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ORIGINAL ARTICLE

Antinociceptive effect of intracerebroventricular administration of D-serine on formalin-induced pain

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Abstract
Purpose In a previous study using the tail-flick test, we found that intracerebroventricular administration of D-serine, an endogenous co-agonist at the glycine sites of *N*-methyl-D-aspartate (NMDA) receptors, elicited an antinociceptive effect on thermal nociception. The purpose of the present study was to evaluate the effect of intracerebroventricular administration of D-serine on nociception induced by tissue damage or inflammation using the formalin test.
Methods Infusion of drugs into the third ventricle in rat was performed via indwelling cannulae. Drugs were infused at a volume of 10 µl over 2 min, and the infusion cannula was left in place for 2 min before removal. The formalin test was performed 10 min after drug administration.
Results Intracerebroventricular administration of D-serine significantly and dose-dependently decreased the number of flinches in both the early and late phases in the formalin test. This antinociceptive effect was antagonized by intracerebroventricular administration of L-701,324, a selective antagonist at the glycine sites of NMDA receptors.
Conclusion The present data suggest that activation of NMDA receptors via glycine sites at the supraspinal level induces an antinociceptive effect on both acute and tonic pain.

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Keywords Antinociception · NMDA receptor · D-Serine · Morphine · Formalin test

Introduction

D-Serine acts as an obligatory ligand for the glycine sites of *N*-methyl-D-aspartate (NMDA) receptors [1–3]. D-Serine is confined to the forebrain, where NMDA receptors are abundantly expressed [2], and has been proposed as an endogenous co-agonist at NMDA receptors in mammalian brain [4, 5].

The NMDA receptor plays an important role in the descending pain modulation pathway from the supraspinal area. In an earlier study, we demonstrated that intracerebroventricular administration of D-serine potentiated antinociception in the tail-flick test [6]. This antinociceptive effect was attenuated by intracerebroventricular administration of a selective NMDA-glycine site antagonist, L-701,324. Several lines of evidence support these results. The injection of NMDA and glutamate into the periaqueductal gray (PAG) and nucleus raphe magnus, respectively, induced antinociception in the tail-flick test [7, 8]. This suggests that activation of NMDA receptors at the supraspinal level activates the descending pain modulation pathway in response to acute thermal stimulation.

The formalin test is used to measure acute pain induced by chemical or physical damage or tonic pain induced by inflammatory stimulation [9]. Whereas most other models, such as the tail-flick test, only allow the threshold for an escape response to be measured, the formalin test permits assessment of the way animals respond to moderate and continuous pain generated by injury to tissue [10]. Thus, it mimics some of the features of the type of pain that naturally occurs following damage to or injury of tissue. This,

then, provides a more accurate model of clinical pain than that achieved with the more typically used analgesic tests such as the tail-flick test [11]. As the type of pain and analgesic response mechanism involved is different to that associated with the tail-flick test [12], it is necessary to investigate the role of supraspinal NMDA receptors in elucidating antinociception against formalin-induced pain. However, the actions of the NMDA receptor at the supraspinal level in formalin-induced pain are diverse. For example, administration of the NMDA receptor antagonist dizocilpine (MK-801) or D-(−)-2-amino-5-phosphonopentanoic acid (AP5) into the cerebroventricular region or PAG induces an antinociceptive effect [13, 14]. For example, administration of the NMDA receptor antagonist dizocilpine (MK-801) and AP5 into the cerebroventricular region and PAG, respectively, induces an antinociceptive effect [13, 14]. On the other hand, administration of NMDA into the cerebroventricular region or PAG also induces an antinociceptive effect [15, 16]. The purpose of the present study was to investigate the effects of intracerebroventricular administration of D-serine, an endogenous co-agonist at NMDA receptors, by measuring formalin-induced acute and tonic nociception to further elucidate the role of supraspinal NMDA receptors in antinociception.

Materials and methods

Animals and drugs

The present animal experiments were performed in strict accordance with the guidelines of Tokai University, and were approved by the Animal Investigation Committee of this institution. Male Wistar rats (Clea Japan Inc., Tokyo, Japan) weighing 180–220 g each were used. The rats were group-housed in laboratory cages and kept in a temperature-controlled room (23 ± 2 °C) under a 12-h light/dark cycle (lights on 07:00) with food and water provided ad libitum. D-Serine (catalogue number S4250) and 7-chloro-4-hydroxy-3-(3-phenoxy) phenyl-2(H)-quinolinone (L-701,324) (catalog number L-0258) were purchased from Sigma (Tokyo, Japan). D-Serine was dissolved in saline. L-701,324, a selective antagonist at the glycine sites of NMDA receptors, was suspended in 0.4 % (w/v) carboxymethylcellulose (CMC) (Fluka Chemie, Tokyo, Japan) solution. All drugs were freshly prepared.

