The allosteric glycine site of the N-methyl-D-aspartate receptor modulates GABAergic-mediated synaptic events in neonatal rat CA3 hippocampal neurons

(development/spontaneous synaptic potentials/synaptic noise/γ-aminobutyric acid)

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ABSTRACT   We report in this study that, in the presence of magnesium, bath application of micromolar concentrations of glycine have prominent effects on synaptic events and N-methyl-D-aspartate (NMDA) responses in neonatal but not in adult hippocampal slices. Intracellular recordings were made from 71 rat CA3 hippocampal neurons in neonatal slices. In keeping with our earlier study, during the first postnatal week, CA3 neurons exhibited giant depolarizing potentials (GDPs). These GDPs are mediated by γ-aminobutyric acid (GABA) acting on type A GABA (GABA$_A$) receptors and modulated presynaptically by NMDA receptors. In the majority of cells (18 out of 31), glycine (10–30 μM) increased the frequency of GDPs (from 0.14 to 0.29 Hz). This effect was mimicked by D-serine (10–20 μM) and blocked by the NMDA receptor antagonists D-(−)-2-amino-5-phosphonovalerate (50 μM) and DL-2-amino-7-phosphonoheptanoate (50 μM) and by the GABA$_A$ antagonist bicuculline (10 μM) but not by strychnine (1 μM). Subthreshold concentrations of glycine (or D-serine) and NMDA, when given together, enhanced synaptic noise and the frequency of GDPs. In the presence of tetrodotoxin (1 μM), glycine and D-serine (up to 50 μM) did not modify the NMDA-induced inward currents in CA3 pyramidal cells. However the reduction of NMDA-mediated currents by 7-chlorokynurenate (10–20 μM) was reversed by glycine and D-serine (100–200 μM). In contrast, glycine (up to 100 μM) had no effect on membrane potential, input resistance, or NMDA responses after postnatal day 10. It is concluded that GABA-mediated events are facilitated by glycine acting on presynaptically located NMDA receptors.

In neonatal hippocampal slices, CA3 pyramidal neurons display spontaneous giant depolarizing potentials (GDPs). These are network-driven events that appear synchronously in a CA3 pyramidal cell population. GDPs are chloride dependent and are mediated by γ-aminobutyric acid (GABA) acting on type A GABA (GABA$_A$) receptors since they are blocked by bicuculline and reverse at the same polarity as GABA responses. GDPs disappear around the end of the first week of life, at a time when the reversal potential of GABA shifts to the hyperpolarizing level. These and other observations (1) suggest that the firing of GABAergic interneurons provides most of the excitatory drive that impinges on pyramidal cells in early postnatal life.

GDPs are also presynaptically modulated by N-methyl-D-aspartate (NMDA) receptors since selective NMDA receptor antagonists and channel blockers reduce (or block) the frequency of GDPs (1). This observation is of particular interest in view of the important role played by NMDA-mediated events in developmental plasticity (2–4).

The NMDA receptor complex possesses an allosteric facilitatory site activated by submicromolar concentrations of glycine (5–7). This site is insensitive to strychnine (5) and therefore is distinct from the classical strychnine-sensitive site associated with a chloride channel and present mainly in hindbrain areas (8). The glycine allosteric site can be demonstrated in cultured neurons (5, 7) or oocyte preparations (9). We now report that in neonatal slices, but not after postnatal day 10 (P10), bath application of micromolar concentrations of glycine enhances the frequency of the GDPs and potentiates NMDA responses. These actions are clearly mediated by the allosteric site of NMDA receptors present on GABAergic interneurons.

MATERIALS AND METHODS

Experiments were performed on CA3 hippocampal neurons in slices obtained from 0- to 18-day-old Wistar rats of both sexes (P0–P18; day 0 was taken as the day of birth). The methods for preparing and maintaining the slices have been extensively reported (1). Briefly, slices (600 μm thick), kept completely submerged in a recording chamber, were superfused (2.5–3 ml/min at 34°C) with artificial cerebrospinal fluid (ACSF) of the following composition: 126 mM NaCl, 3.5 mM KCl, 2 mM CaCl$_2$, 1.3 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, and 11 mM glucose (pH 7.3), gassed with 95% O$_2$/5% CO$_2$. Intracellular recordings were made with glass micropipettes filled with 3 M KCl (resistance of 50–80 MΩ) or 2 M potassium methyl sulfate (resistance of 80–150 MΩ). Current was passed through the recording electrode by means of an Axoclamp-2A amplifier. Bridge balance was checked repeatedly during the experiments, and capacitative transients with the electrode tip outside the neuron were reduced to a minimum by negative capacity compensation. Membrane potential was estimated from the potential observed upon withdrawal of the electrode from the cell. Voltage clamp of the neuronal soma was accomplished by means of an Axoclamp-2A preamplifier, switching between voltage recording and current injection at 3–4 kHz (30% duty cycle). To ensure correct operation of the clamp, the voltage at the headstage amplifier was monitored on a separate oscilloscope. Signals were digitized and displayed on a digital oscilloscope and on a computer-driven chart recorder.

