D-serine in the midbrain periaqueductal gray contributes to morphine tolerance in rats

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Abstract

Background: The N-methyl-D-aspartate subtype of glutamate receptor plays a critical role in morphine tolerance. D-serine, a co-agonist of N-methyl-D-aspartate receptor, participates in many physiological and pathophysiological processes via regulating N-methyl-D-aspartate receptor activation. The purinergic P2X$_7$ receptor activation can induce the D-serine release in the central nervous system. This study aimed to investigate the role of the ventrolateral midbrain periaqueductal gray D-serine in the mechanism of morphine tolerance in rats. The development of morphine tolerance was induced in normal adult male Sprague–Dawley rats through subcutaneous injection of morphine (10 mg/kg). The analgesic effect of morphine (5 mg/kg, i.p.) was assessed by measuring mechanical withdrawal thresholds in rats with an electronic von Frey anesthesiometer. The D-serine concentration and serine racemase expression levels in the ventrolateral midbrain periaqueductal gray were evaluated through enzyme-linked immunosorbent assay and Western blot analysis, respectively. The effects of intra-ventrolateral midbrain periaqueductal gray injections of the D-serine degrading enzyme D-amino acid oxidase and antisense oligodeoxynucleotide targeting the P2X$_7$ receptor on chronic morphine-treated rats were also explored.
Results: We found that repeated morphine administrations decreased the antinociceptive potency of morphine evidenced by the percent changes in mechanical pain threshold in rats. By contrast, the D-serine contents and the expression levels of the serine racemase protein were upregulated in the ventrolateral midbrain periaqueductal gray in morphine-tolerant rats. The development of morphine tolerance was markedly alleviated by intra-ventrolateral midbrain periaqueductal gray injections of D-amino acid oxidase or antisense oligodeoxynucleotide targeting the P2X$_7$ receptor.
Conclusions: Our data indicate that the development of antinociceptive tolerance to morphine is partially mediated by ventrolateral midbrain periaqueductal gray D-serine content, and the activation of the ventrolateral midbrain periaqueductal gray P2X$_7$ receptor is an essential prelude to D-serine release. These results suggest that a cascade involving P2X$_7$ receptor–D-serine–N-methyl-D-aspartate receptor mediated signaling pathway in the supraspinal mechanism of morphine tolerance.

Background

Morphine is an opiate drug that is widely used for the clinical management of moderate to severe pain, but morphine-induced side effects, especially hyperalgesia and tolerance, significantly limit its clinical use. The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor performs critical functions in pain processing including central sensitization that eventually causes hyperalgesia.[1,2] Additionally, the activation of NMDA receptor has been implicated as an anti-opioid system in the development of morphine analgesic tolerance and dependence in the central nervous system (CNS).[3–6]

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Numerous studies have revealed that the opening of the NMDA receptor ion channel requires occupation of two distinct binding sites, the glutamate site and the glycine site.[7,8] As an endogenous ligand for glycine-bind site in NR1 subunit of NMDA glutamate receptors, D-serine (D-ser) is more potent than glycine.[9,10] Besides predominantly expressing in astrocytes, and acting as an important gliotransmitter in the CNS,[11] D-ser is also produced and released by neurons[12] and behaves as a neurotransmitter in the same area of the brain. Accordingly, by modulating neurotransmission through NMDA receptor, D-ser involves in many vital physiological and pathological progresses, such as synaptic plasticity,[13] neural development,[14] social interactions,[15,16] pain,[17] neurotoxicity[18] and is supposed as a potential therapeutic target for the treatment of nervous system diseases such as Alzheimer disease, epileptic seizures, amyotrophic lateral sclerosis,[19] and psychiatric disorders.[20] The D-ser is synthesized from L-serine via racemization of serine racemase (SR), which is a pyridoxal-5′-phosphate-dependent enzyme and regulated by many factors, and, importantly, the regional distribution of SR parallels that of D-ser.[21] In the brain, SR is the major enzyme for D-ser production. Wolosker and Mori [22] reported that SR knockout (SR−/−) mice displayed 90% lower D-ser in the brain and deficits in NMDA receptor activity.

The midbrain periaqueductal gray (PAG) is a strategic site in endogenous nociceptive modulatory system.[23,24] It is also an essential neural circuit for opioid-mediated analgesia,[25–27] especially its ventrolateral PAG (vlPAG) region.[28,29] The upregulation of NMDA receptor expression in the PAG facilitates morphine tolerance development. In the chronic morphine-treated mice, the expression of the NMDA receptor in the PAG was upregulated.[30] A similar study found that epsilon 1 subunit of the NMDA receptor expression dramatically increased in the PAG of morphine-tolerant mice.[6]

Adenosine 5′-triphosphate (ATP) is released by neuronal and nonneuronal cells.[31] In addition to being an intracellular energy source, ATP is an important neurotransmitter or neuromodulator that activates cation-permeable ion channels (P2X receptors) and G-protein-coupled receptors (P2Y receptors) on the cell surface.[32] The $P2X_7$ receptor subtype is widely distributed in glia cells and neuron,[33] and its expression is altered in many pathophysiological processes, such as inflammation, pain, and cancer.[34–36] The $P2X_7$ receptors are also involved in the morphine tolerance. A previous study found that microglial cells in the rat spinal cord expressed the $P2X_7$ receptor, and the protein level of this receptor was upregulated after chronic exposure to morphine. The suppression of the $P2X_7$ receptor activation significantly attenuated the loss of morphine analgesic potency.[37] The activation of $P2X_7$ receptor triggers

the release of D-ser from cultured astrocytes, and a spinal mechanism underlying morphine tolerance has been proposed, in which chronic morphine triggered multiple dialogues between glial and neuronal cells in the spinal cord via a cascade involving a $P2X_7$ receptor–interleukin-18–D-ser–NMDA receptor–protein kinase C gamma (PKCγ)-mediated signaling pathway.[38,39]

Our previous study indicated that the $P2X_7$ receptor expression was more pronounced in the vlPAG in morphine-tolerant rats.[40] The current study aimed to elucidate the role of vlPAG D-ser in morphine tolerance. We explored the levels of D-ser and SR protein expression in the vlPAG of morphine-tolerant and control animals and investigated the effect of the D-ser degrading enzyme D-amino acid oxidase (DAAO) on the behavioral tolerance to morphine in rats. Furthermore, we studied whether the activation of $P2X_7$ receptors in the vlPAG was involved in the mechanisms of morphine tolerance through the secretion of D-ser in rats.

