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Glycine transport accounts for the differential role of glycine vs. D-serine at NMDA receptor coagonist sites in the salamander retina

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Abstract

In this study, we demonstrate that D-serine interacts with *N*-methyl-D-aspartate receptor (NMDAR) coagonist sites of retinal ganglion cells of the tiger salamander retina by showing that exogenous D-serine overcomes the competitive antagonism of 7-chlorokynurenic acid for this site. Additionally, we show that exogenous D-serine was more than 30 times as effective at potentiating NMDAR currents compared with glycine. We thus examined the importance of glycine transport through the application of selective antagonists of the GlyT1 (NFPS) and GlyT2 (ALX-5670) transport systems, while simultaneously evaluating the degree of occupancy of the NMDAR coagonist binding sites. Analysis was carried out with electrophysiological recordings from the inner retina, including whole-cell recordings from retinal ganglion cells and extracellular recordings of the proximal negative field potential. Blocking the GlyT2 transport system had no effect on the light-evoked NMDAR currents or on the sensitivity of these currents to exogenous D-serine. In contrast, when the GlyT1 system was blocked, the coagonist sites of NMDARs showed full occupancy. These findings clearly establish the importance of the GlyT1 transporter as an essential component for maintaining the coagonist sites of NMDARs in a non-saturated state. The normal, unsaturated state of the NMDAR coagonist binding sites allows modulation of the NMDAR currents, by release of either D-serine or glycine. These results are discussed in light of contemporary findings which favor D-serine over glycine as the major coagonist of the NMDARs found in ganglion cells of the tiger salamander retina.

Keywords

GlyT1; PNFP; retinal ganglion cell; whole-cell recording

Introduction

N-methyl-D-aspartate receptors (NMDARs) are excitatory glutamate receptors extensively distributed throughout the nervous system. They contribute to modulation of synaptic transmission in a broad range of physiological events, including long-term potentiation (Martin *et al.*, 2000). In addition, NMDARs have been implicated in clinically relevant disorders such as schizophrenia (Coyle *et al.*, 2003) and in excitotoxic actions of glutamate (Lipton, 2001). In the retina, NMDARs are expressed in amacrine and ganglion cells of the inner retina and contribute to light-evoked activity described in both cell types (Slaughter & Miller, 1983; Massey & Miller, 1990; Mittman *et al.*, 1990; Dixon & Copenhagen, 1992).

Among the unique properties of NMDARs is the requirement for a second agonist, in addition to glutamate, to initiate channel gating (Johnson & Ascher, 1987). Early experiments suggested that glycine was the essential coagonist; however, subsequent studies determined that other amino acids could serve this function, including D-serine (Kleckner & Dingledine, 1988). Both amino acids act at a single, unique binding domain of the NR1 subunit (McBain *et al.*, 1989; Dingledine *et al.*, 1999). The dogma that D-amino acids played no functional role in higher organisms initially made D-serine an unlikely candidate. However, Hashimoto *et al.* (1992), using an HPLC method for separating amino acid enantiomers, measured high levels of D-serine in the brain. Subsequent studies showed that D-serine had a distribution similar to that of NMDARs (Hashimoto *et al.*, 1993) and a synthesizing enzyme, serine racemase, was isolated and characterized (Wolosker *et al.*, 1999). Initially, serine racemase was localized to astrocytes, but more recent studies have suggested that it may also be present in neurons in the brain (Kartvelishvili *et al.*, 2006). The use of D-serine-degrading enzymes has shown that D-serine functions as an endogenous coagonist in the brain and the retina (Mothet *et al.*, 2000; Gustafson *et al.*, 2007).

In the retina, early studies of NMDAR coagonist function, using retina slice preparations, suggested that the coagonist sites were saturated: bath-applied coagonists did not enhance NMDAR currents (Gottesman & Miller, 1992; Lukasiewicz & Roeder, 1995), but more recent studies of the intact retina found that NMDAR coagonist sites were not saturated (Stevens *et al.*, 2003; Gustafson *et al.*, 2007). D-Serine and serine racemase have been identified and localized to Müller cells and astrocytes (Stevens *et al.*, 2003; Williams *et al.*, 2006), and recent evidence found that ganglion cells in the mouse retina may also express serine racemase (Dun *et al.*, 2008; Kalbaugh *et al.*, 2009). Glycine is used as an inhibitory neurotransmitter and is found at relatively high levels in a subset of amacrine cells (Miller *et al.*, 1977; Pourcho & Goebel, 1987; Marc, 1989; Grunert & Wassle, 1993; Menger *et al.*, 1998).

