Contribution of endogenous glycine and D-serine to excitotoxic and ischemic cell death in rat cerebrocortical slice cultures

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Abstract

N-methyl-D-aspartate (NMDA) receptors, whose activation requires glycine site stimulation, play crucial roles in various physiological and pathological conditions in the brain.   We investigated the regulatory roles of potential endogenous glycine site agonists, glycine and D-serine, in excitotoxic and ischemic cell death in the cerebral cortex. Cytotoxicity of NMDA on rat cerebrocortical slice cultures was potentiated by addition of glycine or D-serine.   In contrast, cell death induced by oxygen/glucose deprivation (OGD) was not affected by exogenous glycine or D-serine, although blockade of NMDA receptors by MK-801 abolished cell death.   In addition, higher concentrations of 2,7-dichlorokynurenic acid (DCKA), a competitive glycine site antagonist, were required to suppress OGD-induced cell death than those to suppress NMDA cytotoxicity.   We also found that OGD triggered a robust increase in extracellular glycine.   A glycine transporter blocker ALX 5407 increased the extracellular level of glycine, and the protective effect of DCKA against NMDA cytotoxicity was diminished in the presence of ALX 5407.   Sensitivity of NMDA cytotoxicity to DCKA was also diminished by L-serine that increased the extracellular level of D-serine.   These results indicate that both glycine and D-serine can act as endogenous ligands for NMDA receptor glycine site in the cerebral cortex, and that endogenous glycine may saturate the glycine site.under ischemic conditions.   The present findings are important for the interpretation of the mechanisms of NMDA and OGD cytotoxicity.

Keywords: Cortical neurons; Glutamate; Excitotoxicity; Ischemia

Introduction

Dysregulation of glutamate-mediated excitatory neurotransmission plays a pivotal role in various disorders of the central nervous system.   Particularly, over-activation of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors is implicated in neurodegenerative conditions associated with acute pathological events such as ischemic and traumatic brain injury, and also with chronic neurological disorders such as Huntington disease (Hardingham and Bading, 2003).   In the case of ischemic stroke, massive release of glutamate from neurons and glia results in aberrant stimulation of neuronal NMDA receptors, which constitute a major pathway of Ca^{2+} influx triggering irreversible neuronal injury (Arundine and Tymianski, 2003).   Effectiveness of NMDA receptor blockade against injury induced by focal ischemia underscores the importance of NMDA receptor over-activation (Lipton, 1999), although other Ca^{2+} influx pathways may prevail under severe ischemic conditions (Aarts et al., 2003; Xiong et al., 2004).

Agonists acting on the glycine-binding site are required to activate NMDA receptors, together with glutamate-binding site agonists such as glutamate and NMDA.   Indeed, glycine site antagonists suppress NMDA receptor-mediated neuronal injury in vitro (Patel et al., 1990) and in vivo (Foster et al., 1990).   As the name of the binding site stands for, glycine was originally identified as an endogenous agonist at the glycine site of NMDA receptors (Johnson and Ascher, 1987).   Addition of glycine to dissociated primary neuronal cultures exacerbates NMDA cytotoxicity (McNamara and Dingledine, 1990; Patel et al., 1990).   Conversely, blockade of glycine transporters potentiates NMDA receptor-mediated synaptic responses in cortical neurons (Chen et al., 2003; Eulenburg et al., 2005).

More recently, the role of D-serine has received much attention (Hashimoto et al., 1992a; Martineau et al., 2006).   D-Serine, particularly concentrated in the telencephalon (Hashimoto et al., 1995; Schell et al., 1997), can act as a glycine site agonist showing a higher affinity

than glycine (Matsui et al., 1995).   Endogenous D-serine has been shown to regulate NMDA receptor activity in hippocampal neurons in mixed cell culture and in the cerebellum of immature rats (Mothet et al., 2000), and also in the retina (Stevens et al., 2003).   Endogenous D-serine is also likely to play an important role in neuropathological conditions.   Shleper et al. (2005) have proposed that D-serine is dominant as an endogenous glycine site agonist for induction of NMDA cytotoxiciy in hippocampal slice cultures.   Using acute cortical slice preparation, we have previously shown that endogenous D-serine participates in acute NMDA cytotoxicity and neuronal injury induced by simulated ischemia (Katsuki et al., 2004). However, relative contribution of endogenous glycine and D-serine to neuronal damage in the cerebral cortex is still unclear.