Methods

Animals and ethics

Male Sprague–Dawley rats weighing 220 ± 10 g were purchased from the Center of Laboratory Animals, Third Military Medical University (Chongqing City, China). In a temperature-controlled room ($25 \pm 1°$C), the rats were housed in groups of four or five per cage with a 12 h light-dark cycle (7:00 a.m.–7:00 p.m.). Food pellets and water were given to the rats ad libitum throughout the experiments, except during the experimental periods. All studies were approved by the Institutional Animal Care and Use Committee of the Zunyi Medical University and performed in strict compliance with the Ethical Issues of the International Association for the Study of Pain. All efforts were exerted to minimize the number of animals used and their suffering.

Induction of morphine tolerance

The rat model of morphine tolerance was established as previously described.[39,40] Briefly, morphine was given subcutaneously (s.c.) twice daily (with a 12-h dosing interval) from day 1 to day 9 at 10 mg/kg body weight to establish systemic analgesic tolerance. The drug dosage was based on our previous study.[40] To evaluate the development of morphine tolerance, we assessed morphine antinociception to mechanical stimuli at 30 min after an acute test dose (5 mg/kg) of morphine was intraperitoneally (i.p.) given. Morphine analgesic effects before and after a defined period of tolerance induction were compared. Baseline nociceptive thresholds were measured 15 min before subcutaneous injection of morphine.

Behavioral studies

On each designated test day, the rats were tested for mechanical allodynia after acclimatization to the testing apparatus, and allodynia was tested under nonrestrained conditions. To determine mechanical withdrawal threshold (MWT), we placed each rat in an individual transparent plexiglass cage (18 cm × 12 cm × 12 cm) with a wire mesh floor in a quiet room. The rats were allowed to explore and groom until settling down. The test involved evoking a hind paw flexion reflex (paw withdrawal) with a handheld force transducer (IITC 2390 series electronic von Frey anesthesiometer, Life Science Instruments, Los Angeles, California, USA) equipped with a 0.5-mm^2 contact area polypropylene tip. The investigator was trained to perpendicularly apply the tip to the central area of the hind paw with gradually increasing pressure. Endpoint was characterized by removal of the paw, in which the animal actively lifted the whole paw on the tip of the anesthesiometer. Positive responses included prolonged hind paw withdrawal, licking or biting of the hind paw, or shaking the paw with high amplitude movements in response to the stimulus. Each hind paw was measured five times in grams, and the average values of five measurements were regarded as the paw MWT.[41] The development of morphine-induced tolerance was detected by measuring percent changes in MWT after a challenge injection of morphine (5 mg/kg, i.p.) and calculated as follows: MWT (%) = (tested threshold − basal threshold)/basal threshold × 100%. A higher MWT% represented a better analgesic effect.

Surgical and microinjection procedures

Anesthesia was induced through i.p. injection of 4% chloral hydrate (10 mL/kg body weight). The rats were mounted on a stereotaxic frame (Narishige SR-5R, Tokyo, Japan). The skull was exposed and the bregma was located. A stainless steel guide cannula (0.8 mm o.d.) was inserted unilaterally into the vlPAG and fixed to the skull by using dental zinc cement and jewelers' screws. The stereotaxic coordinates for the vlPAG were 7.90 mm posterior to the bregma, 0.80 mm lateral to the midline, and 6.00 mm ventral to the skull surface. A dummy cannula was inserted into the guide cannula at the time of surgery to minimize occlusion. Skull screws and dental acrylic were used to hold the cannulae securely in place. After removal from the stereotaxic apparatus, the rats were (i.p.) administered with 1 mL of 0.9% sterile saline to prevent dehydration and then placed in a thermally controlled cage to avoid hypothermia until complete anesthetic recovery. Prior to any experiments, the animals were allowed to recover from the implantation surgery for five days and were monitored for signs of motor impairment. Rats with any neurological deficits caused by the surgical procedure were excluded from

the experiments. On the day of intra-vlPAG injection, the rats were transferred from the main holding area to the laboratory and were left undisturbed for 1 h prior to drug administration. Each rat was lightly restrained, and a 32-gauge injection cannula (1.0 mm longer than the guide cannula) was inserted into the guide cannula. The injection cannula was connected to a 5-μL Hamilton microsyringe. A total volume of 0.3 μL was injected over 3 min, and the injector was left in place for an additional 2 min before slow removal to ensure complete drug diffusion. Successful infusion was confirmed by monitoring the movement of a small air bubble in the microsyringe. After the experimental procedures, the animals were anesthetized through i.p. injection of 4% chloral hydrate (20 mL/kg body weight) and then intracardially perfused with physiological saline (0.9% NaCl) and 4% paraformaldehyde solution. The needle position of the cannula was visually confirmed with 0.2 μL of 2% Evans blue infusion through the microinjection cannula. Administration sites were verified through histological examination and plotted on coronal maps adapted from the atlas of Paxinos and Watson.[42] Only rats with whole microinjection sites within the vlPAG were included in analysis. Animals with cannulae located outside the vlPAG (e.g., lateral PAG, dorsal raphe nucleus) were considered "cannulae misses" and discarded (Figure 1a and b).

Enzyme-linked immunosorbent assay

The vlPAG tissue (100 mg) was homogenized in 1 mL lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, and Complete Protease Inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and centrifuged at 3,000 r/min for 20 min at 4°C. The protein concentration of the supernatant (500 μL) was quantified by bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). D-ser was measured using commercially available enzyme immunoassays (rat D-serine enzyme-linked immunosorbent assay (ELISA) set, Shanghai Biological Technology Co., Ltd. enzyme research, China) according to the manufacturer's instructions. The standard curve was included in each experiment, and the protein expression was normalized to the total protein amount per vlPAG tissue and reported as ng/mg wet weight.

