Glycine Tranporter-1 Blockade Potentiates NMDA-Mediated Responses in Rat Prefrontal Cortical Neurons In Vitro and In Vivo

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Chen, Long, Mark Mulhauser, and Charles R. Yang. Glycine transporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. J. Neurophysiol. 89: 691–703, 2003. First published October 30, 2002; 10.1152/jn.00680.2002. The N-methyl-D-aspartate (NMDA) receptor (NMDA-R) has pivotal roles in neural development, learning, memory, and synaptic plasticity. Functional impairment of NMDA-R has been implicated in schizophrenia. NMDA-R activation requires glycine to act on the glycine-B (GlyB) site of the NMDA-R as an obligatory co-agonist with glutamate. Extracellular glycine near NMDA-R is regulated effectively by a glial glycine transporter (GlyT1). Using whole-cell voltage-clamp recordings in prefrontal cortex (PFC) slices, we have shown that exogenous GlyB site agonists glycine and D-serine, or a specific GlyT1 inhibitor HA-966, intravenous NFPS (5 mg/kg), or a GlyB site agonist D-serine (50 mg/kg iv) reversed this (+)-HA-966 block. NFPS may elevate endogenous glycine levels sufficiently to displace (+)-HA-966 from the GlyB sites of the NMDA-R, thus enabling reactivation of the NMDA-Rs by iontophoretic NMDA applications. D-Serine (50–100 mg/kg iv) or NFPS (1–2 mg/kg iv) alone also augmented NMDA-evoked excitatory responses. These data suggest that direct GlyB site stimulation by D-serine, or blockade of GlyT1 to elevate endogenous glycine to act on unsaturated GlyB sites on NMDA-Rs, potentiated NMDA-R-mediated firing responses in rat PFC. Hence, blockade of GlyT1 to elevate glycine near the NMDA-R may activate hypofunctional NMDA-R, which has been implicated to play a critical role in the pathophysiology of schizophrenia.

Introduction

Functional heteromeric N-methyl-D-aspartate (NMDA) receptors (NMDA-R), consisting of an obligatory combination of a NR1 subunit with any of the four NR2 (A–D) subunits (Cull-Candy et al. 2001; Monyer et al. 1992, 1994), are critically involved in neural development, synaptic plasticity, excitotoxicity, learning, and memory (Bliss and Collingridge 1993; Malenka and Nicoll 1999). The ionotropic NMDA-R possesses multiple modulation sites on the receptor subunits: voltage-dependent Mg$^{2+}$ block of the receptor channel pore (Mayer et al. 1984; Nowak et al. 1984), a strychnine-insensitive glycine-B (GlyB) site on the NR1 subunit, a phencyclidine (PCP) site on NR2 subunit, as well as the Zn$^{2+}$, polyamine, glycосylation, and polyamine sites (McBain and Mayer 1994). Extracellular glycine is an obligatory co-agonist for NMDA-R activation (Johnson and Asher 1987; Kleckner and Dingledine 1988; Parsons et al. 1998; Thomson 1990). Only when glycine binds to the GlyB site on the NR1 subunit, with glutamate to the glutamate-binding site on NR2 subunits, do single NMDA-R channels open (Anson et al. 1998; Currás and Pal-lotta 1996; Hirai et al. 1996; Laube et al. 1997). Functionally, this glycine binding allosterically influences NMDA-R to increase the recovery rate from receptor desensitization during synaptic activation (Benveniste et al. 1990; Lester et al. 1993; Mayer et al. 1989; Vylicky et al. 1990).

A considerable debate centers on whether the GlyB site on the NMDA-R is saturated under physiological condition in vivo. Early in vitro studies suggested that endogenous glycine could have saturated the GlyB site on the NMDA receptor (Bashir et al. 1990; Fletcher and Lodge 1988; Kemp et al. 1988). Indeed, extracellular glycine concentration ([glycine]o) is in the micromolar range in vivo, and the measured affinity of the GlyB site for glycine on NMDA-R is in the sub-micromolar range ($K_i = 0.1–0.3 \mu M$) (Baron et al. 1996; Grimwood et al. 1992). However, recent findings suggest that [glycine]o near the NMDA-R in the forebrain is efficiently regulated by a Na$^+$/Cl$^-$/dependent, astroglial, high-capacity glycine transporter (GlyT) adjacent to the NMDA-R (Borowsky et al. 1993; Smith et al. 1992; Zafra et al. 1995). Hence, the transport actions of glycine transporter (GlyT1) and/or intracellular glycine sequestration may exceed the $K_i$ of the glycine-binding site and lead to rapidly keep [glycine]o near the NMDA-R to low levels (e.g., <1 $\mu M$) (Bergeron et al. 1998; Supplisson and Bergman 1997).

GlyTs belong to a superfamily of 12 trans-membrane domains, Na$^+$-dependent, neurotransmitter transporters. Two different genes, GlyT1 and GlyT2, encode GlyT. Transcription of GlyT1 gene resulted in more than or equal to three mRNA isoforms: GlyT1a, GlyT1b, GlyT1c, and with transcription of GlyT1a and GlyT1b mediated by alternative promoter usage (Adam et al. 1995; Borowsky and Hoffman 1998; Kim et al. 1994). While GlyT2 mRNAs are present in axonal terminals of glycineric neurons and specifically regulate strychnine-sensitive glycineric neurotransmission in brain stem and spinal cord, GlyT1 mRNAs are heterogeneously present in frontal cortex and hippocampus, as well as lower brain stem and spinal

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cord (Borowsky et al. 1993; Legendre 2001; Smith et al. 1992; Zafra et al. 1997). GlyT1 can remove glycine efficiently near the NMDA-R, as well as releasing glycine on changes in extracellular glycine and/or ionic composition (Berger et al. 1998; Fedele and Foster 1992; Herdon et al. 2001; Sakata et al. 1997; Supplisson and Bergman 1997). Saturation of the GlyB site by glycine in vivo may depend on the density of GlyT1, as well as the regional differences of local brain glycine levels (see Danyz and Parsons 1998; Wood 1995).

