D-Serine-induced Inactivation of NMDA Receptors in Cultured Rat Hippocampal Neurons Expressing NR2A Subunits is Ca^{2+} -Dependent

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Keywords

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SUMMARY

Aims: Our previous studies indicate that glycine can inhibit N-methyl-D-aspartate receptor (NMDAR) responses induced by high concentrations of NMDA in rat hippocampal neurons. The present study was designed to observe whether D-serine induces inactivation of NMDARs in cultured rat hippocampal neurons and to investigate the underlying mechanisms of this effect. **Methods:** Cell culture, whole-cell patch-clamp electrophysiology, Ca^{2+} imaging, immunohistochemistry, and Western blot analysis were used. **Results:** We found that the peak current and Ca^{2+} influx evoked by 30 μM NMDA were increased by co-application of D-serine, but those evoked by 300 μM NMDA were reduced dose-dependently by co-application of D-serine. However, the inhibitory effect of D-serine on NMDAR responses was reversed by ZnCl_2 (30 nM), an inhibitor of the NR2A subunit, but was less influenced by ifenprodil (10 μM), an NR2B inhibitor. In addition, the inhibitory effect of D-serine was not detected in young hippocampal neurons that expressed less of the NR2A subunits and reversed in the presence of 10 mM BAPTA. **Conclusions:** D-Serine can also induce inactivation of NMDARs, the NR2A subunit is required for the induction of this effect, and this inactivation is Ca^{2+} -dependent in nature. This action of D-serine is hypothesized to play a neuroprotective role upon a sustained large glutamate insult to the brain.

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Introduction

When N-methyl-D-aspartate (NMDA) receptors are activated by L-glutamate and either co-agonist glycine or D-serine, three inactivation courses follow, glycine-dependent desensitization [1,2], glycine-independent desensitization [3,4], and Ca^{2+} -dependent inactivation [5–7]. Moreover, internalization of NMDA receptors (NMDARs) after a conditioning stimulus of NMDA with glycine, or glycine or D-serine alone at a high concentration could also reduce the current induced by subsequent stimuli of NMDA with glycine [8]. We recently identified a novel mechanism of NMDAR inactivation, defined as glycine-dependent inactivation; that is, glycine can dose-dependently reduce the peak current and Ca^{2+} influx evoked by NMDA at concentrations higher than 100 μM [9]. In addition, we found that this inactivation was associated with NR2A, the regulatory subunit of NMDARs [10], and that surface membrane receptor internalization was not involved [9], but the exact mechanisms are unclear. This kind of NMDAR inactivation provides new insight into our knowledge of the complexity of

the functional regulation of NMDARs and glutamatergic synaptic transmission.

D-Serine, another important endogenous co-agonist, is more potent than glycine at the glycine site of NMDARs in the central nervous system [11,12]. D-serine can be produced by both astrocytes and neurons [11–14]. The purpose of this study was to investigate whether D-serine also induced glycine-dependent inactivation of NMDARs in cultured rat hippocampal neurons and whether NMDAR subunit NR2A or NR2B and increase in the intracellular Ca^{2+} concentration were involved in the mechanisms underlying this inactivation.

Materials and Methods

Animals and Chemicals

Sprague-Dawley rats were obtained from the Experimental Animal Centre of Nantong University, Jiangsu, China. All protocols used in this study were in accordance with our institutional

guidelines, which comply with international rules and policies, and were approved by the Animal Care and Use Committee of Nantong University, Nantong, China.

Common inorganic salts were purchased in China; chemicals used for neuronal culture were products of GIBCO (Invitrogen Corporation, Carlsbad, CA, USA); Fluo3-AM was purchased from Dojindo Laboratories (Kumamoto, Japan); other chemicals, except for those indicated elsewhere, were purchased from Sigma-Aldrich Corporation (Saint Louis, MO, USA).