Intracerebroventricular administration

Drug infusion into the third ventricle in each rat was performed via indwelling cannulae, as described previously [6]. The rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and then mounted in a stereotaxic frame (Narishige, Tokyo, Japan). A stainless-steel guide cannula with an outer diameter (o.d.) of 0.55 mm was implanted 3–5 days prior to the day of the experiment. On the day of the experiment, an injection cannula with an o.d. of 0.33 mm was inserted into the third ventricle via the guide cannula (AP −0.8 mm, *V* +7.8 mm, *L* ±0.0 mm) according to the atlas of Paxinos and Watson [17]. Drugs were infused at a volume of 10 µl over 2 min and the infusion cannula was left in place for 2 min before removal. L-701,324 (68.5, or 137 nmol) or saline was administered intracerebroventricularly 10 min before administration of D-serine via the same route, respectively.

Formalin test

The investigators were blind to all drug treatments carried out in these experiments. The formalin test was performed 10 min after drug administration, as described previously [18]. Fifty microliters of 5 % formalin were injected subcutaneously into the dorsal surface of the right hind paw with a 27-gauge needle. Immediately after injection, the rat was placed in an open clear plastic chamber (30 × 30 × 30 cm). Its flinching was counted for 1 min every 5 min. Two phases are usually observed in the formalin test: phase 1, covering the first 6 min after injection; and phase 2, beginning after approximately 10 min, at which time flinching generally shows a decrease. The number of flinches was used to evaluate the antinociceptive effect of each drug.

The 50 % effective dose (ED_{50}) and associated 95 % confidence interval (CI) were generated from a standard nonlinear regression analysis of the log dose–response curve using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Behavioral test

Behavioral tests were performed in a neutral environment by three investigators in a blinded manner. Motor function was assessed with beam balance test and beam walk test [19]. The rat has to remain on an illuminated wooden beam 25 mm in diameter and 900 mm in length [20–22]. Each rat was first given 3 trials to balance and walk on an elevated narrow wooden beam of the same dimensions. The time that the rat remained on the beam was recorded for 30 s. The scoring criteria for beam balance test is based on a rating scale ranging from 0 to 5 [5, balances with a steady posture; 4, hugs the beam, but with one limb slipping off the beam; 3, hugs the beam, but with two limbs slipping off the beam, or spins on beam; 2, attempts to balance on the beam but falls off (>20 s); 1, attempts to balance on the beam but falls off (<20 s); 0, falls off: no attempt to

balance or hang on to the beam (<20 s)], modified briefly from that devised by Ulrich and colleagues [22]. The scoring criteria for beam walk test is based on a rating scale ranging from 0 to 5, where 0 indicates an inability to ambulate beyond the start point, 1–4 corresponds to distal segments of 100, 200, 400, or 800 mm, modified briefly from that devised by Monaco and colleagues [19]. In the righting reflex test, each rat was then left undisturbed on its back. If the rat remained to turn itself upright within 10 s, it was regarded as being sedated [18]. Catalepsy was determined using a box 280 mm in length, 70 mm in width, and 40 mm in height. In the catalepsy test, both the rat's forepaws were placed on the upper surface of the box. If the rat remained immovable for 15 s, it was regarded as being cataleptic. Agitation was judged to be present if the rat spontaneously vocalized or became restless. The presence of allodynia was determined by looking for agitation (escape or vocalization) in response to light stroking of the rat's flank with a pencil. Furthermore, the rats were tested for possible side effects as evidenced by a reduction in corneal or pinna reflexes, as previously described [18].

Data analysis

The results are expressed as the mean ± standard error of the mean (SEM). The statistical analysis was conducted using Prism 6 for a comparison across experimental conditions. When a significant difference among groups was obtained in the one- or two-way (drugs and time) repeated analysis of variance (ANOVA), Dunnett's or Bonferroni's post hoc test was used to define which group contributed to these differences. The level of statistical significance was set at $p < 0.05$. The power of the statistical comparison was assessed using the StatMate 2 program (GraphPad Software Inc.).