Dysfunction of the prefrontal cortex (PFC) and central NMDA-R plays a crucial role in the complex pathophysiology including severe cognitive deficits in schizophrenia (Breier 1999; Goldman-Rakic 1998; Javitt and Zurkin 1991; Tamminga 1998; Tsai and Coyle 2002; Weinberger and Berman 1996; Yang et al. 1999). One therapeutic strategy is to administer daily a large glycine dose (e.g., in grams per day, due to its poor CNS penetrance) (Oldendorf 1973), or GlyB site agonist t-serine, or a partial agonist cycloserine. These treatment approaches led to reported improvement of neuropsychiatric symptoms, presumably via potentiation of NMDA-R functions (Goff et al. 1999; Heresco-Levy and Javitt 1999; Warmerd et al. 1999) and electrophysiological recordings were made at 30–32°C. The temperature of the ACSF entering the recording chamber was rapidly heated to the preset temperature using an in-line heater (SH27B, Warner Instruments). The temperature of the perfusate (30–32°C) was maintained constant via an automatic feedback temperature controller (TC-324B, Warner Instruments).

**Whole cell patch-clamp recordings**

An upright Olympus BX50WI microscope equipped with differential interference contrast optics and infrared videomaging system (DIC-IR, Hamamatsu C2400-07ER) was used to visualize neurons in slices. Layer V–VI PFCpyramidal neurons were easily recognizable via a 40× water-immersion lens by the pyramidal shape of their cell bodies and the presence of a long apical dendrite extending toward superficial layers. In some neurons, the morphology of single neurons from which recordings were made using biocytin (0.2%)-filled patch pipettes was confirmed by streptavidin–horseradish peroxidase staining of biocytin (Yang et al. 1996).

Whole cell patch-clamp techniques were used to study synaptic responses of layers V–VI pyramidal neurons in response to local layer V–VI stimulation in the prelimbic region of the PFC. Patch pipettes (3–5 MΩ) were fabricated from borosilicate tubing (1.5 mm OD, 1.1 mm ID) on a horizontal microelectrode puller (P-97, Sutter Instruments). The internal pipette solution contained the following (in mM): 100 potassium methyl sulfate, 60 sucrose, 10 HEPES, 1 EGTA, 2 MgCl2, 2 Na2ATP, 0.5 Tris–glutamic acid (pH 7.3), and high-K solution (10 MΩ, a noncompetitive selective antagonist of a-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) receptors, formerly known as KYKI 53655) and SCH50911 (10 μM, a selective antagonist for y-aminobutyric acid-B (GABA_B) receptor) to pharmacologically isolate the NMDA-R, as well as releasing glycine on changes in extracellular glycine and/or ionic composition (Berger et al. 1998; Fedele and Foster 1992; Herdon et al. 2001; Sakata et al. 1997; Supplisson and Bergman 1997). Saturation of the GlyB site by glycine in vivo may depend on the density of GlyT1, as well as the regional differences of local brain glycine levels (see Danyz and Parsons 1998; Wood 1995).

**METHODS**

**Brain slice preparations**

The experiments were performed in brain slices prepared from young adult (P25–35) male Sprague-Dawley rats. The euthanasia method used was approved by the Lilly Animal Use Committee, whose policies adhere closely with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Guide). Following decapitation by a guillotine (using a plastic rat restrainer Decapicone, BrainTree Scientific, FL), the brain was quickly removed and placed for 1 min in ice-cold oxygenated (95% O2-5% CO2) artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 NaCl, 26 NaHCO3, 3 KCl, 0.5 CaCl2, 4.0 MgCl2, 0.4 ascorbic acid, 0.8 thiourea, 10 glucose. The temporal lobes of the cortex from both hemispheres were trimmed away, leaving the medial prefrontal PFC of both hemispheres. The prefrontal PFC corresponds to the region outlined in the stereotaxic atlas of Paxinos and Watson (1998) (A-P = 2.2–3.5 mm anterior to the bregma; D-V = 3–5 mm from the cortical surface; M-L = 0.8–0.9 mm from the midline). Bilateral coronal PFC slices (350-μm-thick) were then cut on a vibratome (Vibrorslice, World Precision Instruments). After cutting, the slices were placed in warm (30°C) continuously oxygenated ACSF, containing the following (in mM): 124 NaCl, 26 NaHCO3, 3 KCl, 2.0 CaCl2, 1.3 MgCl2, 10 glucose. After a 30 min incubation, the slices were cooled in the same ACSF to room temperature (22–23°C) for at least 1 h. A single slice was transferred to a submersion recording chamber (Warner Instrument) and electrophysiological recordings were made at 30–32°C. The temperature of the ACSF entering the recording chamber was rapidly heated to the preset temperature using an in-line heater (SH27B, Warner Instruments). The temperature of the perfusate (30–32°C) was maintained constant via an automatic feedback temperature controller (TC-324B, Warner Instruments).