Western blot

Animals were anesthetized by an over dose of chloral hydrate (20 mL/kg body weight, i.p.), and the vlPAG tissue was rapidly removed. The collected tissue samples were homogenized in a lysis buffer containing a mixture of protease inhibitors (Roche Diagnostics) and
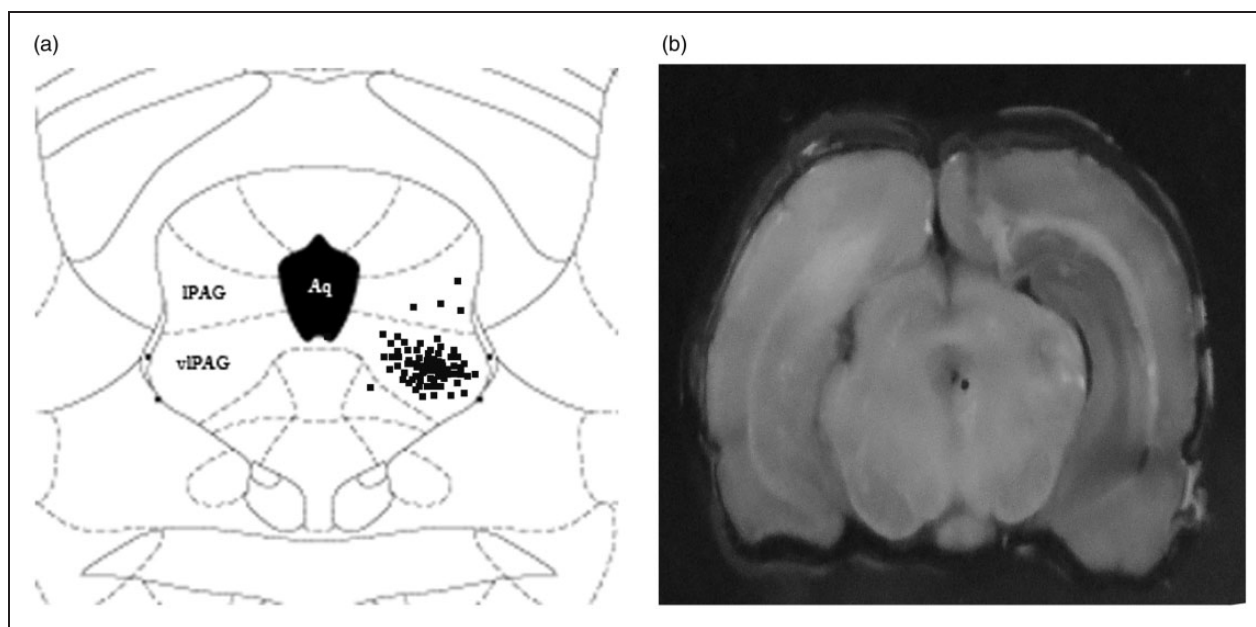
Figure 1. (a) Schematic diagram of the microinjection sites for saline, DAAO, and ODN. Dots represent the corresponding sites identified histologically through Evans blue dye microinjection. (b) Photograph of an injection site in the vlPAG. Microinjection sites were mostly distributed within the vlPAG region. Data from rats with an injection site outside the vlPAG region were discarded.
ODNs: oligodeoxynucleotides; DAAO: D-amino acid oxidase; vlPAG: ventrolateral midbrain periaqueductal gray.

phenylmethylsulfonyl fluoride (Sigma, St Louis, Missouri). Protein samples (20 μg/lane) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis 5–12% gels, Bio-Rad, Canada) and transferred onto polyvinylidene fluoride membranes (Sigma Aldrich, St Louis, Missouri). The membranes were blocked with 5% nonfat milk and then incubated overnight at 4°C with a primary antibody (rabbit anti-rat SR receptor, 1:400; rabbit anti-rat $P2X_7$ receptor, 1:600; Abcam Corporation, Shanghai, China). Mouse anti-rat β-actin primary antibody (1:2,000; Sigma-Aldrich, , St Louis, Missouri) was included as a control for protein loading. The membranes were then incubated in goat anti-rabbit horseradish peroxidase (HRP)- or goat anti-mouse HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Paso Robles, CA) for 2 h at room temperature before the blots were visualized in enhanced chemiluminescence solution (Amersham Pharmacia Biotech, UK) and exposed to X-ray films. The developed X-ray films were scanned for data analysis. Protein levels were normalized to β-actin as the loading control. Relative optical density (ROD) of the protein bands was measured after subtracting the film background. Data are expressed as mean ratio ± S.E.M of the SR/β-actin protein.

Experimental design and drugs

The experiments consisted of four series. In series 1, changes in MWT values were determined after repeated administrations of morphine. The rats were randomly and equally divided into three groups according to a random number table: normal group, saline group, and morphine group ($n = 12$ per group). Morphine (10 mg/kg, s.c.) was administered to rats in the morphine group twice a day for nine days. In the saline group, the rats were injected with 1 mL/kg physiological saline solution (0.9% normal saline [NS]), instead of morphine, with the same schedule. The normal group served as control subjects. All experimental rats were subjected to MWT test daily for nine days.

In series 2, alterations in D-ser concentration and SR expression level in the vlPAG were observed. Six rats from each group in the first series were used for ELISA analysis of D-ser concentrations and the remaining six rats for Western blot analysis of SR expression on day 9 post morphine injection.

In series 3, we observed the effects of intra-vlPAG microinjection of different doses of DAAO. The rats were randomly and equally divided into six groups according to a random number table: normal group, normal + 0.1U DAAO group, morphine group, morphine + 0.001U DAAO group, morphine + 0.01U DAAO group, and morphine + 0.1U DAAO group ($n = 8$ per group). Morphine (10 mg/kg, s.c.) was administered twice a day for six days in all groups, except the normal and normal + 0.1U DAAO groups. DAAO at doses of 0.001U, 0.01U, and 0.1U in 0.3 μL sterile physiological saline (0.9% NS) were intra-vlPAG given on day 1 after morphine injection (s.c.) and once daily

for five days. The experimental rats were subjected to MWT test on day 0 and day 6 after morphine injection (s.c.). The corresponding control (DAAO) was intra-vlPAG given with the same schedule.