Cell Culture

The procedure for producing low-density rat hippocampal neuronal cultures was performed according to Kaech and Banker, as detailed previously [15]. Briefly, the hippocampi were isolated from the embryonic (E17–18 d) rats, cut into pieces, and digested with 0.125% trypsin at 37°C for 12 min. The digested brain tissue pieces were mildly triturated into a single-cell suspension, and the cells were plated at a density of 75,000 cells/ml onto coverslips (1 × 1 cm) coated with poly-L-lysine overnight at 4°C prior to the experiment. The day of plating was counted as day-in-vitro (DIV) 0. The feeding medium was changed every 3 days. Cultures were maintained in a 5% CO₂ incubator at 37°C. The cultured hippocampal neurons on DIV 3 or DIV 11–12 were used for following experiments.

Whole-Cell Electrophysiological Recording of NMDA-Evoked Currents

Current responses (I_{NMDA}) were evoked in cultured hippocampal neurons (DIV 11–12) superfused with bath solution containing NMDA. Normal bath solution was composed of the following (in mM): NaCl 150, KCl 3, HEPES 10, CaCl₂·2H₂O 3, and glucose 8 (pH 7.4, adjusted using 1 M NaOH, and osmolality 300–310 mOsm, adjusted using sucrose). NMDA was dissolved in bath solution immediately before use. I_{NMDA} was recorded via a glass microelectrode filled with the pipette solution containing the following (in mM): CH₃O₃SCs 135, NaCl 8, HEPES 10, EGTA 0.5, Mg-ATP 4, and Na-GTP 0.3 (pH 7.2, adjusted using 1 M CsOH, and osmolality 290 mOsm, adjusted using sucrose). The conventional whole-cell patch-clamp electrophysiological recording technique was used to record I_{NMDA} . A MultiClamp 700A amplifier, a Digidata 1320A AD converter, and pCLAMP 8.0 software (Axon Instruments, Foster City, USA) were used. When filled with pipette solution, the resistance of the microelectrode was 5–8 MΩ. The junction potential between the microelectrode pipette and the bath solution was −9.9 mV. This value was calculated using the junction potential calculation system of Clampex 8.0 in pCLAMP 8.0 and was nullified just before forming each giga-seal. In most experiments, the series resistance (R_s) before compensation was 10–20 MΩ. Routinely, 70–80% of the R_s was compensated. Only neurons with an R_s less than 20 MΩ were selected for further examination. All NMDA currents were recorded while the membrane potential was held at −70 mV.

To determine the influence of D-serine and other agents on I_{NMDA} , NMDA, D-serine, and other agents were all dissolved in bath solution and applied to each neuron using a perfusion

system (DAD-8VC; ALA Scientific Instrument Inc., New York, USA). This perfusion system applies a pressurized injection and allows for complete exchange of solutions in the vicinity of the neuron, with a dead volume of less than 1 μL and without cross-contamination or loss of mechanical stability. The tip (100 μm inner diameter) of the micromanifold was typically placed 50–100 μm away from the recorded cell. The cell recorded was continuously perfused with normal bath solution throughout all experimental procedures, except for the application of NMDA and other agents. NMDA was used for a pulse of 3 ± 1 s with a 2-min interval between two recordings. To obtain stable recordings of I_{NMDA} and following intracellular-free Ca²⁺ imaging, tetrodotoxin was routinely added in the bath solution with a final concentration of 0.5 μM. To inhibit the increase in the intracellular Ca²⁺ concentration, a Ca²⁺ chelator BAPTA was added in the pipette solution with a final concentration of 10 mM. I_{NMDA} was recorded at an ambient temperature of 23–25°C.

Intracellular Ca²⁺ Imaging

For intracellular-free Ca²⁺ imaging, Fluo-3/AM was used as the fluorescent Ca²⁺ indicator. Fluo-3/AM was dissolved in DMSO at a final working concentration of 0.1% DMSO. On DIV 3 or DIV 11–12, the cultured hippocampal neurons were loaded with 5 μM Fluo-3/AM for 45 min at 37°C in bath solution. After washing three times with fresh bath solution, the neurons were incubated at 37°C for another 30 min to completely de-esterify the Fluo-3/AM. Fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 525 nm was recorded every 5–10 s for 5 min using a laser scanning confocal microscope (TCS SP8, Leica Microsystems, Heidelberg, Germany). All image data were collected and analyzed using Leica control software of the microscope. The increase in intracellular-free Ca²⁺ ($[\text{Ca}^{2+}]_i$) was determined according to the following equation: $[\text{Ca}^{2+}]_i$ increase (%) = $(F_{525} - F_{\text{base}, 525}) / F_{\text{base}, 525} \times 100$, where F_{525} represents the fluorescence intensity measured after each treatment, and $F_{\text{base}, 525}$ represents the basal fluorescence intensity.