Results

Induction of antinociceptive effect by D-serine but not L-serine

Injection of formalin elicited a bi-phasic response with a total number of 49.6 ± 2.2 flinches of the injected paw during phase 1 and 161.5 ± 8.9 flinches during phase 2 of the test (Fig. 1). D-Serine (1 μmol), but not L-serine (1 μmol), significantly reduced the number of flinches by 14.5 ± 4.5 and 85.5 ± 14.1 in phase 1 and 2, respectively (Fig. 1). D-Serine dose-dependently reduced the number of flinches in both phase 1 and 2 in the formalin test. The ED_{50} values for phases 1 and 2 were 0.255 μmol (CI 0.1554–0.420 μmol) and 3.242 μmol (CI 0.589–17.85 μmol), respectively (Fig. 2).

D-Serine-induced side effects

Apart from catalepsy and problems in motor function, all rats exhibited normal behavior (including righting reflex, agitation, allodynia, and pinna and corneal reflexes) during the test. One of the 6 rats showed catalepsy after intracerebroventricular administration of 20 μmol D-serine. This catalepsy was reversed, however, within 90 min (Fig. 3). Two of the 6 rats failed to stay on the beam for 30 sec following administration of 20 μmol D-serine (Fig. 4). This side effect was reversed, however, within 90 min. Two of the 6 rats failed to walk 800 mm along the beam following administration of 20 μmol (Fig. 5). This side effect was reversed, however, within 60 min. These results demonstrated that the doses (1–10 μmol) of D-serine induced no behavioral side effects.

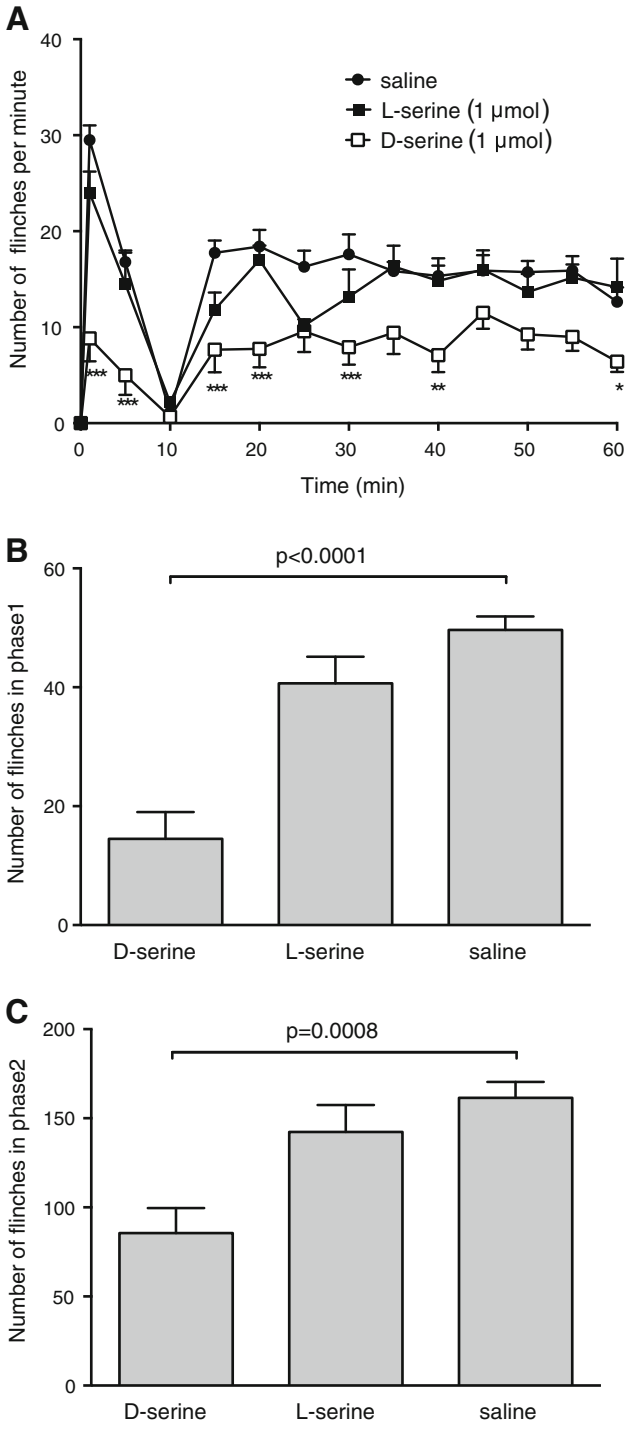
Attenuation of the D-serine-induced antinociceptive effect by L-701,324