To gain further insight into the roles of endogenous glycine site agonists, we performed pharmacological examinations on rat cerebrocortical slice cultures.   Brain slice culture is a suitable preparation for investigations of the actions of intercellular diffusible molecules, because tissue architecture and spatial relationship of cells in vivo are maintained nearly intact.   We evaluated whether alterations of the extracellular levels of glycine and/or D-serine could have any impact on cellular damage induced by excitotoxic and ischemic insults.

Materials and methods

Materials

NMDA and amino acids were obtained from Nacalai Tesque (Kyoto, Japan). 2,7-Dichlorokynurenic acid (DCKA) and *N*-[(*3R*)-([1,1'-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine (ALX 5407) were obtained from Tocris Cookson (Bristol, UK).   MK-801 was purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

Cortical slice cultures

Organotypic slice cultures were prepared according to the procedures described previously (Fujimoto et al., 2004; Shirakawa et al., 2006). All experimental procedures were approved by our institutional animal experimentation committee, and animals were treated in accordance with the guidelines of the U.S. National Institutes of Health regarding the care and use of animals for experimental procedures. Briefly, coronal brain slices of 300 μm thickness of each hemisphere were obtained from 2 or 3 day-old Wistar rats, and six cerebrocortical slices (or twelve slices, for determination of ischemia-induced amino acid release) were transferred onto a Millicell-CM insert membrane (30 mm in diameter; Millipore, Bedford, MA, USA) in six-well plates. Culture medium, consisting of 50% minimal essential medium/HEPES (GIBCO, Invitrogen Japan, Tokyo, Japan), 25% Hanks' balanced salt solution (GIBCO) and 25% heat-inactivated horse serum (GIBCO) supplemented with 6.5 mg/ml glucose and 2 mM glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate (GIBCO), was supplied at 0.7 ml/well. Culture medium was replaced with fresh medium on the next day of culture preparation, and thereafter, every two days. Slices were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 34 °C.

Drug treatment and simulated ischemia

From 9 or 10 days *in vitro* (DIV), cultures were maintained for 24 h in 0.7 ml of serum-free medium consisting of 75% minimal essential medium/HEPES and 25% Hanks' balanced salt solution supplemented with 6.5 mg/ml glucose, 2 mM glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. Then, slices were treated with NMDA (10 or 15 μM) for 24 h in 0.7 ml of serum-free medium containing 5 μg/ml propidium iodide (PI). DCKA (1-10 μM) and amino acids (100-1000 μM) were concomitantly applied

with NMDA for 24 h.   ALX 5407 (30 μM) was applied 24 h prior to NMDA, and was also present during NMDA treatment.

For simulated ischemia, we performed oxygen/glucose deprivation (OGD; Fujimoto et al., 2004).   From 9 or 10 DIV, cultures were maintained for 24 h in 0.7 ml of serum-free medium. At 10 or 11 DIV, slices were pre-incubated for 1 h in 0.7 ml of oxygenated Ringer's buffer composed of 124 mM NaCl, 4.9 mM KCl, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$, 1.3 mM $MgSO_4$ and 10 mM D-glucose.   Then slices were subjected to ischemic treatment for 20, 30 or 60 min by submersion into 1.4 ml of deoxygenated glucose-free Ringer's buffer in a humidified atmosphere of 5% CO_2 and 1% O_2.   After OGD treatment, slices were returned to wells containing 0.7 ml of oxygenated serum-free medium containing 5 μg/ml PI and maintained for 24 h.   MK-801 (10 μM), glycine (100-1000 μM), D-serine (100-1000 μM) and DCKA (10-100 μM) were applied during OGD treatment.

For evaluation of the effects of ALX 5407 (30 μM) and transporter substrates (1 mM) on the extracellular levels and tissue contents of amino acids, slices were pre-incubated for 1 h with oxygenated Ringer's buffer and then treated with drugs in Ringer's buffer for 24 h.

