paradigm for the management of pain in the long term without addiction liability.

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