Immunohistochemistry

The cultured hippocampal neurons were fixed on DIV 12 with 4% paraformaldehyde (PFA) and permeabilized with 0.5% saponin before treating with 10% BSA for 1 h at room temperature. Cultures were then immunolabeled with one of the following antibodies overnight at 4°C: rabbit anti-NR2A (1:100 dilution; Abcam, Hongkong, China), mouse anti-NR1, and mouse anti-NR2B (1:100 dilution; gifts from Prof. Luo Jianhong, Zhejiang University). After washing to remove excess primary antibodies, the cultures were incubated for 1 h at room temperature in one of the following fluorescent-conjugated secondary antibodies: anti-rabbit IgG-Alexa 488 and anti-mouse IgG-Alexa 488 (Jackson ImmunoResearch, West Grove, PA, USA). Excess antibody was removed, and cells were imaged using a Leica confocal microscope (TCS SP8, Leica Microsystems). After acquisition, the images were processed and analyzed using LAS-AF-Lite software (Version 3.1, Heidelberg, Germany).

Western Blot Analysis

To detect the expression of NMDAR subunits at different stages, cultured hippocampal neurons (DIV 3 and DIV 12) were collected and homogenized in lysis buffer (containing 1 mM EGTA, 10 mM Mg^{2+} -ATP, and protease inhibitors; Beyotime, Haimen, China). Homogenized lysates were centrifuged twice at $20,000 \times g$ for 5 min at 4°C. Lysate samples were then denatured, resolved via SDS-PAGE, and subjected to Western blot analysis. Primary antibodies were as follows: rabbit anti-NR2A (1:500 dilution; Abcam), mouse anti- β -actin (1:5000; Sigma), mouse anti-NR1, and mouse anti-NR2B (1:100 dilution; gifts from Prof. Luo Jianhong, Zhejiang University). Secondary antibodies (conjugated to horseradish peroxidase; Jackson ImmunoResearch) were used at dilutions of 1:10,000. After washing steps, the horseradish peroxidase was detected using an Immun-StarTM WesternCTM Kit (Bio-Rad Laboratories, Hercules, CA, USA) and a ChemiDoc XRS imager (Bio-Rad Laboratories). Data were processed and analyzed using Image Lab software (Version 2.0.1, Bio-Rad Laboratories).

Statistical Analysis

All data are presented as the means $\pm$ SE. Data from multiple groups were analyzed using one-way ANOVA and the Newman-Keuls test for *post hoc* comparisons. Other data comparing two

groups were analyzed using Student's *t*-test. Differences were considered statistically significant at a level of $P < 0.05$.

Results

Dose-Dependent Influence of D-serine on NMDA-Elicited Currents

To investigate the influence of D-serine on I_{NMDA} , cultured hippocampal neurons (DIV 11–12) were voltage-clamped at -70 mV in a Mg^{2+} -free extracellular solution and superfused with 30 or 300 μ M NMDA containing 0.1–10 μ M D-serine. Peak current was increased by D-serine in a dose-dependent manner when the hippocampal neurons were exposed to 30 μ M NMDA (Figure 1A,C). However, when NMDA was applied at 300 μ M, D-serine reduced the peak current in a dose-dependent manner (Figure 1B,D). This result suggests that D-serine also induces the inactivation of NMDARs when NMDA is applied at 300 μ M.