Cell death assay

The level of PI uptake into slices was used as a measure of cell death (Fujimoto et al., 2004).   Slices were observed with an inverted fluorescence microscope with a rhodamine filter set.   Fluorescence images were captured through a monochrome chilled CCD camera (C5985, Hamamatsu Photonics, Hamamatsu, Japan), and stored images were analyzed with NIH image 1.62 software.   The average signal intensity in an area of 180×180 μm^2 within the parietal cortex was obtained as the fluorescence value of each slice.   Slice cultures treated with 100 μM NMDA for 24 h were used to determine the degree of the standard injury in each set of experiments.   Fluorescence values were normalized with the intensity of

fluorescence in cultures that received standard injury as 100%.

As another parameter to evaluate the extent of cell death, lactate dehydrogenase (LDH) activity in culture medium was measured with Cytotoxicity Detection LDH kit (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) as described (Shirakawa et al., 2006).

Quantification of amino acids

Extracellular levels and tissue contents of amino acids were determined by the procedures described previously (Hashimoto et al., 1992b).   Tissue-conditioned Ringer's buffer after addition of 5% trichloroacetic acid, or tissues homogenized in 10% trichloroacetic acid, underwent extraction with diethyl ether, and the aqueous layer was harvested as samples. Amino acids in the samples were derivatized with o-phthaldialdehyde and Boc-L-cysteine, and aliquots were analyzed by the HPLC system (Shimadzu, Kyoto, Japan) with a Nova-Pak C_{18} column (Waters, Tokyo, Japan).

Statistics

Data are expressed as means ± S.E.M.   Statistical significance of difference was evaluated with one-way analysis of variance followed by Student-Newman-Keuls test. Probability values less than 5% were considered significant.

Results

Glycine site agonists exacerbate NMDA cytotoxicity

As reported previously (Shirakawa et al., 2006), application of NMDA for 24 h caused cell death in cerebrocortical slice cultures as reflected by an increase in PI fluorescence. NMDA cytotoxicity showed a steep concentration-response relationship: 10 μM NMDA only

modestly increased PI fluorescence, whereas 15 μM NMDA caused a robust increase in the fluorescence.   Similar results were obtained when the extent of injury was evaluated by the amount of LDH released into culture medium (data not shown).

When glycine and D-serine at concentrations of 100 - 1000 μM were applied to slice cultures concomitantly with 10 μM NMDA, these glycine site agonists significantly enhanced NMDA cytotoxicity (Fig. 1A, B).   Although glycine and D-serine are shown to saturate the glycine site of NMDA receptors at concentrations in low micromolar range (Matsui et al., 1995), we used extremely high concentrations of glycine and D-serine to ensure the saturation of the glycine site, because these compounds might be readily wiped out from the extracellular space by cellular uptake systems.   The effect of D-serine was more prominent than that of glycine.   Exogenous glycine at tested concentrations did not affect cell death induced by 15 μM NMDA, whereas D-serine (300-1000 μM) was again effective in enhancing the cytotoxic effect of NMDA at this concentration (Fig. 1C, D).   These results suggest that the glycine site of NMDA receptors in the cerebral cortex is not normally saturated by endogenous agonists.

Glycine site agonists do not affect ischemic cell death

Slice cultures that received a brief period of OGD treatment exhibited cell death after 24 h of post-incubation in normal medium, as described (Fujimoto et al., 2004).   The extent of cell death was dependent on the duration of OGD treatment.   Injury induced by 20 min OGD was modest, whereas 30 min OGD induced substantial cell death as shown by an increase in PI fluorescence.   LDH assay gave similar results (data not shown).

A striking difference was observed between OGD-induced cell death and NMDA cytotoxicity with respect to the effects of glycine site agonists.   That is, neither glycine nor D-serine showed any significant effects on cell death induced by OGD for 20 and 30 min (Fig.

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2A-D).　MK-801, a non-competitive NMDA receptor antagonist, almost completely inhibited the induction of cell death by 30 min OGD, indicating that NMDA receptors indeed play a critical role in cell death under these conditions.　These results imply that endogenous supplies of glycine agonists reach a saturated level during OGD treatment.