The sarcosine derivative N[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS, also known as ALX-5407) represents a useful tool that has been shown to block glycine uptake by glial GlyT1 (Kᵢ = 5 nM; IC50 = 0.03–0.22 μM) (Atkinson et al. 2001; Aubrey and Vandenberg 2001; Bergeron et al. 1998; Herdon et al. 2001) and to augment hippocampal NMDA-R-mediated synaptic responses in vitro (Bergeron et al. 1998). Although NFPS can elevate [Glycine]o levels in PFC and hippocampus in vivo (Atkinson et al. 2001; G. Nomikos and K. Johnson, personal communication), it is not known whether NMDA receptor functions can be potentiated in vivo. Since PFC is a key brain region where NMDA-R dysfunction in schizophrenia has been implicated (Tsai and Coyle 2002), the present electrophysiological study aims to determine whether stimulation of GlyB site by d-serine, glycine, or blockade of GlyT1 native to the PFC by NFPS will augment evoked NMDA excitatory postsynaptic currents (EPSCs) in PFC slices. In addition, by using the GlyB site antagonist (+)HA-966 to block NFPS-evoked firing in vivo, we also determined the site of action of d-serine and elevated [glycine]o following blockade of native GlyT1 by NFPS in vivo. Our data have provided key evidence to show that in vivo augmentation of endogenous [Glycine]o in PFC can lead to a potentiation of NMDA-R-mediated neuronal excitability. Preliminary results have been reported in an abstract form (Chen et al. 2001).

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NMDA EPSCs. Alteration of [glycine]$_o$ concentration for each series of experiments is specified in the appropriate sections of the text. Neurons were voltage clamped at a $V_{	ext{holding}}$ of $-75$ to $-80$ mV. At the beginning of the experiments, whole-cell evoked NMDA EPSCs were examined at different holding voltages (from $-100$ to $-50$ mV). Only cells with little or no evoked γ-aminobutyric acid-A (GABA$_A$) outward current were selected. At the end of experiments (80% of all experiments), the competitive NMDA antagonist d-2-amino-5-phosphonovaleric acid (APV; 50 μM) was added to ensure that all inward currents evoked and potentiated were NMDA receptor mediated (see Fig. 1 and 3B).

Synaptic stimulations

Electrical stimulation was delivered via a concentric bipolar metal-stimulating electrode (MCE-100X, David Korp) placed in layer V, approximately 200–300 μm from the adjacent recorded neuron to activate local afferent synaptically. Programmed monophasic square-pulse (0.1 ms, 50–200 μA) at 30-s inter-stimulus intervals) were delivered via a Master-8 programmable pulse-generator to an optically isolated stimulator (Isolflex, A.M.P.I., Israel).

Extracellular single-unit recording with microiontophoresis

Male Sprague-Dawley rats (250–320 g, >P45) were anesthetized with urethane (1.5 g/kg, ip) and mounted in a stereotaxic frame (Stoelting, Harvard Apparatus). Core temperature was monitored by a rectal probe and maintained at 37°C by a heating pad (Frederick Haer, Brunswick, NJ). Burr holes were drilled through the skull over the PFC (stereotaxic coordinates for the PFC: A–P = 2.7–3.0 mm anterior to the bregma, L–M = 0.8–1.0 mm, D–V = 2.0–3.5 mm from the cortical surface; Paxinos and Watson 1998). A venous catheter, made of PE-10 tubing, was inserted into the jugular vein for intravenous syringe infusion of drugs.

Conventional extracellular single-unit recordings with iontophoresis were made using five-barrel glass micropipettes. The multi-barrel pipette blanks (World Precision Instruments) were pulled by a vertical Narishige pipette puller (PE-2, Narishige, Tokyo, Japan). The recording barrel was filled with 0.5% sodium acetate in 2% Pontamine Sky blue mixed with bicuculline methide (0.5 μM). A slow leak of bicuculline was used to partially block local GABA$_A$ receptor-mediated tonic inhibitory responses (Gigg et al. 1994). The side-barrels were filled with NMDA (2–20 mM, pH 8; Sigma, St. Louis, MO), the Gly-B site antagonist (+/HA-966 (0.2 mM, pH 4), and NaCl (200 mM, for current balancing). The electrode was advanced by a single-axis hydraulic micromanipulator (MHW-40-1, Narishige) mounted onto the stereotaxic frame.

Extracellular single-unit activity was amplified by a Xcell-3 Preamp amplifier (Frederick Haer). Amplified (gain: 100,000×; low-pass filter at 5 kHz, high-pass filter at 500 Hz) single-unit activity was isolated using a window discriminator (model 74-60-3, Frederick Haer). The output signals from the window discriminator were digitized and multiplexed by an A/D converter (1401 mini, Cambridge Electronics Design, CED, Cambridge, UK) and were sampled at 10 kHz by a PC-based computer using Spike 2 software (Version 4, Cambridge Electronics Design). Programmed NMDA pulses (−20 to −40 nA) were iontophoretically applied repeatedly (Dagan 6400, Dagan) for 10–25 s every 45–60 s to evoke firing of single PFC neurons.

**FIG. 1.** Pharmacological isolation of synaptically evoked N-methyl-D-aspartate (NMDA) excitatory postsynaptic current (EPSC). A: evoked synaptic responses over time showing the gradual pharmacological isolation of the NMDA component of the evoked EPSC. Electrical stimulation was delivered once every 30 s (i.e., at 0.033 Hz) to layer V–VI adjacent to the recorded pyramidal neuron in prefrontal cortex (PFC). The gray bar on top indicates the time when a mixture of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist LY303168 (50 μM), glycine (10 μM), and Mg$^{2+}$ (0.1 μM) were added to perfuse with the above drug cocktail. 4: (top) Pharmacologically isolated NMDA EPSC. (bottom) Application of competitive d-2-amino-5-phosphonovaleric acid (APV; 50 μM) or noncompetitive 5,7-dichlorokynurenic acid (5,7-DCK) 50 μM NMDA receptor antagonist completely blocked the EPSC, suggesting that it is an NMDA EPSC. B: normalized NMDA EPSC (integrated area) evoked at different holding potentials in the presence of low Mg$^{2+}$ and [glycine]$_o$ (10 μM) (filled circles). Note that the voltage-dependent augmentation of NMDA EPSC responses was enhanced at all holding potentials in low Mg$^{2+}$/[glycine]$_o$ condition. In the graphical plot the peak NMDA EPSC responses were observed at a $V_m$ $\approx$ −50 mV and displays a region of negative slope conductance. We chose a holding potential of −75 to −80 mV for evoking our control NMDA EPSC in this study.