L-701,324 significantly and dose-dependently attenuated the antinociceptive potency of D-serine in both phases in the formalin test compared with in the 0.4 % CMC-pretreated group at the same dose of D-serine (Fig. 6). L-701,324 (137 nmol) caused a significant increase in the number of flinches in phase 1 compared with in the 0.4 % CMC-pretreated group (Fig. 6). L-701,324 (137 nmol) caused an increase, but not significantly, in the number of flinches in phase 2 of the formalin test.

Discussion

The results of the present study demonstrated that intracerebroventricular administration of D-serine, but not L-serine, produced a dose-dependent antinociceptive effect on both acute and tonic formalin-induced pain. The effective doses (0.5–10 μmol) induced no behavioral side effects. The antinociceptive effect of D-serine, but not L-serine, may be related to stimulation of the glycine sites of NMDA receptors, as this stereoselectivity corresponds well with the potency of these stereoisomers as agonists at glycine sites. In fact, D-serine has a 100-fold greater potency in displacing [^{3}H] glycine binding to glycine sites compared with L-serine [1]. Further support for this comes from the fact that a selective antagonist at glycine sites on NMDA receptors, L-701,324, antagonized the antinociceptive effect induced by D-serine in a dose-dependent manner.

These observations suggest that activation of NMDA receptors at the supraspinal level may lead to potentiation of the antinociceptive effect in the formalin test. Taken together with the present results, several lines of evidence



◀ **Fig. 1** Effects of intracerebroventricular administration of D-serine on the formalin-induced flinching response. **a** Time course of the change in the number of flinches per min after formalin injection in rat administered with saline (10 μl, $n = 20$), D-serine (1 μmol in 10 μl saline, $n = 12$), or L-serine (1 μmol in 10 μl saline, $n = 6$). Significantly different from saline-treated control by Dunn's post hoc test following two-way repeated measures ANOVA; $^{**}p < 0.01$ and $^{***}p < 0.001$. **b**, **c** Number of flinches in phases 1 and 2 of the formalin test, respectively. Significantly different from values in the group receiving saline according to Dunnett's post hoc test following one-way ANOVA ($F = 31.92$, $p < 0.0001$, and $F = 11.87$, $p = 0.0008$, for phases 1 and 2, respectively)