The present study examined NMDAR coagonist function in the salamander retina by comparing the efficacy of D-serine and glycine. We first investigated what until now has been taken for granted, i.e. that in the retina, D-serine and glycine affect NMDAR currents through direct interaction with the coagonist binding site. Subsequently, we examined the efficacy with which NMDAR currents in retinal ganglion cells are enhanced by exogenous D-serine or glycine. Finally, we took a first step in addressing the mechanisms through which NMDAR coagonist occupancy is determined by evaluating the role of high-affinity glycine uptake in coagonist availability. The findings are discussed in light of the evidence that D-serine plays a major role in NMDAR coagonist function.

Methods

Animal maintenance and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Animals were killed by decapitation, which was followed by double pithing. Tiger salamanders were purchased from a dealer (Charles D. Sullivan Co., Nashville, TN, USA) and were maintained in circulated cold-water tanks (4°C) with a 12-h room light/dark cycle.

Whole-cell recordings (WCRs)

Two unique retinal preparations from tiger salamanders (*Ambystoma tigrinum*) were utilized: (i) an isolated flatmount retina (Stevens *et al.*, 2003) and (ii) an intact retina-eyecup preparation. The experiments utilizing the flatmount preparation (see Figs 1 and 2) began by removing the cornea and lens and draining the vitreous (Miller & Dacheux, 1976). The internal limiting membrane was then removed either mechanically, by removing atomized

alumina particles adherent to the surface of the retina with jewelers' forceps, or by enzymatic digestion with collagenase and hyaluronidase. No physiological differences were noted between the two methods of vitreous removal. The retina was then removed from the pigment epithelium by use of a blunted, fire-polished glass micropipette and placed, ganglion cells up, over a 1-mm hole in the center of a 13-mm-diameter nitrocellulose filter paper (Millipore, Billerica, MA, USA). Eyecup preparations began with enucleation of the eye and placing a crystal of dextran-conjugated tetramethylrhodamine (10 000 mol. wt; Invitrogen, Carlsbad, CA, USA) onto the severed optic nerve. The eyecup was then half immersed, cornea up, in a 6-mm-diameter, 2-mm-deep well filled with warmed 4% agarose (Type IX-A) in normal amphibian Ringer solution containing (in mM) 110 NaCl, 2.5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 HEPES and 5 D-glucose (pH 7.8). After cooling, the cornea, lens and vitreous were removed as above, and the eyecup was treated with collagenase and hyaluronidase. The eyecup (in agarose) was then placed in a 15-mL plastic centrifuge tube half filled with normal Ringer solution, secured in a light-tight container on ice, and bubbled with oxygen for 2–5 h while the dye diffused into the ganglion cells. The eyecup, still in agarose, was removed from the oxygenated Ringer solution, placed on a tissue slicer and a vertical cut was made which removed a side of the eyecup, roughly one-quarter to one-third of the diameter of the intact eyecup. This was done to permit recording micropipettes to access the retina under the water-immersion objective. At this point, the retina preparations were placed in custom-designed chambers mounted on the stage of an Olympus BX50WI microscope and connected to a gravity-fed perfusion system at a flow rate of 1–1.5 mL/min with a cooled (18°C), oxygenated, normal amphibian Ringer solution. Isolated retinas were transilluminated with infrared light (780 nm) and eyecup preparations were visualized with fluorescent microscopy. Each preparation was visualized with a CCD camera attached to a small LCD screen or video monitor. Isolated retina preparations were used for experimental data presented in Figs 1 and 2, whereas the retina–eyecup preparation was used for data presented in Fig. 3.

All whole-cell recordings were obtained from identified retinal ganglion cells. In retina–eyecup preparations, ganglion cells were observed via retrograde label as described above, and in isolated retinas, 5, 6-carboxyfluorescein (Invitrogen) was added to the pipette solution to allow cell and axon visualization following each experiment. Patch electrodes (5–15 MΩ) were pulled using a P-97 Brown Flaming Pipette Puller (Sutter Instruments, Novato, CA, USA) and filled with an intracellular solution containing (in mM): 98.0 KCH₃SO₄, 3.5 NaCH₃SO₄, 3.0 MgSO₄, 1.0 CaCl₂, 11.0 EGTA, 5.0 HEPES, 2.0 D-glucose, 1.0 glutathione, 1.0 MgATP, 0.5 NaGTP (pH 7.4). Positive pressure was applied to the solution in the recording pipette to access the surface of the ganglion cell under the enzymatically loosened inner limiting membrane. The pipette was held and manipulated with an MP-285 micromanipulator (Sutter Instruments). Dimpling of the fluorescently labeled ganglion cells in the eyecup preparation indicated the proximity of the pipette (not directly visualized) and its penetration through the inner limiting membrane in the eyecup preparation. After achieving a high-resistance seal onto the cell, the capacitance of the pipette was compensated by utilizing the automated circuitry of the Axoclamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA), or manually with the Dagan 3900 (Dagan Corporation, Minneapolis, MN, USA), each configured to record with a 10-kHz low-pass Bessel filter at a sampling frequency of 10 kHz. Signals were digitized with a Digidata 1320 and recorded in PClamp 9.0 (all Molecular Devices). Breakthrough to WCR was achieved using gentle negative pressure to the pipette. Each cell was characterized in current clamp mode with input resistance determined using small negative current injections, and the light-evoked response of the cell was determined by stimulating with a 2-s spot (diameter 250 μm) or bar (100 μm) of light centered on the recording electrode. The solution was then switched to the control solution, which consisted of (unless noted) a nominally magnesium-free version of the extracellular Ringer solution containing tetrodo-