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neurons. (+)HA-966 was iontophoretically applied (30–80 nA) continuously to block the NMDA-evoked firing responses. An additional barrel in the five-barrel pipette contains saline (0.9% NaCl). Automatic ejection of currents in opposite polarity with respect to drug ejection current applied was made for current balancing to eliminate possible current artifacts.

To verify the position of the microelectrode, DC current (10 μA, for 15 min) was delivered to iontophoretic Pontamine Sky blue through the recording electrode to mark the recording site. The animal was then perfused transcardially with saline, followed by buffered formalin. Brain sections (70 μm) containing the PFC were cut using a freezing microtome (Leitz, Germany) and the sections were washed, dehydrated with alcohol, and stained with cresyl violet to permit examination of the recording sites.

Drug applications

For in vitro brain slice experiments, all drugs used were bath-applied by gravity. Stock solutions of APV, bicuculline, 5,7-dichlo-roxyneurine, (5,7-DCK), (+)HA-966, and d-serine were prepared in de-ionized water. All other drugs including the selective AMPA antagonist LY300168, GlyT1 inhibitor NFPS, were dissolved in DMSO and stored as frozen aliquots at −20°C and diluted to appropriate concentrations in ACSF for slice perfusion.

For in vivo experiments, NFPS was first dissolved in 200 μl ethanol. An appropriate amount of a hydroxy-β-cyclodextran solution (50%, HBC; Sigma) was added and sonicated briefly. Deionized water was then added to make a final concentration of HBC to 15%. Intravenous administration of drug vehicle alone showed no change in NMDA-evoked firing.

Data analyses

For in vitro brain slice experiments, the integrated areas and amplitudes of evoked EPSCs were measured using pClamp 8.0 software (Axon Instruments). The decay time constant of the averaged NMDA EPSC was fitted for two exponentials from the peak of the EPSC to the end of the trace using standard exponential fitting formula in pClamp 8.0 (Axon Instruments)

\[ f(t) = \sum A_i e^{-t/\tau_i} + C \]

where \( i \) is the current as a function of time (t), \( A_i \) and \( \tau_i \) are the amplitude and time constant of each component of the current, respectively, and C is the constant y-offset for each component i. The NMDA-EPSC trace recorded following different doses of GlyT inhibitor NFPS, glycine, or d-serine was scaled to the same peak amplitude of the predrug control EPSC. All group data were presented as mean ± standard error (SE). Analysis of variance (ANOVA) and post hoc Dunnett’s test was used to compare differences between group mean data with control group mean. Differences between control and experimental responses with \( P < 0.05 \) were deemed significant. Student’s t-test was used for group comparison to determine exogenous glycine and d-serine effects on evoked NMDA EPSCs.

For in vivo iontophoretic data, the control NMDA-evoked firing measured from five to six repeated stable baseline responses were averaged and analyzed using Spike 2 software (version 4, CED). Following systemic drug injections, the mean firing rates evoked by NMDA application that consist of responses at 20% or greater than baseline NMDA-evoked responses were taken for comparison with the baseline mean data. Group data comparisons were made using one-way ANOVA, followed by post hoc Dunnett’s test (GraphPad Prism Software).

RESULTS

In vitro experiments

CHARACTERIZATION OF SYNAPTICALLY EVOKED NMDA-EPSC. Electrical stimulation of layer V–VI synaptically evoked a mixed EPSC in layer V–VI pyramidal neurons. The EPSC was dominated by a large, fast AMPA receptor component. Bath application of the drug cocktail containing AMPA and GABA_B antagonists in low \([\text{Mg}^{2+}]_0\) superfusate resulted in a gradual blockade over time of the prominent AMPA EPSC. This was followed by a slow emergence of the NMDA EPSC, which was pharmacologically confirmed by its sensitivity to blockade with the competitive NMDA antagonist APV (50 μM, \( n = 3 \)), or the GlyB site NMDA antagonist 5,7-DCK (50 μM, \( n = 3 \)) (Fig. 1). This suggests that the evoked EPSC is NMDA receptor mediated.

By varying the steady-state holding potentials prior to each stimulation, we have examined the voltage-dependence of the evoked NMDA EPSC. Low \([\text{Mg}^{2+}]_0\) (0.1 mM) perfusate in the presence of LY300168 and SCH52911 was used to isolate the NMDA EPSC. In low \([\text{Mg}^{2+}]_0\), elevating [glycine] \((10 \mu M)\) potentiated the NMDA EPSC integrated areas (Fig. 1B). The increase in evoked NMDA EPSC began at approximately −75 mV and gradually, the evoked inward current increased with more positive holding potentials to a maximal current evoked at −50 mV. There was a voltage-dependent reduction of the evoked NMDA EPSC with holding potentials more positive than −50 mV (Fig. 1B). Furthermore, the selected holding potentials of −75 to −80 mV (for recording NMDA EPSCs in low \([\text{Mg}^{2+}]_0\) are far from the activation voltage whereby the evoked EPSC could trigger dendritic \(\text{Ca}^{2+}\) current (Seamans et al. 1997), which can obscure the synaptic current.