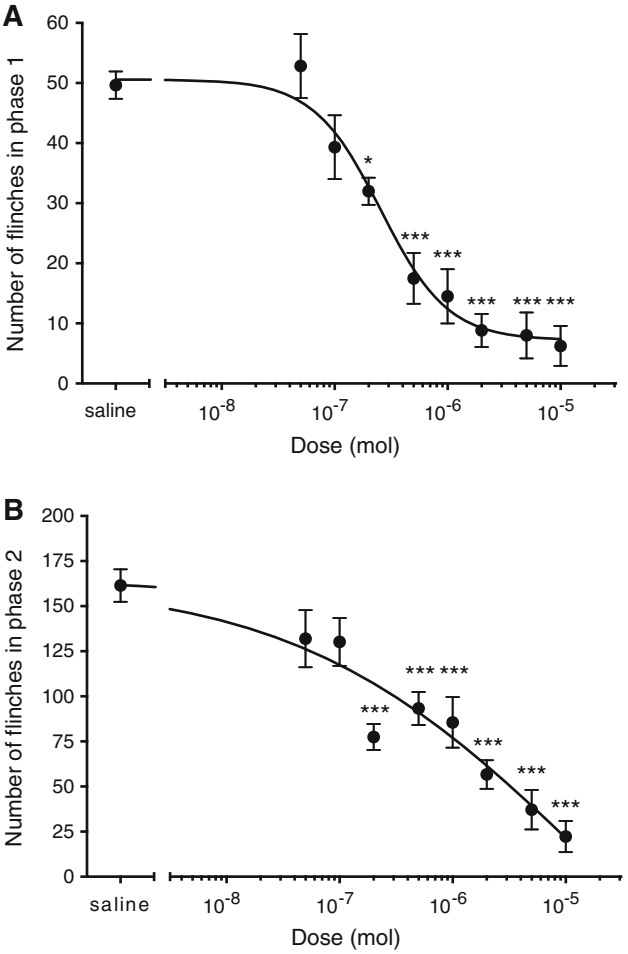


Fig. 2 Dose-dependent effect of intracerebroventricular administration of D-serine (0.05, 0.1, 0.2, 0.5, and 2 μmol, $n = 6$; 1 and 10 μmol, 12; 5 μmol, $n = 11$, saline, $n = 20$) on formalin-induced flinching response. **a**, **b** Number of flinches in phases 1 and 2 of the formalin test, respectively. Significantly different from values in the group receiving saline according to Dunnett's post hoc test following one-way ANOVA ($F = 26.52$, $p < 0.0001$, and $F = 20.79$, $p < 0.0001$, for phases 1 and 2, respectively) $^{***}p < 0.001$

also support this possibility: first, administration of NMDA into the cerebroventricular region or PAG induced an antinociceptive effect in the formalin test [15, 16]; second, in the present study, intracerebroventricular administration of L-701,324 alone increased paw flinch latencies in phases 1 and 2; third, again, in this study, intracerebroventricular administration of D-serine induced the antinociceptive effect of a selective antagonist for glycine sites on NMDA receptors in an L-701,324-reversible manner; and fourth, intracerebroventricular administration of D-serine induced the antinociceptive effect in the tail-flick test [6]. Interestingly, it has also been reported that intracerebroventricular administration of D-serine induced the antinociceptive

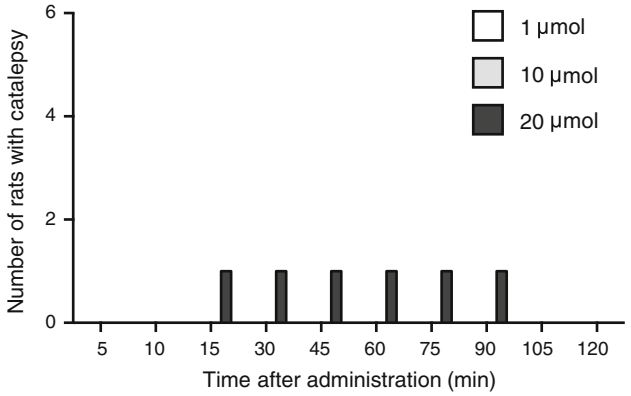


Fig. 3 The cataleptogenic effect of D-serine (1, 10, and 20 μmol). Results represent the number of rats that exhibited catalepsy from 6 rats in each group. Time 0 indicates the pre-injection values

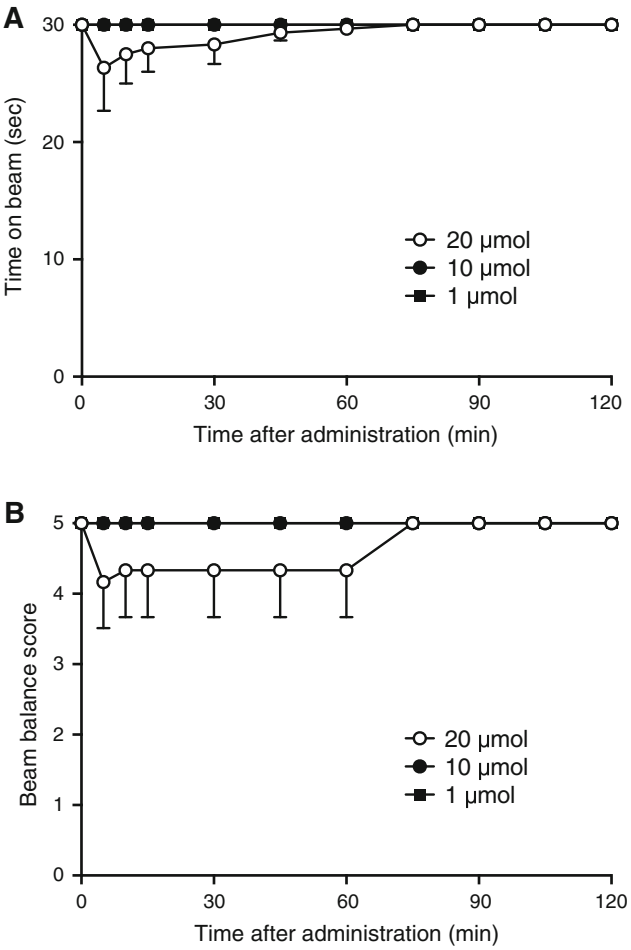


Fig. 4 Beam balance test. **a**, **b** Times that rats maintained their balance on an elevated narrow beam, and scores before and after administration of D-serine (1, 10, and 20 μmol), respectively. Results represent the mean with the SEM of data from 6 rats in each group. Time 0 indicates the pre-injection values