In series 4, changes in contents of D-ser in the vlPAG in response to oligodeoxynucleotides (ODNs) targeted against $P2X_7$ receptor were determined. A total of 36 normal rats were divided into three groups: saline + morphine group, mismatch ODN + morphine group (MM ODN + morphine), and antisense ODN + morphine group (AS ODN + morphine; $n = 12$ per group). The MM ODN + morphine and AS ODN + morphine groups were given ODN (15 nmol/0.3 μL) through vlPAG microinjection (from day 5 after morphine injection (s.c.), once daily for 5 days) and morphine (10 mg/kg, s.c.) twice daily for nine consecutive days. In the saline + morphine group, the rats were injected with 0.3 μL of 0.9% NS, instead of ODN, with the same schedule. At the end of the experiment (on day 9 after chronic morphine treatment), six rats from each group were used for Western blot analysis for $P2X_7$ receptor expression and the remaining six rats for ELISA analysis for D-ser levels.

Morphine hydrochloride was purchased from Shenyang First Pharmaceutical Factory (Shenyang City, China). DAAO (Sigma-Aldrich, France) was used at a dose of 0.001U, 0.01U, and 0.1U in 0.3 μL sterilized normal saline. Phosphorothioate-modified oligonucleotides of the rat $P2X_7$ receptor were synthesized and purified by Sangon Biological Engineering Technology Co. (Shanghai City, China). According to our previous report [40], the sequences were designed as follows: $P2X_7$ receptor antisense ODN: 5′-TTG ATG GTG CCG TAA TTC ACG CTC T-3′ targeted to the nucleotide sequence 186 through 210 that directly follows the initiation codon of the rat $P2X_7$ receptor; and mismatch ODN: 5′-AAT TAC ACA GTA AGC GAA CTT AGC C-3′. A database search using the BLAST program indicated that the antisense sequence was specific for the rodent $P2X_7$ receptor. Nevertheless, the search did not identify the corresponding rodent sequence for the mismatch sequence. Antisense and mismatch ODNs for the $P2X_7$ gene were dissolved in double distilled water to a concentration of 50 nmol/μL. ODNs were aliquoted and stored at −20°C, and oligonucleotide treatments were performed as previously described.[43]

Statistical analysis

Experimental data were processed using GraphPad Prism (version 6.01; GraphPad Software, Inc., San Diego, CA) and SPSS 17.0 (SPSS Inc., Chicago, IL). All data were presented as mean ± standard deviation. Results from the Western blot work and ELISA test were tested using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Two-way ANOVA followed by Tukey's post hoc test were used to analyze the data from the time course of morphine tolerance and the effects of drugs on the development of morphine tolerance. A p value < 0.05 was considered to be statistically significant in this study.

Results

Development of tolerance to analgesia produced by repeated morphine injections in rats

Before subcutaneous injection of morphine (day 0), the percentage change in MWT was not significantly different among the normal, saline, and morphine groups ($p > 0.05$, $n = 12$ in each group). In the morphine group, the analgesic effect induced by a test dose (5 mg/kg, i.p.) of morphine gradually decreased after the rats received multiple subcutaneous injections of 10 mg/kg morphine twice daily and was nearly abrogated on day 9. This phenomenon was considered as morphine tolerance. In contrast, the percentage changes in MWT in the saline group did not significantly change compared with that in the normal group at all observation time points (all $p > 0.05$, $n = 12$ in each group). Moreover, basal MWT did not significantly change during this time period (data not shown; Figure 2).

Increased release of D-ser in the vlPAG induced by chronic administrations of morphine

By using the ELISA method, we detected D-ser release levels in the vlPAG in three experimental groups (normal, saline, and morphine group) of rats. As shown in Figure 3, there was a low level of D-ser in the vlPAG of normal rats and no detectable change in D-ser level following saline injection on day 9 time point ($p > 0.05$, $n = 6$, Figure 3). In contrast, D-ser content was significantly elevated on day 9 of chronic morphine treatment (10 mg/kg, s.c., twice daily for nine days consecutively; $p < 0.001$, $n = 6$, Figure 3) along with the development of tolerance to morphine analgesia, suggesting an involvement of D-ser release in the vlPAG in the development of morphine tolerance.

Upregulation of SR expression in the vlPAG in morphine-tolerant rats

Immunoblots from vlPAG homogenates revealed the presence of an immunopositive band of the SR in the normal, saline, and morphine groups. The results were quantified based on the ROD of the immunoblot bands compared with β-actin calculated from the densitometric quantification of the bands. No significant difference was detected between the normal and saline
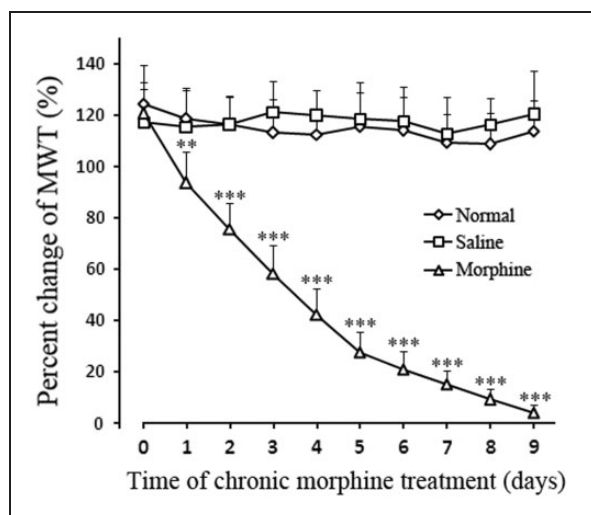
Figure 2. Percentage changes in MWT of the experimental rats subjected to repeated morphine/saline injections. Rats were administered with morphine (10 mg/kg, s.c.) twice daily for nine days. The antinociceptive effect of morphine was measured through the paw withdrawal test after treatment with a test dose of morphine treatment (5 mg/kg, i.p.). The analgesic efficacy of the test dose of morphine significantly decreased after repeated morphine administration. Results are means $\pm$ SEM ($n = 12$). **$p < 0.01$ and ***$p < 0.001$ versus data from the normal group. MWT: mechanical withdrawal threshold.