The decay time constants of the NMDA EPSC represent a complex interaction of at least four factors. These factors include the following: slow dissociation of glutamate from the NMDA receptor (deactivation) (Hestrin et al. 1990; Lester and Jahr 1992), intrinsic channel kinetics (periodic cluster bursts of channel opening and closing in the presence of glutamate binding on the receptor) (D’Angelo et al. 1990, 1994; Gibb and Colquhoun 1991; Lester et al. 1990), intrinsic properties of the combined NMDA receptor subunits (Cull-Candy et al. 2001; Monyer et al. 1994; Vinci et al. 1998), and \(\text{Ca}^{2+}\)-mediated receptor desensitization (Clark et al. 1990; Lerma et al. 1990; McBain and Mayer 1994; Tong et al. 1995; Zilberter et al. 1991). The decay time-constant (\(\tau\)) of the NMDA-EPSC in PFC neurons could be fitted by two exponentials (D’Angelo et al. 1990, 1994); a fast, and a slow component, typically found in >P22-day-old rats as used in this study. The mean decay time of the slow \(\tau\) reduces with age and this may be due to an age-dependent switch of the NMDA receptor subunit from NR2B to NR2A (Carmignoto and Vicini 1992; Flint et al. 1997), which can obscure the synaptic current.

Both glycine and d-serine dose-dependently potentiated synaptically evoked NMDA EPSC. Next, incremental doses of GlyB agonists glycine or d-serine were bath-applied to determine the extent to which these amino acids potentiate the evoked NMDA-EPSCs. Increasing the concentration of gly-
cine and d-serine (from 0.1 to 100 µM) increased both the peak amplitude, as well as the integrated area [glycine: $F(3,28) = 3$, $P < 0.05$; d-serine: $F(3,22) = 14$, $P < 0.001$] of the NMDA EPSCs (Fig. 2, A and B). Group data summarized in Fig. 2C show that at 0.1, 1 µM d-serine induced a significantly greater potentiation of NMDA EPSC integrated area compared with that induced by glycine. However, at 10 and 100 µM glycine and d-serine, there was a considerable cell-to-cell variation in the NMDA EPSC potentiation effects, e.g., potentiation by glycine at 100 µM was in the range of 17–127%, while potentiation by d-serine at 100 µM was in the range of 20–190%. These large variations of NMDA EPSC modulation by the GlyB site agonists suggest a heterogeneous degree of GlyB site saturation perhaps due to different NR subunit combinations. Although each of the GlyB site agonist potentiated individual NMDA EPSC significantly, the potentiation of the NMDA EPSC by glycine versus d-serine (at 10 and 100 µM) did not reach statistical significance ($P > 0.05$).

One action of glycine is to accelerate the recovery of NMDA receptors from desensitization (Benveniste et al. 1990; Lerma et al. 1990; Mayer et al. 1989; Vyckicky 1993). As a result, glycine lengthens the duration of the decay time constants ($\tau$) (Fig. 2B, top) of the evoked NMDA EPSCs. However, we found that glycine at 1, 10, and 100 µM exerted an inverted-U dose-response profile for the NMDA EPSC in PFC slices (Fig. 2D, right). While there was no overall change in the mean $\tau_{\text{Fast}}$ at any dose of [glycine]$_o$, the $\tau_{\text{Slow}}$ was significantly ($P < 0.02$) enhanced by glycine only at 10 µM (control = 182 ± 6.2 ms; 10 µM glycine = 274 ± 34.1 ms; $F(3,41) = 4.58$; $P < 0.01$; Dunnett’s test, $P < 0.01$). A further increase of [glycine]$_o$ to 100 µM did not further increase the $\tau_{\text{Slow}}$. Hence, the mean $\tau_{\text{Slow}}$ at 100 µM glycine did not differ from the control value ($P > 0.05$).

The GlyB site agonist d-serine (Brugger et al. 1990) potentiated the peak amplitude and $\tau_{\text{Slow}}$ of the NMDA EPSCs in individual neurons (Fig. 2B), thus yielding a net dose-dependent increase in the integrated area (Fig. 2C). In some individual PFC neurons, although d-serine showed a dose-dependent

**FIG. 2.** Dose-dependent potentiation of the NMDA EPSC by glycine and d-serine and prolongation of mainly the slow decay time constant of the NMDA EPSC. **A–C:** increase of extracellular glycine (**A**), d-serine (**B**) dose dependently augments the peak and integrated area (**C**) of the evoked NMDA EPSCs in a PFC neuron (*$P < 0.05$; **$P < 0.005$). The superimposed gray traces in the last trace of **A** and **B** are control traces that are used for comparison. Control traces were obtained in nominally glycine-free (<100 nM from high-performance liquid chromatography (HPLC) analysis). ACSF: D; NMDA EPSC decay time constant typically show 2 components (fast and slow $\tau$). There was no change in the fast $\tau$ with elevation of extracellular glycine concentrations. Increased concentration of extracellular glycine increases the slow $\tau$ $\approx$ 10 µM compared with the control ($*P < 0.05$). However, at 100 µM, there was no change in the slow $\tau$ with respect to the control. **Inset:** average trace of the NMDA EPSC in control and in glycine (10 µM). The traces are scaled to be the same as the control peak. Glycine moderately increases the slow decay time constant of the NMDA EPSC. **E:** in individual neurons an increase concentration of extracellular d-serine also augments the slow component of the decay time constant of the NMDA EPSCs but there is no effect on the fast decay time constant. However, group data analysis by analysis of variance (ANOVA) shows no dose-dependent change in the NMDA EPSC $\tau_{\text{Slow}}$ with incremental dose of d-serine. **Inset:** average trace of the NMDA EPSC in control and in d-serine (10 µM). The traces are scaled to be the same as the control peak and show that d-serine (10 µM) induced a moderate increase in the $\tau_{\text{Slow}}$ of the NMDA EPSC.
trend in enhancing $\tau_{\text{slow}}$ of the NMDA EPSC (Fig. 2D). ANOVA test [$F(3,21) = 0.8; P = 0.5$] applied to group data failed to show a statistical dose-dependent change in the $\tau_{\text{slow}}$ of the NMDA EPSC ($P > 0.05$; Fig. 2E). Similar to glycine, d-serine at 1, 10, and 100 $\mu$M also did not change the $\tau_{\text{fast}}$ of the evoked NMDA-EPSC (Fig. 2E).