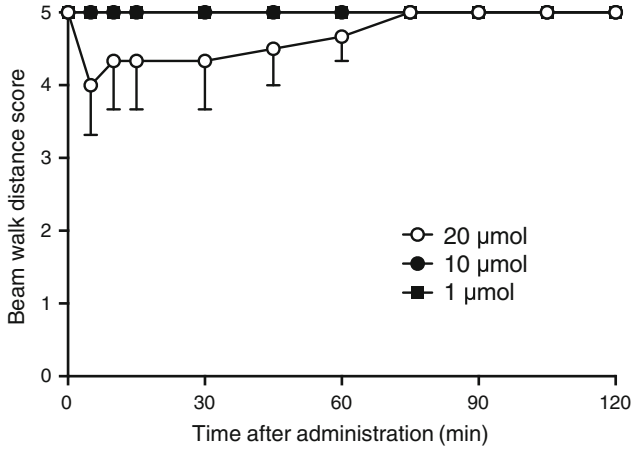


Fig. 5 Beam walk test. Scores for the mean distance traveled along an elevated narrow beam before and after administration of D-serine (1, 10, and 20 μmol). Results represent the mean with the SEM of data from 6 rats in each group. Time 0 indicates the pre-injection values

effect produced by morphine in both the formalin [23] and tail-flick test [6]. These studies indicate functional interactions between NMDA and μ-opioid receptors in the regulation of the antinociceptive effect at the supraspinal level. The mechanisms underlying these interactions between supraspinal NMDA and μ-opioid receptors remain to be determined. However, activation of supraspinal NMDA receptors may lead to opioid-induced activation of the descending inhibitory system and produce an antinociceptive effect, as off-cell activation is known to be necessary for the descending inhibitory effects of supraspinal administration of morphine; and because administration of AP5 into the rostral ventromedial medulla blocked the morphine-induced activation of off-cells, significantly reducing the antinociceptive effect of this drug [24, 25]. Alternatively, the antinociceptive effect of D-serine could be due to the release of endogenous opioid peptides at the supraspinal level, as administration of NMDA and L-glutamate into the hypothalamus or striatum leads to the release of methionine-enkephalin and β-endorphin, respectively, in an NMDA receptor-antagonist-reversible manner [7, 8, 26].

In previous studies, administration of AP5 or MK-801 (NMDA receptor antagonists) into the cerebroventricular region or PAG induced an antinociceptive effect in the formalin test [13, 14]. In previous studies, administration of AP5 and MK-801 into the cerebroventricular region and PAG, respectively, induced an antinociceptive effect in the formalin test [13, 14]. In the present study, however, intracerebroventricular administration of L-701,324 (137 nmol) significantly induced nociception in phase 1 of the formalin test. L-701,324 (137 nmol) caused an increase, but not significantly so, in the number of flinches in phase 2 of the formalin test. One reason for this inconsistency in the results may lie in differences in the recognition sites for these antagonists. Both competitive