Ischemic cell death is less sensitive to glycine site blockade than NMDA cytotoxicity

To add support for the idea that the extracellular levels of endogenous glycine site agonists are elevated during OGD conditions, we examined the effect of DCKA.　Because DCKA is a competitive antagonist at the glycine site of NMDA receptors (Baron et al., 1990), the potency of the effect of this drug on NMDA receptor-mediated events should be influenced by the levels of competing glycine site agonists.　As shown in Fig. 3, both NMDA cytotoxicity and OGD-induced cell death were inhibited by DCKA in a concentration-dependent manner.　Consistent with our assumption, the range of effective concentrations of DCKA was different between these two cytotoxic conditions.　Cytotoxicity of 15 μM NMDA was significantly attenuated by DCKA at a concentration as low as 1 μM, and was completely abolished by 10 μM DCKA (Fig. 3A and C).　In contrast, OGD-induced cell death was not affected by 10 μM DCKA, and was significantly inhibited by DCKA at concentrations of 30 μM or higher (Fig. 3B and D).

Extracellular levels of glycine and D-serine are elevated during ischemia

Next we directly assessed whether OGD was accompanied by increased levels of extracellular glycine site agonists.　We analyzed the levels of several amino acids released during exposure of slices to deoxygenated glucose-free Ringer's buffer, with an established HPLC method (Hashimoto et al., 1992b).　Consistent with the crucial role of NMDA glutamate receptors in OGD-induced cell death, the extracellular concentration of glutamate

was markedly increased by OGD treatment for 30 min (Table 1).   Notably, a robust increase of extracellular glycine was also observed in response to 30 min OGD.   Prolongation of the OGD period to 60 min resulted in a further increase in extracellular glycine.   In addition, the extracellular concentration of D-serine increased significantly in response to 60 min OGD, but the absolute change in D-serine concentration was much smaller than that in glycine concentration.   We also observed that other small neutral amino acids, including L-alanine, L-serine and L-threonine, were released at significant amounts during 60 min of OGD treatment (Table 1).

Manipulations that increase extracellular glycine or D-serine affect NMDA cytotoxicity

Because the extracellular levels of endogenous glycine site agonists were considered to be insufficient to saturate the glycine site of NMDA receptors during NMDA treatment (Fig. 1), experimental manipulations that affect the release and/or uptake of these agonists may influence the cytotoxic consequences of NMDA.   To address this issue, we carried out two sets of experiments.

First, we examined the effect of blockade of glycine transporters.   Of two known glycine transporters, GLYT1 is widely distributed in the central nervous system and is involved in glial uptake of glycine (Cubelos et al., 2005; Eulenburg et al., 2005).   ALX 5407, a potent and selective GLYT1 blocker, showed no significant effect on the cytotoxicity of NMDA applied at 15 µM (Fig. 4A) or 10 µM (data not shown).   However, when combined with DCKA, ALX 5407 caused a rightward shift of the concentration dependency of the effect of DCKA.   That is, the protective effect of 3 µM DCKA against 15 µM NMDA was largely abrogated in the presence of 30 µM ALX 5407 (Fig. 4A).   Measurement of amino acid levels by HPLC revealed that 24 h treatment of slice cultures with 30 µM ALX 5407 caused a marked increase in extracellular glycine, whereas the concentration of extracellular D-serine

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exhibited no significant change (Table 2).

Second, we sought to determine the influence of a selective increase in extracellular D-serine.   Unlike in the case of glycine, specific transporters for D-serine uptake have not been described.   Instead, transporters for small neutral amino acids, such as Asc-1 and ASCT, recognize D-serine as a substrate, and several reports have suggested that these transporters are involved in regulation of the extracellular levels of D-serine (Fukasawa et al., 2000; Ribeiro et al., 2002; Helboe et al., 2003).   Accordingly, we examined the effects of excess amount of amino acid transporter substrates, because ASCT transporters can mediate heteroexchange of amino acids between extracellular and intracellular compartments (Zerangue and Kavanaugh, 1996), and also because small neutral amino acids have been reported to enhance D-serine release from cultured astrocytes (Ribeiro et al., 2002). Application of 1 mM L-serine for 24 h resulted in a significant increase in extracellular D-serine, but had no significant effect on extracellular glycine (Table 2).   L-Alanine (1 mM) also caused a significant but small increase in extracellular D-serine, whereas L-threonine (1 mM) showed no significant effect.   Neither L-alanine nor L-threonine affected the level of extracellular glycine.   Measurement of tissue contents of free amino acids showed that the effects of exogenous amino acids on extracellular D-serine coincided with the effects on intracellular levels of D-serine.   That is, L-serine caused a robust increase in intracellular D-serine, whereas L-alanine caused a significant but small increase, and L-threonine had no significant effect (Table 3).