Drugs were dissolved in ACSF and superfused by means of a three-way tap system. Drugs used were NMDA (CRB), GABA (Sigma), glycine (Sigma), D-serine and L-serine (gift of

Abbreviations: NMDA, N-methyl-D-aspartate; GABA, γ-aminobutyric acid; AP-5, D-(−)-2-amino-5-phosphonovalerate; AP-7, DL-2-amino-7-phosphonoheptanoate; TTX, tetrodotoxin; GDP, giant depolarizing potentials; Px, postnatal day x; LHP, large hyperpolarizing potential; ACSF, artificial cerebrospinal fluid.
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P. Ascher), D-(−)-2-amino-5-phosphonovalerate (AP-5; CRB or Tocris), DL-2-amino-7-phosphonoheptanoate (AP-7; gift of P. L. Herrling, Sandoz), strychnine (Sigma), tetrodotoxin (TTX; Sigma), bicuculline (Sigma), and 7-chlorokynurenate (gift of P. L. Herrling).

RESULTS

Long-lasting intracellular recordings were made from 71 immature (P0–P18) CA3 pyramidal neurons. In agreement with a previous report (1), immature cells had mean resting membrane potentials of −62 ± 8 mV (mean ± SD, ranging from −55 to −75 mV), action potentials >55 mV, and resting input resistance from 80 to 200 MΩ. As already reported (1), >90% of the neurons recorded during the first postnatal week exhibited GDPs. These consisted of a brief, high-frequency burst of spikes riding on a large depolarizing potential (25- to 50-mV amplitude, 300- to 500-ms duration; Fig. 1A).

Glycine Enhances the Frequency of GDPs. Between P0 and P6, bath application of glycine (10–30 μM) greatly enhanced the frequency of GDPs (in 18 out of 31 cells; the average frequency increased from 0.14 to 0.29 Hz; $P < 0.05$, paired t test; Fig. 1A) without changing their amplitude or duration. Glycine-sensitive and -insensitive cells were found in the same slice. The effect of glycine, which was strychnine insensitive, was rapid in onset (30–60 sec) and washed out in a few minutes (1–3 min). In 10 out of 18 cells, glycine did not affect resting membrane potential and/or input resistance of the cells. The effects of glycine were mimicked by D-serine (10–20 μM, $n = 14$; Fig. 2A), which was often more effective than glycine itself on the same cells. In 4 out of 7 cells, L-serine (≤50 μM) had no effect on GDPs (Fig. 2B); in the remaining 3 cells, L-serine increased the frequency of GDPs but at concentrations higher than D-serine (20–40 μM). The effects of glycine and D-serine are mediated by NMDA receptors since they were prevented by specific antagonists of the NMDA receptor. Thus AP-5 or AP-7 (50 μM) blocked or reduced the frequency of GDPs and fully prevented the effects of glycine (5 out of 6 cells) or D-serine (Fig. 2C; $n =$
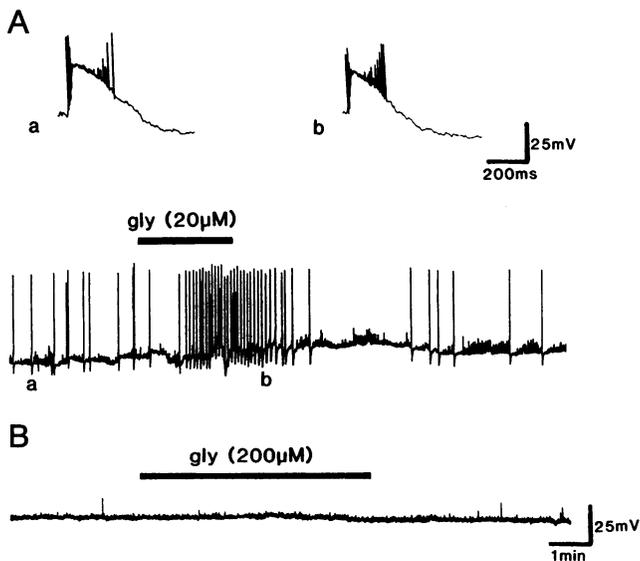
gly (20µM)

gly (200µM)