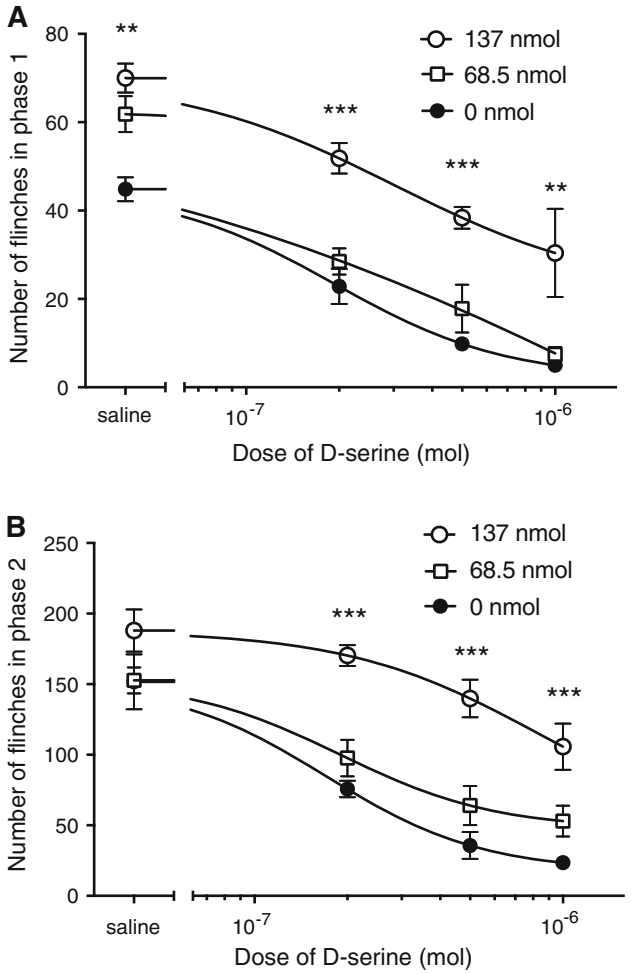


Fig. 6 Dose-dependent attenuation of D-serine-induced antinociceptive effect by L-701,324. **a**, **b** Effects of D-serine (0, 0.2, 0.5, or 1 μmol in 10 μl saline) on the number of flinches in rat pretreated with 10 μl of 0.4 % CMC (0 nmol) and L-701,324 (68.5 or 137 nmol) in phases 1 and 2 of the formalin test, respectively. Results represent the mean with the SEM of data from 6–8 rats in each group, and these results were significantly different from the values in the group receiving 0.4 % CMC (0 nmol) at the same dose of D-serine according to Bonferroni's post hoc test following two-way ANOVA. Bonferroni's test showed a significant difference between CMC (0 nmol) and L-701,324 (137 nmol) in phases 1 and 2. [Phase 1 $t = 3.980$, $p < 0.01$ (0 nmol D-serine), $t = 4.586$, $p < 0.001$ (0.2 nmol), $t = 4.825$, $p < 0.001$ (0.5 nmol), and $t = 4.173$, $p < 0.01$ (1.0 nmol); phase 2 $t = 1.988$, $p > 0.05$ (0 nmol D-serine), $t = 5.180$, $p < 0.001$ (0.2 nmol), $t = 6.096$, $p < 0.001$ (0.5 nmol), and $t = 4.669$, $p < 0.001$ (1.0 nmol)]. $^{**}p < 0.01$, and $^{***}p < 0.001$. Bonferroni's test showed no difference between CMC (0 nmol) and L-701,324 (68.5 nmol) in phases 1 and 2

antagonists at glutamate recognition sites, such as AP5, and noncompetitive antagonists at phencyclidine sites on NMDA receptors, such as MK-801, are reflected in neurotoxic, psychotomimetic, and other functional actions. Compared to these two types of antagonist, antagonists at the glycine sites of NMDA receptors, such as L-701,324, display a more benign side-effect profile in terms of a relative lack of neurotoxic action, including motor dyscoordination and psychotomimetic properties, while retaining neuroprotective and anticonvulsive properties [27]. The safety profile of antagonists at the glycine sites of NMDA receptors may be superior to those of both competitive and non-competitive antagonists [28]. In fact, a previous study using the tail-flick test demonstrated that intracerebroventricular administration of L-701,324 induced nociception [6].

In an earlier study using the tail-flick test, we showed that intracerebroventricular administration of D-serine elicited an antinociceptive effect on acute thermal nociception, and the ED_{50} (7.660 μmol) was higher than that on formalin-induced nociception in the present study (0.255 and 3.242 μmol in phases 1 and 2, respectively) [6]. These data suggest that different pain tests reveal different pain modulation systems or different characteristics of a single analgesic system. Indeed, the antinociceptive effect produced by stimulation of PAG requires significantly less current intensity in the formalin test than in the tail-flick test [29]. The results of the present study suggest that different neural systems are involved in the antinociceptive effect of D-serine in tests of thermal and formalin-induced pain. Further study is necessary, however, to determine the reason for these different potencies.

In conclusion, intracerebroventricular administration of D-serine elicited an antinociceptive effect on formalin-induced nociception in an L-701,324-reversible manner. Taken together with those of our earlier study [6], these findings suggest that activation of the glycine sites of NMDA receptors at the supraspinal level by the endogenous ligand D-serine induces an antinociceptive effect on both acute and tonic pain.

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