Figure 3. Alterations in D-ser levels in the vlPAG in experimental rats. The multiple morphine treatments induced an increased D-ser level in the vlPAG in morphine-tolerant rats. ($n = 6$). ***$p < 0.001$ versus normal or saline group at the same time point.
vlPAG: ventrolateral midbrain periaqueductal gray.

groups ($p > 0.05$, $n = 6$). The protein level of the SR was significantly higher in the morphine group than those in the normal and saline groups on nine days after chronic morphine treatment (all $p < 0.001$, $n = 6$; Figure 4a and b).

Effect of microinjection of DAAO on MWT in morphine-tolerant rats

To address the importance of vlPAG D-ser content in morphine tolerance, we undertook intra-vlPAG administration of DAAO, an enzyme that selectively degrading D-ser, on morphine-tolerant rats for six days. The administration of DAAO resulted in a markedly decrease in D-ser levels in the vlPAG and alleviated the morphine-tolerant behaviors ($p < 0.001$, $n = 8$). Moreover, DAAO acted in a dose-dependent manner, as the highest dose (0.1U) induced a more significant diminution in D-ser levels in the vlPAG and morphine-tolerant behaviors than a moderate dose (0.01U; $p < 0.001$, $n = 8$). The use of low dose (0.001 U) DAAO revealed no significant changes in D-ser level in the vlPAG and morphine-tolerant behaviors compared with morphine group ($p > 0.05$, $n = 8$). The percentage changes in MWT in the normal + 0.1U DAAO group did not significantly change in D-ser levels in the vlPAG and morphine-tolerant behaviors compared
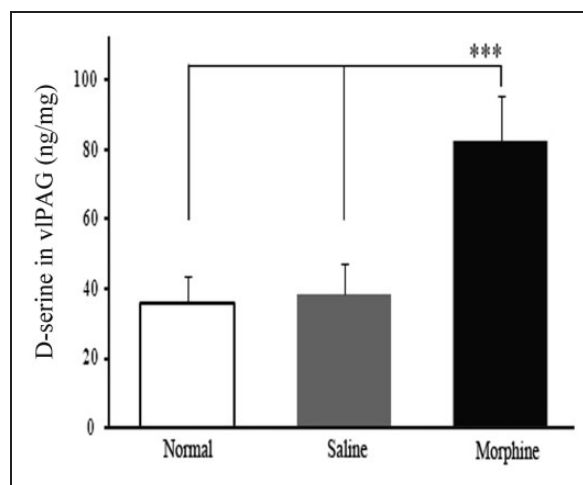
with that in normal group ($p > 0.05$, $n = 8$; Figure 5a and (b)).

Effect of ODN against P2X₇ receptor on D-ser level in the vlPAG

In this experimental procedure, a reversal effect of intra-vlPAG injection of AS ODN targeting the P2X₇ receptor on D-ser level was observed. Western blot analysis indicated that delivery of AS ODN (15 nmol/0.3 μL) for five consecutive days in AS ODN + morphine group significantly downregulated the expression of the P2X₇ receptor in the vlPAG compared with saline + morphine group or MM ODN + morphine group (all $p < 0.001$, $n = 12$). The expression levels of P2X₇ receptor in the vlPAG were not significantly different between the saline + morphine and MM ODN + morphine group ($p > 0.05$, $n = 12$; Figure 6a and b). Using the ELISA method, we found that there was a lower level of D-ser in the vlPAG in AS ODN + morphine group compared with saline + morphine and MM ODN + morphine group ($p < 0.001$, $n = 12$). The difference in the level of D-ser in the vlPAG in MM ODN + morphine group, when compared with the saline + morphine group, was insignificant ($p > 0.05$, $n = 12$; Figure 6c).

Discussion

The present set of experiments attempted to testify the hypothesis that D-ser in the vlPAG contributes to the development of chronic morphine tolerance in rats. The major findings in the current study are as follows:
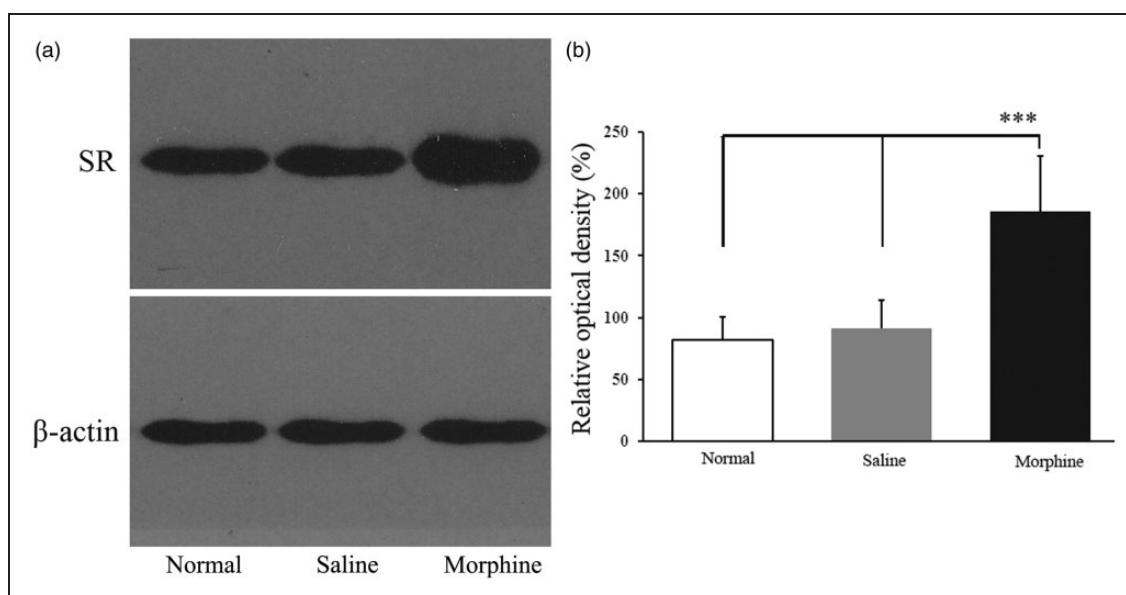
Figure 4. (a) Upregulation of SR protein level in the vlPAG induced by chronic morphine treatment. Western blot analysis detected a protein band of approximately 40 kDa, which coincides with the known molecular weight of the SR. β-actin was used as the loading control. (b) The protein levels of the SR in different groups were expressed as ROD. ($n = 6$ in each group). ***$p < 0.001$ versus normal or saline group.
SR: serine racemase; vlPAG: ventrolateral midbrain periaqueductal gray; ROD: relative optical density.