The influences of exogenous L-alanine, L-serine and L-threonine on NMDA cytotoxicity were examined.   These amino acids alone at 1 mM had no significant effect on NMDA cytotoxicity.   However, L-serine caused a rightward shift of the concentration dependency of the protective effect of DCKA against NMDA cytotoxicity, in a similar manner to ALX 5407. L-Alanine and L-threonine did not modulate the effect of DCKA (Fig. 4B-D).

Discussion

The present study was aimed to obtain insights into the roles of glycine and D-serine in the regulation of excitotoxic events associated with neurodegenerative conditions.   Results suggest that the changes in the extracellular levels of endogenous glycine and D-serine can influence the extent of cellular damage in the cerebral cortex.   Although in this study we did not discriminate the types of cells damaged by NMDA and OGD, previous studies have demonstrated that neurons are vulnerable to these insults (Shirakawa et al., 2002; Hassen et al., 2004; Wilkins et al., 2006).

NMDA cytotoxicity was markedly enhanced by exogenous addition of glycine and D-serine, which suggests that the glycine site of NMDA receptors in the cerebral cortex is not saturated by endogenous glycine site agonists.   This is consistent with a previous study showing that NMDA receptor-mediated synaptic transmission in the prefrontal cortex can be enhanced by exogenous glycine and D-serine (Chen et al., 2003).   D-Serine was found to be more potent than glycine in enhancing NMDA cytotoxicity.   This difference may be in part attributable to the fact that added glycine can be cleared efficiently from the extracellular space by high-affinity glycine transporters, whereas the extracellular level of D-serine remains elevated because of the lack of specific transporters for this amino acid.   In addition, D-serine exhibits a higher affinity to the glycine site of NMDA receptors than glycine (Matsui et al., 1995), which may also contribute to the potent effect of exogenous D-serine.   In our previous study using acute cortical slice preparation, addition of glycine and D-serine had no effect on NMDA-induced cellular damage (Katsuki et al., 2004).   In that study, cell death was evaluated at 6 h after 30 min exposure to NMDA.   Thus, assessment of cell death at longer term (24 h exposure to NMDA) in the present study may have improved detection of

the influences of altered levels of the extracellular glycine site agonists.

In contrast to NMDA cytotoxicity, ischemic cell death induced by OGD was not affected by exogenous glycine or D-serine, although OGD-induced cell death was definitely dependent on NMDA receptor activation as shown by the protective effect of MK-801. A reasonable explanation is that the extracellular levels of glycine site agonists are elevated under ischemic conditions, which lead to saturation of the glycine site. Supporting this notion, the results of the experiments using DCKA showed that much higher concentrations of a competitive glycine site antagonist were required to inhibit OGD-induced cell death, than those to inhibit NMDA cytotoxicity. It should be cautioned that several variables such as pH and the redox status might also be involved in the alterations of the pharmacological profile of NMDA receptors under ischemic conditions (Herin and Aizenman, 2004). Therefore, the low sensitivity to DCKA of OGD-induced cell death might not be totally attributable to the increase in glycine site agonists. Despite these caveats, however, we indeed detected a significant increase in extracellular glycine and D-serine during OGD, by an HPLC analysis.