**GlyT1 inhibitor potentiates synaptically evoked NMDA current in PFC neurons in vitro**

In the absence of added extracellular glycine ($\leq 100$ nM glycine present in the solution), NMDA-EPSCs were also evoked by electrical stimulation of the local afferents. This suggests that a trace amount of glycine is likely to be present in the tissue and/or the perfusate (as trace contaminant or metabolic product). When the GlyT1 inhibitor NFPS was bath-applied (0.01 $\mu$M), it moderately potentiated the evoked NMDA EPSC ($\sim 15\%$). However, further increases in the concentration of NFPS (from 0.1 to 10 $\mu$M) failed to cause further increases in the evoked NMDA EPSCs, suggesting that the effects of glycine on NMDA EPSC was at its maximum when there was no added extracellular glycine present in the media.

Since extracellular glycine levels in the PFC in vivo are approximately 10 $\mu$M (Hashimoto and Oka 1997), we then included 10 $\mu$M glycine in our low Mg$^{2+}$ perfusate in the experiments to determine the effects of GlyT inhibitor NFPS on evoked NMDA EPSCs. Application of NFPS (from 0.01 to 10 $\mu$M) dose-dependently augmented the integrated area of the evoked NMDA-EPSCs (Fig. 3, B and C). Group data analysis of the decay $\tau$ of the NMDA EPSC shows that similar to glycine and d-serine, NFPS did not change the mean fast decay $\tau$ of the NMDA EPSC. Group analyses of the slow decay $\tau$ show that NFPS $\leq 1$ $\mu$M showed a trend in causing a dose-dependent lengthening of the NMDA EPSC [$F(4,31) = 3.43, P < 0.01$]. Only at 1 $\mu$M did NFPS show a statistically significant increase of the slow decay $\tau$ with respect to the control (Dunnett’s test, $P < 0.01$). Increasing the NFPS concentration to 10 $\mu$M (with 10 $\mu$M glycine) had no effect on the slow decay $\tau$ of the NMDA EPSC.

To rule out the possibility that NFPS might potentiate the NMDA EPSC via other mechanisms, we have conducted an additional experiment. In the presence of a saturating concentration of GlyB site agonist d-serine (100 $\mu$M) that is not normally transported by GlyT1 (Broer et al. 1990; Ribeiro et al. 2002; Schell et al. 1995, 1997; Snyder and Ferris 2000; Snyder and Kim 2000; Supplisson and Bergman 1997), the fully potentiated NMDA EPSC could not be further changed by the GlyT1 inhibitor NFPS (in 10 $\mu$M glycine) (Fig. 3E). This result suggests that NFPS did not have any additional unanticipated properties on NMDA EPSC.

It is difficult to absolutely rule out an increase in NMDA EPSC decay time constant by d-serine, glycine, or NFPS may also be due to a possible degraded voltage control under voltage-clamp when the EPSC has been greatly increased. However, activation of the GlyB site of the NMDA receptor by glycine or d-serine has been shown to prolong the decay kinetics of the NMDA EPSC or miniature EPSC, and likewise, the decay time constant of NMDA EPSC can be reduced by the GlyB site antagonist HA966 (e.g., Berger et al. 1998; Lester et al. 1993). Our findings are consistent with the findings from these studies.

**In vivo experiments**

**EFFECTS OF D-SERINE AND NFPS ON IONTOPHORETIC NMDA-EVOKED EXCITATORY RESPONSES IN PFC NEURONS IN VIVO.** Systemic injection of the GlyT inhibitor NFPS (1–2 mg/kg iv) significantly enhanced the NMDA-evoked spike firing ($+76 \pm 10\%$; $P < 0.05$, $n = 6$; Fig. 4, A–D) in single PFC neurons in vivo (Fig. 4). Since NFPS does not interact with NMDA-R directly (e.g., NFPS does not displace MDL105519 or MK-801 binding of the glycine, or the channel pore sites, respectively, of the NMDA receptor, Bergeron et al. 1998; D. Calligaro, personal communication), the most plausible explanation would be that NFPS blocked the GlyT1 to enable sufficient glycine accumulate near NMDA-R to potentiate the NMDA-evoked firing. This finding may further support the suggestion that the GlyB site of the NMDA-R is not saturated in PFC.

D-Serine is moderately better than glycine in penetrating the blood-brain barrier when administered systemically (Oldendorf 1973). In our in vivo studies, we tested whether the GlyB site of the NMDA receptor in the PFC was saturated by using the GlyB site agonist d-serine to probe at this issue. Iontophoretically application of NMDA (20–60 nA) at an inter-application interval of approximately 1 min reliably elicited a significantly greater ($+89.77 \pm 8.75\%$, $P < 0.05$) number of spikes in these same neurons ($n = 6$). Since glycine and d-serine interact at the same GlyB site on the NMDA receptor, the enhancement of NMDA-evoked firing response by d-serine suggests that the GlyB site on the NMDA receptor, similar to that in visual cortex and hippocampus (Czepita et al. 1996; Dalkara et al. 1992; Mizutani et al. 1991; Salt 1989), is not saturated by endogenous glycine in rat PFC in vivo (Figs. 4 and 5).