(a) repetitive applications of morphine could gradually induce morphine tolerance in rats; (b) the chronic morphine administration dramatically elevated the D-ser concentration and SR expression levels in the vlPAG in morphine-tolerant rats; (c) the analgesic effect of morphine was partly but significantly maintained by the vlPAG microinjection of DAAO; (d) intra-vlPAG injection of the ODN against P2X$_7$ receptor markedly decreased D-ser level in the vlPAG and inhibited the development of morphine tolerance. These results demonstrated the important role of vlPAG D-ser in morphine-tolerant rats and suggested a direct implication of P2X$_7$ receptor-D-ser pathway in the formation of morphine tolerance in rats.

D-amino acids are stereoisomers of naturally occurring L-amino acids. They are initially considered as unnatural amino acids and were thought to be only existed in bacteria and invertebrate species. However, the development and improvement of analytical methods and instruments have revealed the presence of D-amino acids such as D-ser and D-aspartate even in mammalian brains.[44] It has been reported that D-ser immunoreactivity is abundant in the cerebral cortex, hippocampus, anterior olfactory nucleus, and amygdala of the rat brain.[45] In the current study, we also found that a relative low level of D-ser content presented in the vlPAG in normal or saline-treated rats. Contrarily, after nine days of morphine treatment, the D-ser content in the vlPAG was obviously elevated, and the rats developed substantial tolerance to morphine analgesia, implicating an

involvement of D-ser release in the vlPAG in the development of morphine tolerance.

D-ser has been initially reported to be released from astrocytes via large vesicles[46] or exocytosis.[47–49] It must be noted that several recent reports have described that neurons may also release D-ser and glycine.[50,51] By using a conditional cell-specific SR-knockout mice, a lower forebrain D-ser level along with long-term potentiation (LTP) deficits were observed when SR gene was deleted in neurons while deletion in astrocytes only leads to a minimal decrease in forebrain SR expression and no significant change in D-ser level and NMDA receptor activity.[52] Based on these findings, an elegant hypothesis of a serine shuttle between neurons and astrocytes was established.[53] Namely, neuronal D-ser depends on the production of L-serine by astrocytes because the 3-phosphoglycerate dehydrogenase which catalyzes the production of L-serine from glucose is exclusively located in astrocytes.[51,54,55] L-serine is then exported and shuttles to neurons to fuel the synthesis of D-ser by SR. Finally, D-ser is released by neurons and accumulates back in astrocytes.[53] As a result, neurons and glial cells all play an important role in the metabolism of D-ser.

The glutamatergic NMDA receptor signaling pathways have been studied as targets for intervention in a variety of neuropathological conditions like neurodegenerations, epilepsy, neuropathic pain, drug addiction, and schizophrenia. In morphine tolerance, activation of the NMDA receptor has been implicated as an anti-opioid system in the development of morphine analgesic tolerance and
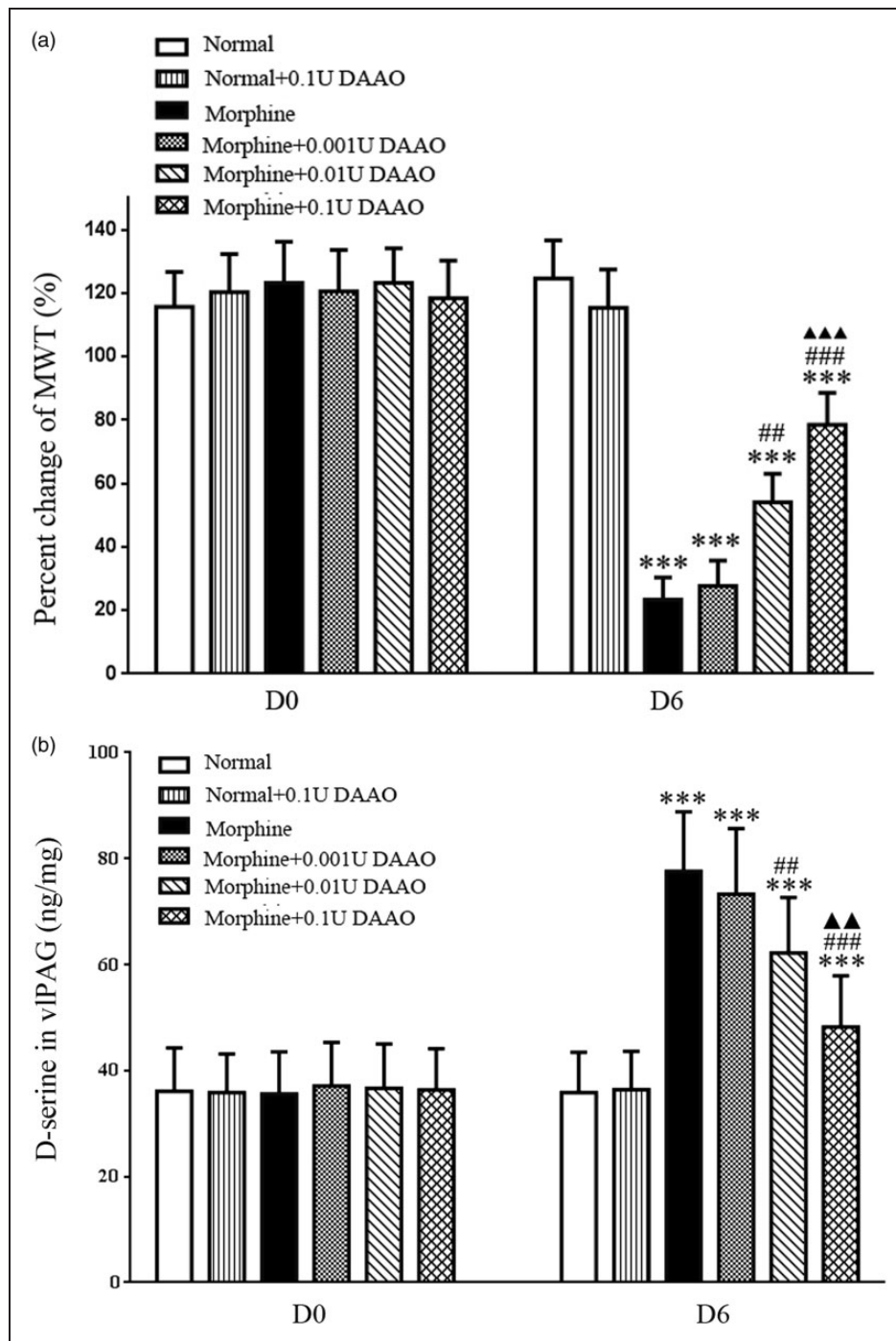
Figure 5. Attenuation of the development of morphine tolerance by intra-vlPAG injection of DAAO. The D-ser levels in the vlPAG and chronic morphine-induced analgesic tolerance were attenuated in mechanic withdrawal tests by the intra-vlPAG pretreatment of the DAAO. ***$p < 0.001$, versus normal group; ##$p < 0.01$, ###$p < 0.001$ versus morphine group; ▲▲$p < 0.01$, ▲▲▲$p < 0.001$ versus morphine + 0.01U DAAO group.