FIG. 1. Prominent effects of glycine at P6 and lack of effects of glycine at P13. (A) Lower trace, superfusion of glycine increased the frequency of GDPs. The resting membrane potential was −60 mV. Upper traces, GDPs taken at the time indicated by letters (a, control; b, glycine) are shown with an expanded time scale. (B) Bath application of a higher concentration of glycine for a longer period had no effect. The resting membrane potential was −68 mV. KCl-filled electrodes were used in this figure and Figs. 2, 4, and 5.
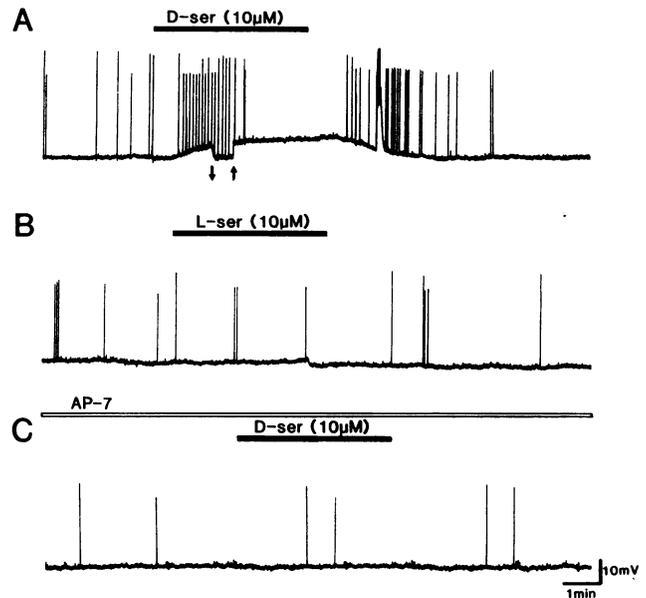
FIG. 2. D-Serine increases the frequency of GDPs in a P4 neuron (resting membrane potential = −75 mV). (A) Bath application of D-serine (filled bar) induced small depolarization and increased the frequency of GDPs. The increase in frequency of GDPs was independent of membrane depolarization since it was still present when the membrane was manually clamped to the initial potential, by injecting a steady current through the recording electrode (arrows). Note the inactivation of GDPs during application of D-serine and their reappearance during the wash. (B) The same concentration of L-serine had no effect. (C) The effects of D-serine (filled bar) on GDPs were prevented by AP-7 (50 μM, open bar).

6). Since GDPs are mediated by GABA (1), they were fully blocked by bicuculline, which also prevented the effect of glycine or D-serine (10 μM, $n = 6$).

Between P6 and P9 there is a transitional period during which GDPs are replaced by spontaneous large hyperpolarizing potentials (LHPs). LHPs are also mediated by GABA acting on GABA$_A$ receptors since they reversed at the same potential as the response to exogenous applied GABA (−62 mV with methyl sulfate electrodes) and were blocked by bicuculline (1). Bath application of glycine (10–20 μM) or D-serine (10–30 μM) increased the frequency of LHPs ($n = 3$). This effect, which was strychnine insensitive (Fig. 3B; 1 μM, $n = 3$), was fully prevented by AP-5 (50 μM, $n = 3$).

After P10, the LHPs were not present, and bath applications of high concentrations of glycine (200 μM, $n = 5$) or D-serine (200 μM, $n = 4$) had no effect on resting membrane potential (Fig. 1B), input resistance, or synaptic noise, recorded in the absence or presence of TTX (1 μM).