**D-SERINE OR GLYT1 INHIBITOR REVERSES THE BLOCKADE OF GLYB SITE OF NMDA RECEPTOR BY (+)HA-966 IN PFC NEURONS IN VIVO.** To further determine how elevation of extracellular glycine may enable the endogenous glycine to interact with the GlyB site of the NMDA receptor and potentiate NMDA-evoked spike firing, we continuously applied iontophoretically a selective GlyB antagonist (+)HA-966 (Foster and Kemp 1989) to block the NMDA-evoked firing response first. We then administered d-serine to compete with HA-966 at the GlyB sites of the NMDA receptor, or the GlyT1 inhibitor NFPS to block GlyT1 (Atkinson et al. 2001).

In separate series of experiments using continuous iontophoretic application of (+)HA-966, we aimed to block the NMDA-evoked firing responses by $\sim 50\%$, or by $\sim 80\%$ to determine the ability of the GlyB site agonist d-serine or NFPS to reverse the two levels of NMDA-R blockade. After achieving steady-state blockade of the NMDA evoked responses, we injected intravenous d-serine (50 mg/kg) or NFPS (5 mg/kg). In the group of PFC neurons with a partial (52 $\pm 15.6\%$ of control) blockade of the NMDA-evoked responses, both d-serine (87.5 $\pm 5.5\%$ of control; $P < 0.05$; Fig. 5B) or NFPS (113.8 $\pm 14.4\%$ of control; $P < 0.05$; Fig. 6, A–C) signifi-
cantly reversed the blockade \((n = 6\) in each group). In the other group of PFC neurons with a greater blockade of the NMDA-evoked response (down to \(23.86 \pm 3\% \) of control) by continuous iontophoretic application of \((-\)HA-966), intravenous D-serine (50 mg/kg iv) was able to reverse significantly \((P < 0.05)\) the blockade to 60.3 \pm 16\% of control. These findings suggest that, while D-serine directly competes with \((+\)HA-966 at the GlyB site to restore NMDA-evoked responses, NFPS may have enhanced extracellular glycine levels sufficiently to displace \((-\)HA-966 and hence restore NMDA-evoked firing responses.

**DISCUSSION**

Evoked NMDA-EPSCs were potentiated dose-dependently by GlyB site agonist glycine and D-serine, and by the GlyT inhibitor NFPS (in the presence of 10 \(\mu\)M extracellular glycine).
cine) in vitro. In vivo electrophysiological data show that excitatory responses to microiontophoretic application of NMDA were potentiated by intravenous administration of the GlyT inhibitor NFPS alone, or by the GlyB site agonist d-serine. Partial blockade of the GlyB site by continuous (+)-HA-966 was reversed by intravenous d-serine or NFPS. Some of

FIG. 4. The GlyT1 inhibitor NFPS potentiated NMDA-evoked firing of single PFC neurons in vivo. A: continuous voltage trace illustrating spike firing evoked by direct iontophoretic application of NMDA onto the PFC neuron. B: the same NMDA-evoked firing was enhanced 15 min following intravenous administration of NFPS (accumulative dose of 2 mg/kg). C: frequency-time histogram (binwidth = 10 s) showing a gradual enhancement of the NMDA-evoked firing following intravenous administration of NFPS. Note that the same NMDA iontophoretic pulses (~45 nA, 7 s applied iontophoretically every minute) were applied throughout the entire experiment. D: histograms summarizing the group data and showing a significant enhancement of the NMDA-evoked firing following NFPS administrations. *P < 0.05.

FIG. 5. The GlyB agonist d-serine augmented NMDA-evoked firing in PFC neuron and reversed partial blockade of the NMDA-evoked firing by iontophoretic application of the GlyB site NMDA antagonist HA-966. A: frequency-time histogram showing dose-dependent potentiation of NMDA-mediated firing responses in a PFC neuron. Binwidth = 5 s. In this PFC neuron intravenous administration of 50 mg/kg of d-serine induced a 45.3% increase in NMDA-evoked firing. An additional 50 mg/kg d-serine (= accumulated dose of 100 mg/kg) induced a 114% increase in NMDA-evoked firing. B: iontophoretic NMDA pulse evoked firing in a PFC neuron was blocked partially by continuous iontophoretic application of (+)-HA-966 (60 nA). Intravenous d-serine (50 mg/kg) was able to reverse the partial blockade of the NMDA-evoked firing, suggesting that d-serine acts by competing with the HA-966 at the unsaturated GlyB site of the NMDA receptor in PFC neurons in vivo.
these results suggest that the GlyB site on the NMDA receptor in PFC are likely to be unsaturated in vivo and can be modulated by manipulating extracellular glycine levels near the NMDA-R at the glutamate synapses.

In nominal absence of extracellular glycine [high-performance liquid chromatography (HPLC) analysis of ACSF showed <100 nM glycine present], NMDA EPSC can clearly be synaptically evoked and pharmacologically isolated in our study. Endogenous GlyB site NMDA agonists such as D-serine might be present in the slices and acted as a co-agonist. Since D-serine is not taken up by GlyT1 (Broer et al. 1990; Ribeiro et al. 2002; Schell et al. 1995, 1997; Snyder and Ferris 2000; Snyder and Kim 2000), it can positively modulate evoked NMDA EPSCs in the absence of glycine. When no glycine is included in the perfusate, NFPS potentiated the NMDA EPSCs only at a concentration of 0.01 μM. When a physiological level of extracellular glycine concentration (10 μM) is present in the ACSF, NFPS dose-dependently augments synaptically evoked NMDA EPSC in PFC slices. Further increase in the concentration of NFPS (0.1–1 μM) did not additionally augment the NMDA EPSCs. This suggests that NFPS may only block the uptake of a steady-state level of a trace amount of endogenous glycine present in the slice (<100 nM measured) to augment the NMDA EPSC.