The increase in the extracellular levels of glycine in response to OGD treatment was much more robust than that of D-serine. Similar results have been reported in the rabbit cerebral cortex during transient focal ischemia in vivo (Lo et al., 1998). We observed a significant increase in extracellular glycine concentration immediately after 30 min OGD. Because cell death was not yet induced at this time point (data not shown), leakage of glycine from damaged cells is unlikely to contribute to this initial increase in extracellular glycine. The pathways for glycine efflux into the extracellular space are unclear, but GLYT1 is a potential candidate. GLYT1 mediates symport of glycine with Na^+ and Cl^-, and the direction of transport may be reversed by alterations of transmembrane ion gradients and membrane potential that should occur under ischemic conditions (Eulenburg et al., 2005). Other candidates for glycine efflux pathway are several families of amino acid transporters with

broad specificity that transport glycine as well as other amino acids (Hyde et al., 2003). For example, Asc-1 is a Na^+-independent exchanger of small neutral amino acids including glycine, L-alanine, L-serine and L-threonine, which can mediate both influx and efflux of these amino acids. The fact that the extracellular levels of L-alanine, L-serine and L-threonine were also elevated in response to a prolonged period of OGD is consistent with possible involvement of amino acid transporters with a broad spectrum.

We could selectively increase the extracellular levels of endogenous glycine and D-serine, by application of ALX 5407 and L-serine, respectively. That is, a selective GLYT1 blocker ALX 5407 induced a significant increase in extracellular glycine but had no effect on extracellular D-serine. On the other hand, L-serine increased the extracellular level of D-serine but not of glycine. Because we could only analyze amino acids escaped from the tissues into Ringer's buffer, the obtained values of concentrations may not faithfully reflect the levels of these amino acids in narrow extracellular space within the cortical tissue. However, both ALX 5407 and L-serine caused a rightward shift of the concentration dependency of the protective effect of DCKA against NMDA cytotoxicity, indicating that these agents increased the concentrations of endogenous glycine site agonists in the vicinity of NMDA receptors.

Because neutral amino acid transporters have been implicated in the efflux pathway for D-serine (Ribeiro et al., 2002), we expected that application of excess amounts of the substrates for these amino acid transporters increase the extracellular level of D-serine by countertransport (Zerangue and Kacanaugh, 1996). Contrary to the expectation, L-threonine, a potent inducer of D-serine efflux from cultured astrocytes (Ribeiro et al., 2002), did not affect the extracellular level of D-serine, although a large amount of L-threonine itself was incorporated into the slices as reflected by the increase in tissue content. L-Serine and L-alanine indeed caused a significant increase in extracellular D-serine, but this effect is likely

to result from accelerated synthesis of D-serine.　L-Serine is a direct precursor of D-serine whose production is catalyzed by serine racemase (Wolosker et al., 1999), and in the present study, L-serine treatment induced a robust increase in the tissue content of L-serine and D-serine.　We observed that L-alanine treatment also resulted in an increase in tissue D-serine pool.　The mechanisms of L-alanine-induced increase in D-serine are unclear, although L-alanine and L-serine share pyruvate as a common metabolic product.　Interestingly, changes in the extracellular concentrations of D-serine after treatment with L-alanine, L-serine and L-threonine, roughly paralleled those in the tissue levels of D-serine and L-serine.　These results suggest that the availability of the precursor L-serine is an important determinant of the extracellular level of D-serine.　In sharp contrast, the extracellular level of glycine did not fluctuate significantly, despite neutral amino acids, particularly L-serine, caused a remarkable increase in the tissue content of glycine (Tables 2 and 3).　This result suggests tight regulation of glycine levels by glycine transporters.

Our results do not suggest that amino acid transporters are the major routes for D-serine release.　As an alternative route, exocytotic release machinery has been proposed to mediate D-serine release from cultured cortical astrocytes (Mothet et al., 2005).　On the other hand, recent evidence has demonstrated that cortical neurons can synthesize D-serine, and neuronal release of D-serine may not involve exocytotic pathway (Kartvelishvily et al., 2006).　Hence, multiple routes can mediate D-serine efflux into the extracellular space, and their relative contributions may be varied depending on different physiological and pathological conditions.

Conclusion

The present study showed that both glycine and D-serine could act as endogenous ligands for NMDA receptor glycine site and influence excitotoxic consequences in the cerebral cortex.