Glycine Enhances the Effects of NMDA on GDPs. As shown in Fig. 4, at P5, bath application of NMDA (1 μM) had little effect on synaptic noise and on the frequency of GDPs. In contrast, application of NMDA in the presence of glycine (30 μM), which *per se* did not affect spontaneous synaptic activity, greatly enhanced the synaptic noise and the frequency of GDPs (Fig. 4 A and B). Similar potentiations of NMDA responses were found in seven neurons with concentrations (20–30 μM) of glycine ($n = 5$) or D-serine ($n = 2$) that were by themselves inactive on these neurons. The potentiating effect of glycine or D-serine was unaffected by strychnine (Fig. 4C; 1 μM, $n = 3$) but was blocked by AP-5, AP-7 (Fig. 4D; 50 μM, $n = 3$), or bicuculline (10 μM, $n = 3$; data not shown).

Glycine or D-serine also potentiated the effects of subthreshold concentrations of NMDA on the LHPs recorded at the end of the first postnatal week. Thus, as shown in Fig. 3A, a combined application of subthreshold concentrations of
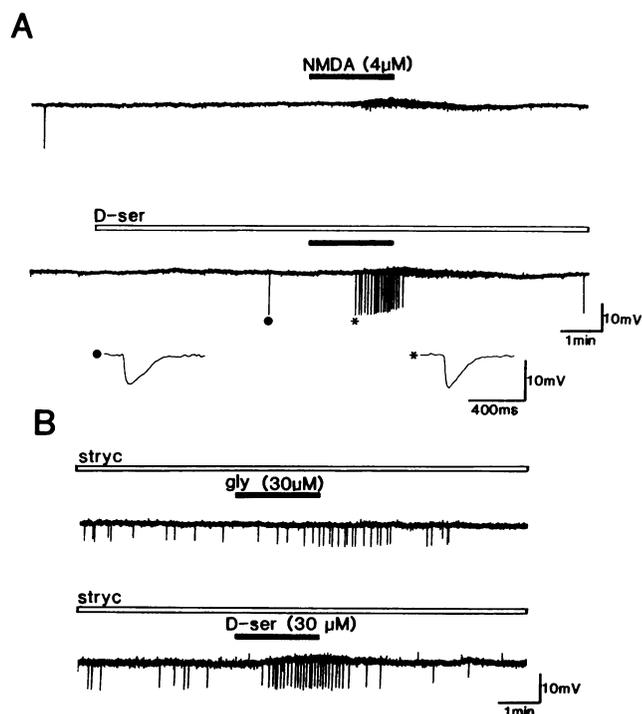
A

NMDA (4μM)

B

D-ser

10mV
1min

10mV
400ms

B

stryc
gly (30uM)

stryc
D-ser (30 μM)

10mV
1min

FIG. 3. Glycine and D-serine enhance the effects of NMDA and the frequency of large hyperpolarizing potentials during the transitional period. (*A*) Sporadic spontaneous hyperpolarizing potentials were recorded with potassium methyl sulfate electrodes in a P7 neuron. Bath application of NMDA (filled bars) in control conditions induced an increase in synaptic noise. In the presence of D-serine (30 μM, open bar), which *per se* had no effect, NMDA strongly enhanced the frequency of the hyperpolarizing potentials. In the lower traces, two spontaneous hyperpolarizing potentials are shown with an expanded time scale. The resting membrane potential was −58 mV. (*B*) The frequency of the spontaneous hyperpolarizing potentials was increased by glycine and D-serine in a P6 neuron. Strychnine (stryc, 1 μM, open bars) was present in the bath throughout the experiment. Potassium methyl sulfate-containing electrodes were used. The resting membrane potential was −55 mV.

NMDA and D-serine produced a burst of LHPs. These effects were also strychnine insensitive. Therefore glycine and D-serine modulated NMDA receptors, which controlled GABA-mediated events.

After P10 when neither GDPs nor LHPs were present, we failed to detect any effect of glycine (up to 100 μM) on the membrane depolarization or the inward current produced by bath application of NMDA (10 μM).

Glycine Does Not Enhance NMDA Responses in the Presence of TTX. In the presence of TTX (1 μM) to block propagated synaptic activity, glycine or D-serine (≤50 μM) induced no change in membrane potential or input resistance (*n* = 5) and in voltage-clamp experiments did not generate inward or outward currents (Fig. 5*A*; *n* = 3). In the presence of TTX, glycine or D-serine (up to 50 μM) did not potentiate the AP-5-sensitive inward current generated by NMDA (Fig. 5*A*; *n* = 5). In three of these neurons, prior to TTX application, glycine induced a clear facilitation of GDPs. However, as in cortical neurons (6), the reduction of NMDA responses induced in the presence of TTX by the selective glycine antagonist 7-chlorokynurenate (10–20 μM) was reversed by glycine (Fig. 5*B*; 100–200 μM, *n* = 5). Therefore, although glycine modulatory sites are present on pyramidal cells, they clearly do not mediate the prominent effects of glycine or D-serine on GDPs or NMDA responses.