At high doses of glycine (100 μM) or NFPS (10 μM; in the presence of 10 μM glycine), the NMDA EPSC peak was potentiated, but the slow decay time constant did not differ from the control. One possibility is that the enhanced NMDA EPSC will increase enough Ca$^{2+}$ influx via NMDA-R to activate calcineurin to cause a glycine-insensitive NMDA-R desensitization (Legendre et al. 1993; Rosenmund et al. 1995; Tong et al. 1995). Thus at a higher concentration of extracel-
lular glycine, the secondary events (i.e., onset of Ca\textsuperscript{2+}-mediated NMDA-R desensitization) can prevent this glycine prolongation of the late $\tau_{\text{slow}}$, while the high glycine continued to increase early NMDA receptor channel openings (see Fig. 2 and Table 1 in Parsons et al. 1993).

The issue regarding a glycine saturation of the high-affinity GlyB ($K_0 = 0.1 - 0.3 \mu M$) site of the NMDA-R in vivo has been controversial (see INTRODUCTION). Although microdialysis studies in freely moving animals have shown that the extracellular concentration of glycine in the rat PFC may be as high as approximately 10 $\mu M$ (Hashimoto et al. 1995), glycine levels at the GlyB site of the NMDA-R at the synapse may be much less than 1 $\mu M$ due to the efficient glycine uptake by the GlyT1 (Supplisson and Bergman 1997). Our in vivo electrophysiological recordings showed that by blocking the GlyT1 using NFPS, NMDA-evoked firing of PFC neurons was progressively potentiated. Furthermore, systemic injection of the GlyB site agonist $\alpha$-serine alone also enhanced the NMDA-evoked firing of single PFC neurons.

Our iontophoretic experiments also showed that the target site of $\alpha$-serine is likely to be the GlyB site of the NMDA receptor. When the GlyB site antagonist (+) HA-966 was iontophoretically applied continuously to partially block (50 or 80%) the excitatory responses to iontophoretic NMDA applications, intravenous injection of either the GlyB site agonist $\alpha$-serine or the GlyT1 inhibitor NFPS reversed the blockade of NMDA-evoked firing by (+)HA-966 in single PFC neurons. $\alpha$-Serine may directly displace (+)HA-966 from their GlyB site occupancy and stimulate the NMDA GlyB site. $\alpha$-Serine is known to penetrate the blood-brain barrier moderately better than glycine (Oldendorf 1973). In our present study, intravenous administration of $\alpha$-serine enabled co-activation of the GlyB site when exogenous NMDA was iontophoretically applied, thus functionally potentiating NMDA-evoked firing and reversed GlyB antagonist (+)HA-966 partial blockade of the NMDA-evoked responses in vivo. On the other hand, NFPS may block GlyT1, resulting in elevation of sufficient endogenous glycine levels near the NMDA receptor to displace (+)HA-966 from the NMDA GlyB site, thus allowing the endogenous glycine to stimulate GlyB site and potentiate NMDA-induced firing. It is likely that the transport actions of GlyT1 and/or intracellular glycine sequestration may exceed the $K_0$ of the glycine-binding site and help to rapidly keep [glycine]o near the NMDA-R to low levels (e.g., <1 $\mu M$) (Supplisson and Bergman 1997). Taken together, this evidence suggests that the GlyB sites on NMDA receptor are not likely to be saturated by endogenous glycine in vitro and in vivo in the PFC.

Although it is used as an exogenous GlyB site agonist in this study, endogenous $\alpha$-serine is also synthesized in glia cells and is highly concentrated in forebrain areas enriched in NMDA-R (Schell et al. 1995, 1997). $\alpha$-Serine is not a substrate for the GlyT1, but is transported by the ASC2 system of transporters (Broer et al. 1990; Ribeiro et al. 2002). Synaptically released glutamate may activate ionotropic glutamate receptors on astroglia to release $\alpha$-serine. In turn, the released endogenous $\alpha$-serine can serve to co-activate NMDA-R on adjacent postsynaptic neurons (Baranano et al. 2001; Snyder and Kim 2000). However, during repeated iontophoretic NMDA application to establish a stable baseline prior to any systemic drug injections, we did not observe a gradual increase in NMDA-evoked firing over time. Hence, it is unlikely that the iontophoretic application of exogenous NMDA stimulates glia $\alpha$-serine release in vivo.

Hypofunction of the glutamate/NMDA receptor system has been implicated in the pathophysiology of schizophrenia (Javitt and Zurkin 1991; Tsai and Coyle 2002). The clinical finding that GlyB site stimulation following administration of a large quantity of exogenous glycine (because of its poor CNS penetration) or $\alpha$-serine as an adjunct to atypical antipsychotics improves schizophrenic symptoms supports the hypothesis that hypo-NMDA system is likely to be involved in the complex pathophysiology of schizophrenia (Javitt et al. 1994; Tsai et al. 1998). The current finding that blocking the GlyT1 may augment endogenous glycine to a level sufficient to potentiate NMDA-R function in vivo may provide a good rationale to
implement this kind of strategy for treating schizophrenia. Perhaps the more important question remaining is to determine to what extent that potentiation of NMDA-R function is beneficial and to address possible excitotoxicity. Animal studies show a lack of neurotoxic damage following long-term pharmacological glycine exposure (Patel et al. 1990; Shoham et al. 1999) and that other glycine transporter inhibitors reverse behavioral hyperactivity caused by psychotomimetic NMDA receptor blocker ketamine (Javitt et al. 1999). These findings further provide a compelling rationale for using GlyT1 inhibitors to indirectly potentiate NMDA receptor functions safely in schizophrenia.

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REFERENCES