Particularly, massive release of glycine may lead to saturation of the glycine site of NMDA receptors and drive excitotoxic events under ischemic conditions.   These findings provide important information for the interpretation of the results of various in vitro experiments on NMDA cytotoxicity and ischemic neuronal death.   Identification of cellular origins and efflux pathways for glycine and D-serine requires further investigations.

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Figure Legends

Fig. 1. Exogenous glycine and D-serine exacerbate cellular damage induced by NMDA. Glycine (A, C) or D-serine (B, D) at indicated concentrations was concomitantly applied with 10 μM (A, B) or 15 μM (C, D) of NMDA for 24 h.   The extent of cell death at 24 h was evaluated by the level of PI fluorescence.   n = 18 slices for all groups.   *** P < 0.001 vs. control; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. NMDA alone. $$, P < 0.01, $$$, P < 0.001 vs. NMDA plus the corresponding concentration of glycine (columns in panel A or C).

Fig. 2. Exogenous glycine and D-serine do not affect cellular damage induced by OGD. (A-D) Slices were treated with deoxygenated glucose-free Ringer's buffer for 20 min (A, B) or 30 min (C, D), and further incubated for 24 h in oxygenated medium.   Glycine (A, C) or D-serine (B, D) at indicated concentrations were applied during OGD treatment.   n = 12 - 18 slices for all groups, except for the groups treated with glycine or D-serine alone (n = 6).   (E) MK-801 prevents cellular damage by OGD.   Slices were treated with 10 μM MK-801 in deoxygenated glucose-free Ringer's buffer for 30 min and incubated for further 24 h in oxygenated medium.   n = 13 for the group treated with MK-801 alone, and n = 30 - 36 for all other groups.   The extent of cell death at 24 h was evaluated by the level of PI fluorescence.   *** P < 0.001 vs. control; ### P < 0.001 vs. OGD alone.

Fig. 3. Different sensitivity of NMDA cytotoxicity and OGD-induced cell death to DCKA, a competitive glycine site antagonist.   (A) Representative images of PI fluorescence of a control slice (left), a slice treated with 15 μM NMDA for 24 h (middle) and a slice treated with 15 μM NMDA and 10 μM DCKA for 24 h (right).   (B) Representative images of PI fluorescence of a control slice (left), a slice incubated for 24 h after 30 min of OGD treatment (middle) and a slice incubated for 24 h after 30 min of OGD treatment in the presence of 10

22

μM DCKA (right).   Scale bar, 100 μm.   (C) Summary of the effect of DCKA against

NMDA cytotoxicity.   DCKA at indicated concentrations was concomitantly applied with 15

μM NMDA for 24 h.   n = 43 - 45.   (D) Summary of the effect of DCKA against

OGD-induced cell death.   Slices were treated with deoxygenated glucose-free Ringer's

buffer for 30 min, and further incubated for 24 h in oxygenated medium.   DCKA at indicated

concentrations was applied during OGD treatment.   n = 6 for the group treated with DCKA

alone, and n = 24 - 30 for all other groups.   *** $P < 0.001$ vs. control; ## $P < 0.01$, ### $P <$

0.001 vs. NMDA or OGD alone.

Fig. 4. Effects of a GLYT1 blocker and amino acid transporter substrates on NMDA

cytotoxicity.   (A) Slices were treated with 15 μM NMDA and indicated concentrations of

DCKA for 24 h, in the absence (open columns) or the presence (shaded columns) of 30 μM

ALX 5407.   ALX 5407 was applied 24 h before and during treatment with NMDA and

DCKA.   n = 12 for all groups.   (B-D) Slices were treated with 15 μM NMDA and indicated

concentrations of DCKA for 24 h, in the absence (open columns) or the presence (shaded

columns) of L-alanine (1 mM; B), L-serine (1 mM; C) and L-threonine (1 mM; D).   n = 12

for the groups treated with L-alanine, L-serine and L-threonine alone, and n = 17 - 30 for all

other groups.   The extent of cell death at 24 h was evaluated by the level of PI fluorescence.

*** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$.   ALX 5407 and L-serine caused a

rightward shift of the concentration dependency of the effect of DCKA against NMDA

cytotoxicity, whereas L-alanine and L-threonine had no effect.