Similar Endogenous Glycine Levels Are Present in Adult and Neonatal Preparations. Adult (*n* = 6) or neonatal (*n* = 6, P5) slices were incubated in ACSF, and the glycine content of
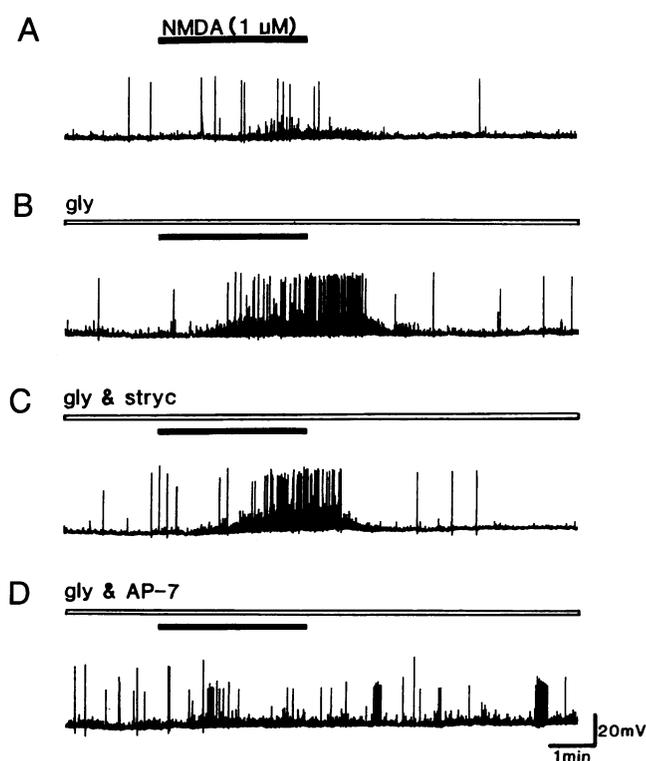
A   NMDA (1 uM)

B   gly

C   gly & stryc

D   gly & AP-7

20mV
1min

FIG. 4. The enhancement of NMDA-mediated events by glycine is blocked by AP-7 but not by strychnine in a P5 neuron. (*A*) NMDA (1 μM, filled bars) in control conditions induced an increase in synaptic noise and in the frequency of GDPs. (*B*) This effect was potentiated by glycine (30 μM, open bar). (*C*) The potentiating effect of glycine was insensitive to strychnine (stryc, 1 μM), but it was prevented by AP-7 (50 μM) (*D*). The resting membrane potential was −56 mV.

ACSF was measured by HPLC. In keeping with a recent study (10), in the control ACSF, the glycine levels were 0.045–0.002 μM; in the effluent of adult or immature slices, the concentrations were 0.168–0.02 and 0.289–0.08 μM per mg of protein, respectively.

DISCUSSION

These results suggest that, in hippocampal slices, micromolar concentrations of glycine potentiated spontaneous synaptic activity and the effects of NMDA until P8–P10 but not after that time. The effects of glycine were mediated by the allosteric site of the NMDA receptor since (*i*) they are mimicked by D-serine known to act on this site (5, 9, 11), (*ii*) they are insensitive to strychnine, and (*iii*) they are prevented by selective antagonists of the NMDA receptors, indicating that activation of the latter receptor is required for the action of glycine. These glycine sites are not located on the pyramidal cells but presynaptically, most likely on GABAergic interneurons, since (*i*) in the presence of bicuculline or TTX, glycine failed to enhance NMDA currents or potentials recorded in pyramidal cells and (*ii*) large GABA-mediated hyperpolarizing potentials recorded during the transient phase (P6–P9) are also potentiated by glycine and D-serine. These and other observations (12, 13) suggest that in neonatal brain slices, the control of GABA release from interneurons is modulated by glycine-sensitive NMDA receptors.