Dose-Dependent Influence of D-Serine on NMDA-Elicited Ca^{2+} Influx

To verify the above results obtained using the whole-cell recording technique, we measured changes in intracellular Ca^{2+} in cultured hippocampal neurons (DIV 11–12) activated by 30 or

Figure 1 Dose-dependent effects of D-serine on NMDA-elicited peak currents. **(A)** Examples of the effect of D-serine on 30 μ M NMDA-elicited currents. Scale bar: 200 pA, 2 s, except that specified (+D-serine 10 μ M). **(B)** Examples of the effect of D-serine on 300 μ M NMDA-elicited currents. Scale bar: 500 pA, 2 s. **(C)** D-serine dose-dependently increased the peak currents (I_p) of I_{NMDA} elicited by 30 μ M NMDA ($n = 8$). **(D)** D-serine dose-dependently inhibited the 300 μ M NMDA-elicited I_p ($n = 8$). *, $P < 0.05$; **, $P < 0.01$ vs. control current elicited by 30 or 300 μ M NMDA without addition of D-serine. # $P < 0.05$ vs. treatment with 0.1 μ M D-serine.

300 μM NMDA in the presence of 0.1–10 μM D-serine via a free- Ca^{2+} imaging technique. Identical to the results of I_{NMDA} recording shown in Figure 1, D-serine increased Ca^{2+} influx in a dose-dependent manner when 30 μM NMDA was applied to the neurons (Figure 2A,B). However, Ca^{2+} influx was reduced dose-dependently by D-serine when the neurons were exposed to 300 μM NMDA (Figure 2C,D). Therefore, the Ca^{2+} imaging results also indicate that D-serine induces the inactivation of NMDARs when NMDA is applied at 300 μM .

Ifenprodil Does Not Influence the Synergistic or Inhibitory Effect of D-Serine on 30 or 300 μM NMDA-Elicited Responses

It is generally understood that the composition of the NMDAR subunits will influence the function of the receptor. Therefore, we determined whether inhibition of NR2A or NR2B subunit would interfere with the effects of D-serine on NMDAR responses in cultured rat hippocampal neurons (DIV 11–12).

In the presence of ifenprodil (10 μM), an inhibitor of the NR2B subunit [16], NMDA-elicited Ca^{2+} influx was reduced. However, D-serine still dose-dependently increased Ca^{2+} influx induced by 30 μM NMDA (Figure 3A,B). When 300 μM NMDA was applied to the neurons, ifenprodil also reduced Ca^{2+} influx, but ifenprodil did not influence the inhibitory effect of D-serine on 300 μM NMDA-elicited responses; that is, D-serine still reduced Ca^{2+} influx in a dose-dependent manner (Figure 3C,D).

Zinc Does Not Influence the Synergistic Effect of D-Serine on 30 μM NMDA-Elicited Responses but Reverses the Inhibitory Effect of D-Serine on 300 μM NMDA-Elicited Responses

In cultured hippocampal neurons (DIV 11–12), Ca^{2+} influx elicited by 30 μM NMDA was reduced in the presence of ZnCl_2 at 30 nM (Figure 4A,B), a concentration of ZnCl_2 that selectively antagonizes the NR2A subunit [16]. However, treatment with ZnCl_2 did not change the dose-dependent effect of D-serine to increase

Figure 2 Dose-dependent effects of D-serine on NMDA-elicited Ca^{2+} influxes. **(A)** Examples of the effect of D-serine on Ca^{2+} influx elicited by 30 μM NMDA. **(B)** Mean values of the initial increase in Ca^{2+} influx elicited by 30 μM NMDA ($n = 20$). **(C)** Examples of the effect of D-serine on Ca^{2+} influx elicited by 300 μM NMDA. **(D)** Mean values of the initial increase in Ca^{2+} influx elicited by 300 μM NMDA ($n = 20$). ** $P < 0.01$ vs. NMDA without addition of D-serine. ## $P < 0.01$ vs. treatment with 0.1 μM D-serine.

Figure 3 Effect of D-serine on NMDA-elicited Ca^{2+} influxes in the presence of 10 μ M ifenprodil. **(A)** Examples of the effect of D-serine on 30 μ M NMDA-elicited Ca^{2+} influx. **(B)** Mean values of the initial increase in 30 μ M NMDA-elicited Ca^{2+} influx ($n = 20$). **(C)** Examples of the effect of D-serine on 300 μ M NMDA-elicited Ca^{2+} influx. **(D)** Mean values of the initial increase in 300 μ M NMDA-elicited Ca^{2+} influx ($n = 20$). * $P < 0.05$; ** $P < 0.01$ vs. NMDA without addition of D-serine or ifenprodil; # $P < 0.05$; ## $P < 0.01$ vs. NMDA with ifenprodil without addition of D-serine.