Table 1. OGD-induced changes in the extracellular concentrations of amino acids (in μM)

Treatment	L-Glutamate	Glycine	D-Serine	L-Alanine	L-Serine	L-Threonine
Control (7)	0.62 ± 0.11	2.35 ± 0.78	0.38 ± 0.17	9.13 ± 0.85	4.46 ± 0.82	22.22 ± 2.69
30 min OGD (7)	10.04 ± 2.11***	8.55 ± 1.06***	0.55 ± 0.19	16.23 ± 0.80***	5.86 ± 0.92	26.18 ± 2.86
Control (4)	1.70 ± 0.40	4.49 ± 1.09	0.65 ± 0.17	7.62 ± 1.66	6.18 ± 0.87	20.60 ± 4.17
60 min OGD (4)	60.93 ± 6.27***	67.46 ± 11.32**	1.37 ± 0.18*	28.23 ± 3.57**	17.00 ± 1.55***	39.59 ± 8.12*

Cortical slice cultures were treated with deoxygenated glucose-free Ringer's buffer for 30 or 60 min.   Then the buffer was harvested, and amino acid levels in the buffer were determined.   The number of samples for each condition is given in parentheses.   * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.

Table 2. Effects of a GLYT1 blocker and ASCT transporter substrates on the extracellular concentrations of amino acids (in μM)

Treatment	L-Glutamate	Glycine	D-Serine	L-Alanine	L-Serine	L-Threonine
Control (6)	0.50 ± 0.04	2.84 ± 0.27	1.27 ± 0.07	8.47 ± 1.22	14.22 ± 2.01	19.32 ± 2.40
30 μM ALX 5407 (5)	0.73 ± 0.21	27.47 ± 2.90***	1.48 ± 0.07	14.42 ± 2.26	19.82 ± 0.66	46.47 ± 10.46*
1 mM L-alanine (3)	0.76 ± 0.03	2.07 ± 0.03	1.68 ± 0.04*	25.20 ± 0.44	16.98 ± 0.72	22.77 ± 0.64
1 mM L-serine (3)	0.81 ± 0.03	2.58 ± 0.12	3.12 ± 0.05***	10.70 ± 0.64	Not measurable	24.95 ± 1.08
1 mM L-threonine (3)	0.71 ± 0.04	2.35 ± 0.50	1.40 ± 0.12	9.18 ± 0.99	14.03 ± 1.40	Not measurable

Cortical slice cultures were treated with respective drugs in Ringer's buffer for 24 h. Then the buffer was harvested, and amino acid levels were determined. The number of samples for each condition is given in parentheses. * $P < 0.05$, *** $P < 0.001$ vs. control.

Table 3. Effects of ASCT transporter substrates on the tissue levels of amino acids (in nmol/mg protein)

Treatment	Glycine	D-Serine	L-Alanine	L-Serine	L-Threonine
Control (6)	24.43 ± 0.92	0.78 ± 0.08	9.35 ± 0.13	5.30 ± 0.41	8.79 ± 0.36
1 mM L-alanine (3)	60.05 ± 8.68	2.72 ± 0.41*	24.20 ± 2.43	15.74 ± 1.27	21.55 ± 2.56
1 mM L-serine (6)	83.42 ± 16.68*	6.80 ± 0.48***	29.55 ± 6.10	43.37 ± 9.16***	30.33 ± 5.74
1 mM L-threonine (3)	35.82 ± 7.79	1.39 ± 0.26	22.72 ± 4.64	6.09 ± 1.01	78.21 ± 17.55

Cortical slice cultures were treated with respective drugs in Ringer's buffer for 24 h.   Then the cortical tissue was harvested, and amino acid contents were determined.   The number of samples for each condition is given in parentheses.   * P < 0.05, *** P < 0.001 vs. control.

A control | NMDA | NMDA + DCKA

B control | OGD | OGD + DCKA

C
PI fluorescence (%)

120
100
80 ***
60 ##
40 ###
20 ###
0

Cont. | 1 | 3 | 10 | 10
DCKA (μM)
NMDA (15 μM)

D
PI fluorescence (%)

120
100
80
60 ***
40 ##
20 ###
0

Cont. | 10 | 30 | 100 | 100
DCKA (μM)
OGD (30 min)