The contribution of the glycine allosteric site to ongoing synaptic activity *in vivo* or in slices is controversial. Binding assays in membrane preparations from adult rats (14) and electrophysiological studies on cell cultures (5, 6) or oocytes (9) suggest that the glycine allosteric site is a high affinity site (K_d in the submicromolar range). The levels of endogenous
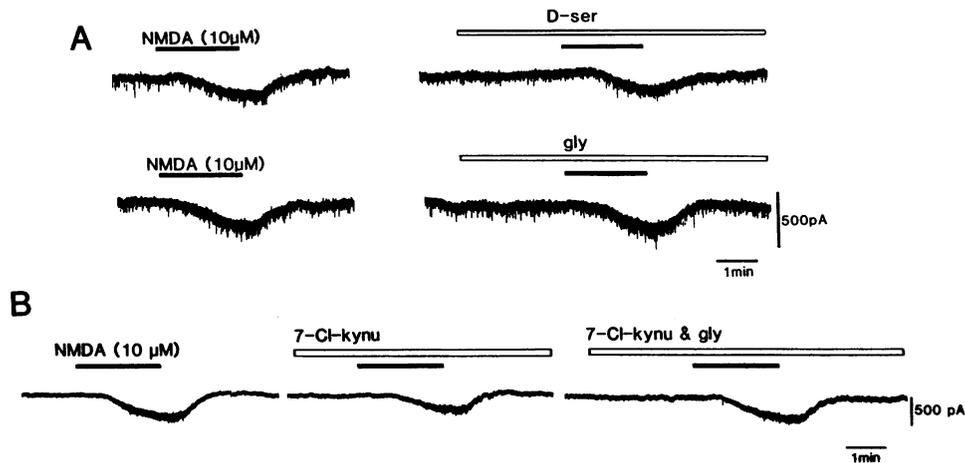
FIG. 5.   In the presence of TTX, glycine and D-serine have no effect on inward currents generated by NMDA, but they reversed the effects of 7-chlorokynurenate (7-Cl-kynu). (*A*) Inward currents induced by bath application of NMDA (10 μM, filled bars) in control conditions and in the presence of D-serine (20 μM, open bar) or glycine (50 μM, open bar). Note that D-serine and glycine had no effect *per se*. A P2 neuron was used; the cell was voltage-clamped at a holding potential of −40 mV in the presence of 1 μM TTX. (*B*) In the presence of TTX (1 μM), bath application of NMDA (10 μM, filled bars) induced an inward current in control conditions. This NMDA-mediated current is reduced by 7-chlorokynurenate (20 μM, open bar). Glycine (200 μM) reversed the effect of 7-chlorokynurenate (right trace). A P5 neuron was used; the cell was voltage clamped at a holding potential of −45 mV.

glycine *in vivo* (15) or in slices (6, 10) therefore should saturate this site and thus prevent any attempt to detect a modulation of NMDA receptors by exogenous glycine in physiological conditions. In keeping with this, bath application of glycine to adult slices (refs. 10 and 16; present study) has no effect on NMDA responses; glycine sites are present, however, since addition of glycine displaces the effect of 7-chlorokynurenate on NMDA responses (6). A recent study suggests, however, that local iontophoretic application of glycine enhances NMDA-mediated synaptic events (17).

The lack of effects of glycine after the second week of postnatal life could be due to the disappearance of NMDA receptors located presynaptically on GABAergic interneurons. This phenomenon might coincide with the synaptic rearrangements known to occur in the hippocampus at this developmental stage (18) and with the reduction in the density of NMDA binding sites observed in rat (19) and human (20) hippocampus. It is also in keeping with the preferential contribution of NMDA-mediated events in developmental plasticity, notably in the visual system (2–4).

Several hypotheses can be put forward to explain the difference between pre- and postsynaptic effects of glycine in neonatal neurons. Regional differences of glycine levels in neonatal slices (i.e., higher levels in the pyramidal layer than in the immediate vicinity of GABAergic cell bodies) could enable a preferential effect of exogenous glycine on an allosteric site located on interneurons. This possibility is perhaps unlikely since a significant percentage of GABAergic neurons is in fact adjacent to pyramidal cells (21, 22). Alternatively we would like to suggest the existence of an additional, low-affinity, strychnine-insensitive glycine site located on GABAergic neurons during early developmental stages. Stimulation of this site by exogenous glycine would facilitate the activation of NMDA receptors, leading to the increase in frequency of spontaneous GDPs, by means of an enhancement of GABA release. Evidence in favor of two glycine sites has recently been reported in biochemical (23) and release experiments (24).

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