vlPAG: ventrolateral midbrain periaqueductal gray; DAAO: D-amino acid oxidase; D0: day 0; D6: day 6.

dependence. At the supraspinal sites, both the NMDA receptor and μ-opioid receptor (MOR) are present in the PAG neurons, and the activation of NMDA receptor restricts the activity of the MOR. The cross talk between these receptors is sustained by the MOR-associated

histidine triad nucleotide binding protein 1.[56] In general, the activation of NMDA receptor can be an important factor for promoting the morphine tolerance.

As the activation of NMDA receptor is involved in above-mentioned disorders, high activity NMDA-
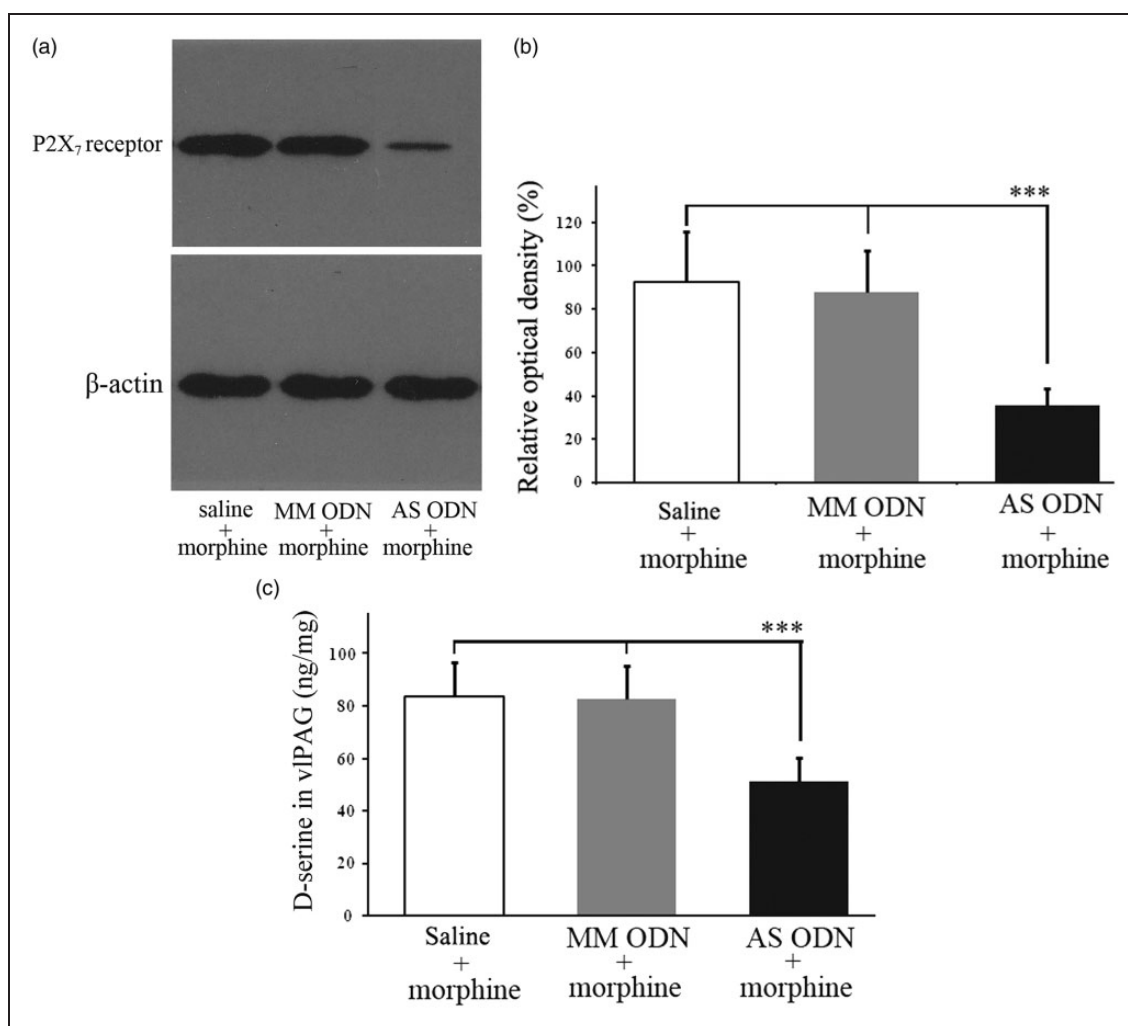
Figure 6. (a) and (b) P2X$_7$ receptor antisense ODN, but not mismatch ODN, at 15 nmol/0.3 μL intra-vlPAG injection once daily for five days reduced P2X$_7$ receptor expression in the vlPAG in rats. ***$p < 0.001$ versus data from the saline + morphine group or MM ODN + morphine group ($n = 12$ in each group). (c) ELISA showed the D-ser level in the AS ODN + morphine group was reduced compared with saline + morphine group (***$p < 0.001$). Conversely, the D-ser levels in the MM ODN + morphine group were not significantly different compared with that in the saline + morphine group ($p > 0.05$).
vlPAG: ventrolateral midbrain periaqueductal gray; MM ODN: mismatch oligodeoxynucleotide; AS ODN: antisense oligodeoxynucleotide