30 μ M NMDA-elicited Ca^{2+} influx (Figure 4A,B). When the neurons were exposed to 300 μ M NMDA, Ca^{2+} influx was also reduced in the presence of 30 nM $ZnCl_2$ (Figure 3C,D). However, D-serine did not reduce, but rather dose-dependently increased Ca^{2+} influx elicited by 300 μ M NMDA (Figure 4C,D).

Summarizing the above Ca^{2+} imaging results in Figure 5, we could more clearly determine the influence of NMDAR subunit inhibitors on the effect of D-serine to alter 30 and 300 μ M NMDA-elicited responses in cultured hippocampal neurons (DIV 11–12). These results suggest that addition of $ZnCl_2$ could reverse the dose-dependent inhibitory effect of D-serine on 300 μ M NMDA-elicited Ca^{2+} influx; that is, the NR2A subunit is likely involved in the regulation of glycine-dependent inactivation of NMDARs induced by D-serine when cultured rat hippocampal neurons are exposed to 300 μ M NMDA. However, the NR2B subunit is not directly involved in the inactivation induced by D-serine.

To further verify the involvement of NR2A subunits in regulation of glycine-dependent NMDAR inactivation induced by

D-serine, we utilized cultured rat hippocampal neurons at DIV 3, when NR2A subunits were less strongly expressed due to developmental differences in the composition of the NMDAR subunits. We compared the responses of these neurons to 300 μ M NMDA in the presence of D-serine at different concentrations to the 300 μ M NMDA-elicited responses of neurons at DIV 12.

NR2B subunits were notably expressed in cultured hippocampal neurons at DIV 3, but NR2A subunits were less strongly expressed (Figure 6). However, both NR2A and NR2B subunits were expressed in cultured hippocampal neurons at DIV 12 (Figure 6). In contrast to neurons at DIV 12, D-serine did not dose-dependently reduce Ca^{2+} influx in neurons at DIV 3 when they were exposed to 300 μ M NMDA, but D-serine dose-dependently increased Ca^{2+} influx (Figure 7). Therefore, NR2A subunits are suggested to be potentially responsible for glycine-dependent NMDAR inactivation induced by D-serine.

As shown in Figure 7, we found that 300 μ M NMDA elicited Ca^{2+} influx in neurons at DIV 12 greater than that in neurons at

Figure 4 Effect of D-serine on NMDA-elicited Ca^{2+} influxes in the presence of 30 nM ZnCl_2 . (A) Examples of the effect of D-serine on 30 μ M NMDA-elicited Ca^{2+} influx. (B) Mean values of the initial increase in 30 μ M NMDA-elicited Ca^{2+} influx (n = 20). (C) Examples of the effect of D-serine on 300 μ M NMDA-elicited Ca^{2+} influx. (D) Mean values of the initial increase in 300 μ M NMDA-elicited Ca^{2+} influx (n = 20). ** P < 0.01 vs. NMDA without addition of D-serine or Zn^{2+} . ## P < 0.01 vs. NMDA with Zn^{2+} without addition of D-serine.

Figure 5 Ca^{2+} responses for increasing concentrations of D-serine in the presence of NMDA and NMDA subunit selective antagonists (NR2A inhibitor, ZnCl_2 , 30 nM and NR2B inhibitor, ifenprodil, 10 μ M). (A) NMDA was applied at 30 μ M (n = 20). (B) NMDA was applied at 300 μ M (n = 20).

DIV 3 when D-serine was not added. Does this great increase in the intracellular Ca^{2+} concentration in neurons at DIV 12 induce a Ca^{2+} -dependent inactivation of NMDARs that might be responsible for the glycine-dependent inactivation of NMDARs induced by D-serine? To clarify this issue, we used a Ca^{2+} chelator

BAPTA to inhibit the increase in the intracellular Ca^{2+} concentration of neurons at DIV 12. As a result, in the presence of BAPTA, D-serine did not dose-dependently reduce, but dose-dependently increased the current responses elicited by 300 μ M NMDA (Figure 8).

Figure 6 Expression of NMDAR subunits in cultured hippocampal neurons at DIV 3 and DIV 12. **(A)** Immunofluorescence staining examples of the expression of NR1, NR2A, and NR2B; scale bar 20 μ m. **(B)** Examples of the expression of NR1, NR2A, and NR2B measured via Western blot analysis. **(C)** Mean values of the expression of NR1, NR2A, and NR2B quantified via Western blot analysis (n = 3). ***P* < 0.01 vs. DIV 3.

Discussion

The present study revealed that D-serine dose-dependently increased the peak current and Ca^{2+} influx in cultured rat hippocampal neurons (DIV 11–12) when exposed to 30 μ M NMDA but reduced the peak current and Ca^{2+} influx when exposed to 300 μ M NMDA. These results suggest that D-serine also induces the inactivation of NMDARs when NMDA is applied at a concentration of 300 μ M. This effect of D-serine is consistent with that of glycine, as we reported previously [9,10].

To investigate whether the inactivation of NMDARs induced by D-serine was associated with particular regulatory subunits of NMDARs, we examined the influences of NR2A and NR2B subunit inhibitors on cultured rat hippocampal neurons (DIV 11–12). We found that inhibiting the NR2B subunit using ifenprodil (10 μ M) did not interfere with the synergistic or inhibitory effects of D-serine on the 30 or 300 μ M NMDA-elicited responses, respectively. On the other hand, inhibiting the NR2A subunit using ZnCl_2 (30 nM) did not alter the synergistic effect of D-serine on the 30 μ M NMDA-elicited response, but it reversed the dose-dependent effect of D-serine on the 300 μ M NMDA-elicited response from an inhibitory effect to a synergistic effect, suggesting that the NR2A subunit is likely involved in the regulation of D-serine-induced inactivation of NMDARs when neurons are exposed to 300 μ M NMDA. However, the NR2B

subunit is apparently not involved in this inactivation induced by D-serine.

In addition, we did not detect an inhibitory effect of D-serine on the 300 μ M NMDA-elicited response in cultured rat hippocampal neurons at DIV 3, when the NR2B subunits were principally expressed but the NR2A subunits were less strongly expressed, suggesting that the NR2A subunits are required for the induction of glycine-dependent inactivation by D-serine. Furthermore, we found that 300 μ M NMDA elicited Ca^{2+} influx in neurons at DIV 12 greater than that in neurons at DIV 3. When BAPTA was added in the pipette solution to inhibit the increase in the intracellular Ca^{2+} concentration of neurons at DIV 12, D-serine did not dose-dependently reduce, but dose-dependently increased the current responses elicited by 300 μ M NMDA. These results suggest that D-serine- and glycine-induced inactivation of NMDARs found in the present study and reported previously by us [9,10] is Ca^{2+} -dependent, that is, greater increase in the intracellular Ca^{2+} concentration in the presence of increasing doses of D-serine or glycine in neurons that express NR2A subunits can induce a Ca^{2+} -dependent inactivation of NMDARs, being consistent with previous studies [5–7].

Why does at 30 μ M NMDA D-serine only display a potentiation effect and at 300 μ M NMDA D-serine exert different effects on NMDARs with different subunit compositions? Comparing result in Figure 7B with that in Figure 7C, when the neurons were

Figure 7 Dose-dependent effect of D-serine on 300 μM NMDA-elicited Ca^{2+} influx in cultured hippocampal neurons at DIV 3 and DIV 12. **(A)** Examples of Ca^{2+} imaging in neurons at DIV 3 and DIV 12, respectively. **(B)** and **(C)** Mean values of Ca^{2+} influx in neurons at DIV 3 and DIV 12, respectively ($n = 12$).

exposed to 300 μM NMDA without addition of D-serine, we found that more Ca^{2+} entered the cell across the membrane in the cell expressing more NR2A subunits. Moreover, according to the data in Figure 8, we consider that more Ca^{2+} influx is potentially responsible for D-serine-induced dose-dependent inhibition on NMDAR responses because use of 10 mM BAPTA reversed this effect. Therefore, difference in the Ca^{2+} influx induced by 300 μM NMDA in neurons with different subunit compositions of NMDARs could account for the difference in D-serine effects on NMDAR responses to 300 μM NMDA.