blocking agents have been designed to treat some of these disorders; however, their effect is often compromised by undesirable side effects. Therefore, alternative ways of modulating NMDA receptor function need to be sought after. Numerous studies have revealed that D-ser is more effective than glycine as an NMDA receptor co-agonist[9,10,57] and the distribution pattern of D-ser closely resembles that of NMDA receptors, therefore D-ser and its metabolism-related enzymes became the potential candidates for this purpose.

It has been reported that chronic administration of morphine produced a significant elevation of both the mRNA and protein expressions of SR in all the brain regions, whereas no significant change in the protein expression of DAAO was observed in all the brain

regions.[58] But SR is a very difficult target, with only few compounds so far identified exhibiting weak inhibitory activity,[59] therefore we used DAAO in the current study and found that the vlPAG microinjection of DAAO was effective in restoring morphine analgesic effect, indicating the involvement of D-ser and glycine site in NMDA receptor in morphine tolerance. Together, our findings support the idea that endogenous D-ser in the vlPAG is a key co-agonist of NMDA receptor overactivation in the induction of morphine tolerance.

On the basis of the signal transduction mechanisms and characteristic molecular structures, the P2 purinoceptor can be divided into the P2X receptors (P2X$_{1-7}$) and P2Y receptors (P2Y$_{1,2,4,6,11,12,13,14}$).[60–62] P2X

receptors are cationic-selective ion channels gated by extracellular ATP. With techniques, results confirmed that the $P2X_7$ receptors are localized in the rodent CNS neurons.[63–65] Previous works have also suggested the expression of $P2X_7$ receptor on CNS glial cells including astrocytes and microglial cells.[66–68] Compared with other P2X receptors, $P2X_7$ receptor have a lower affinity for ATP,[69] indicating that their activation mostly occurs in pathological conditions associated with enhanced extracellular ATP levels. In peripheral tissues, $P2X_7$ receptor mediates inflammation, cancer, cell proliferation, and apoptosis.[70] In the nervous system, it modulates neurotransmitter release, as well as microglial and astroglial activation.[33] The activation of $P2X_7$ receptor on neuronal or nonneuronal cells is related to many brain disorders such as trauma,[71,72] Alzheimer's disease,[73,74] Parkinson's disease,[75,76] and multiple sclerosis.[77] In morphine tolerance, after chronic exposure to morphine, the protein expression of the $P2X_7$ receptor in spinal microglia was upregulated and morphine tolerance was developed. Intrathecal administration of Brilliant Blue G, a potent $P2X_7$ receptor inhibitor, or RNA interference targeting the spinal $P2X_7$ receptor significantly attenuated the loss of morphine analgesic potency, upregulated $P2X_7$ receptor expression, and activated microglia.[37] So far, two mechanisms have been proposed to explain the $P2X_7$ receptor activation-induced D-ser release. One is the pannexin-1 hemichannel, the $P2X_7$ receptor-mediated D-ser release by the $P2X_7$ receptor–pannexin-1 complex formation; the other one is the activated $P2X_7$ receptor per se is also functioned as a permeation channel to release D-ser in part.[38] In this study, we found that the use of AS ODN against $P2X_7$ receptor reduced the level of D-ser in the vlPAG, so we speculated that under the circumstance of morphine tolerance, $P2X_7$ receptor can promote the release of serine. As $P2X_7$ receptor formed a channel or a $P2X_7$ receptor–pannexin-1 complex in the D-ser-releasing mechanism, further investigations are demanded.

The racemase activity was independently stimulated by both Mg^{2+} and ATP.[78] In our previous report, we found the expression of $P2X_7$ receptor in the vlPAG was increased in morphine-tolerant rats. Combined with the findings from the current study that the expression of SR protein in the vlPAG was upregulated, we believed that an increased extracellular ATP level was presented in morphine-tolerant rat which is sufficient for the activation of both the $P2X_7$ receptor and SR.

In our previous study, we found that the expression of the vlPAG $P2X_7$ receptor significantly increased in morphine-tolerant rats, but $P2X_7$ receptor antagonist A-740003 reduced the development of chronic morphine tolerance in rats.[40] In the current study, we further revealed that intra-vlPAG injection of AS ODN against $P2X_7$ receptor prominently decreased the $P2X_7$ protein expression and D-ser concentration in the vlPAG in morphine-tolerant rats. Herein, the activation of $P2X_7$ receptor leaded to an increased D-ser release in the vlPAG and thus at least in part contributed to the NMDA receptor activation-induced morphine tolerance in rats. The D-ser in the vlPAG is a potential molecule for the treatment of morphine tolerance.

Author contributions

SC, ZX, YL, and MS performed the experiments. ZX analyzed the data and drafted the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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