Among L-glutamate-activated ion channels, NMDARs have received special attention because of their distinct role in the regulation of synaptic plasticity [17–19] and because of their critical roles in neurological and psychiatric disorders [20,21]. Functional modulation of NMDARs in the central nervous system is complex [22–24]. Three inactivation courses have been found when NMDARs are activated [1–7]. Among these inactivation processes of NMDARs, Ca^{2+} -dependent inactivation is a reversible decrease in peak current that can be induced by a rise in extracellular Ca^{2+} concentration [5–7]. Second, desensitization is a decrease in the current response induced in the persistent presence of a glutamate

site agonist [1–4]. Glycine- and D-serine-induced inactivation of NMDARs, which was discovered in our previous studies [9,10] and further confirmed in the present study, is a Ca^{2+} -dependent inactivation of NMDARs in nature. Desensitization and inactivation of NMDARs are thought to shape neuronal responses upon repeated stimulation [25,26] and to be neuroprotective during a sustained glutamate insult by limiting Ca^{2+} influx [9,27,28].

It is generally understood that functionally and pharmacologically distinct receptor subtypes can be generated from different combinations of the NR1 and NR2 subunit families [29]. Many studies have indicated that there are subunit-specific differences in the desensitization properties of NMDARs [5,30]. Both NR2A and NR2B subunits are highly expressed in the mature forebrain, where NMDARs containing NR1/NR2B predominate at nonsynaptic sites in the neuronal plasma membrane, while NR2A-containing NMDARs are enriched at the synapses [31–34]. Therefore, glycine- or D-serine-induced NMDAR inactivation we reported may exert some influence on synaptic plasticity and may also help to limit the hyperactivity of these synapses. However, results reported by Thomas *et al.* [35] indicate that NR2A- and NR2B-containing receptors can be located at either the synaptic or the

Figure 8 Dose-dependent effect of D-serine on 300 μM NMDA-elicited current in the presence of Ca^{2+} chelator BAPTA in cultured hippocampal neurons at DIV 12. **(A)** Examples of NMDA current. **(B)** Mean values of NMDA current ($n = 8$). $**P < 0.01$ vs. NMDA with addition of 0.1 μM D-serine.

extrasynaptic compartments. Synaptic and extrasynaptic NMDARs are all involved in the excitotoxicity induced by application of exogenous NMDA or L-glutamate [28]. Thus, glycine- or D-serine-induced NMDAR inactivation is potentially neuroprotective by inhibiting hyperactivation of hippocampal neurons.

Zhou et al. have reported that developmental neurons predominantly expressing NR1/NR2B NMDARs [36] were more vulnerable to NMDA excitotoxicity than the adult hippocampal

neurons predominantly expressing NR1/NR2A NMDARs [37]. Some other reports suggest that the CA1 area of the hippocampal formation is more vulnerable to excitotoxic or ischemic insults than the CA3 area and the dentate gyrus [38–41]. This is likely related to differences in the distribution of NR2 subunits in the hippocampal formation; that is, the expression of NR2B subunits is higher in the CA1 area than in the CA3 area and the dentate gyrus, but the expression of NR2A subunits in the CA1 area is lower than in the CA3 area and the dentate gyrus [38,42–44]. Glycine- or D-serine-induced inactivation of NMDARs in hippocampal neurons that express more NR2A subunits may contribute to their higher resistance to such detrimental insults.

In conclusion, the present results suggest that D-serine can also induce glycine-dependent inactivation when NMDARs are activated by NMDA at 300 μM, the NR2A subunit is potentially required for the induction of this effect because the effect of D-serine was not detected in hippocampal neurons when the NR2A subunit was less strongly expressed and because inhibiting the NR2A subunit reversed the effect of D-serine, and this inactivation is Ca^{2+} -dependent in nature. This action of D-serine is hypothesized to play a neuroprotective role upon sustained high glutamate insults in the central nervous system.

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Conflict of Interest

The authors declare no conflict of interest.

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