toxin (TTX, 0.5 μM) to block action potentials and strychnine (10 μM) to block glycine receptors. Voltage-clamp studies were carried out after adjusting the clamp voltage to provide a standing current of zero (average membrane potential = -64.9 ± 4.8 mV), and series resistance was generally compensated 50–95%. Because of the small size of currents, the relatively low pipette resistance and the high input resistance of tiger salamander retinal ganglion cells (742.3 ± 183 M Ω), we did not correct for voltage errors associated with uncompensated series resistance. Voltages reported here are also not corrected for the liquid junction potential (calculated at -8.8 mV).

Light stimulation was provided by a computer-controlled LCD projector system using a tungsten-halogen light source (Burkhardt *et al.*, 1998) projected onto the retina through a 20 $\times$ microscope objective (Olympus, Melville, NY, USA) and focused on the plane of the ganglion cell layer. A Fish-Schurmann 6143 heat filter (New Rochelle, NY, USA) and neutral density wedges (Kodak, Rochester, NY, USA) were used to attenuate the full intensity of the lamp to a background intensity of 9 Cd/m². Unless indicated otherwise, light stimuli were centered on the recording electrode and consisted of a 2-s, 250- μm -diameter spot at a 1.0 log unit increase in contrast. The interstimulus interval was 20 s. Direct application of NMDA was achieved by applying a brief pressure pulse with a Picospritzer II (Genreal Valve Corp., Fairfield, NJ, USA) to a micropipette with a low-resistance tip filled with 1 mM NMDA in normal Ringer solution with an interstimulus interval of 1 min. In all experiments, 3–4 data traces were signal averaged in each stimulus condition and voltage clamp responses were evaluated as the total charge (integrated current) entering the cell during the 2-s light stimulus (ON response) and 2 s following the cessation of the stimulus (OFF response).

Extracellular recordings

Recordings of the proximal negative response (Burkhardt, 1970), or proximal negative field potential (PNFP), were obtained from retina-eyecup preparations, as described previously (Gustafson *et al.*, 2007). Briefly, following removal of the cornea and lens, the vitreous was drained and the eyecup was placed in a perfusion chamber. The eyecup was continuously perfused with chilled (19°C), oxygenated Ringers solution. Glass microelectrodes, pulled and beveled to a tip resistance of a few megaohms, were manipulated into the retina to a depth of maximum PNFP amplitude, approximately 50 μm from the retinal surface (Burkhardt, 1970). Alternating with a diffuse full-retina background light, a 110- μm -diameter spot of light from a 12-V tungsten-iodide lamp was positioned over the electrode tip and the intensity was adjusted using neutral density filters. The PNFP was amplified via a Grass P16 amplifier, A/D converted (DigiData 1200), and recorded on a PC using PClamp software (9.0). To maximize the NMDAR component of the PNFP, a control cocktail solution was used: (in μM) 10 NBQX, 10 strychnine, 50 picrotoxinin, 50 mecamylamine in nominally magnesium-free Ringer solution. The peak amplitudes of the responses in each experimental condition were compared.

Chemicals and agents

We obtained two glycine transporter blockers from NPS Allelix Corp (Toronto). (R)-(N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)pro-pyl])sarcosine (NFPS) is a GlyT1 antagonist (Aubrey & Vandenberg, 2001), and a potent GlyT2 antagonist, (2S,3R)-2-amino-3-[-(9-phenyl-9H-fluoren-9-yl)oxy]butanoic acid (ALX-5670), was kindly provided by Dr Methvin Isaac of Allelix (Mississauga, Canada). All other chemicals and agents used in these experiments were purchased from Sigma Chemical (St. Louis, MO) with the exception of hyaluronidase and collagenase (Worthington Chemical, Lakewood, NJ, USA); TTX (Alamone labs, Jerusalem, Israel); picrotoxin (Fluka, Neu-Ulm, Switzerland); KCH₃SO₄ (Pfaltz & Bauer); D,L-2-amino-phosphonoheptanoate (AP7); 5, 7-dichlorokynurenic acid

(DCK); and 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxalinedione (NBQX; Tocris, Ellisville, MO, USA).

Statistics and fitting

Electrophysiological data were analysed in Clampfit 9.0 (Molecular Devices) to determine peak amplitude and charge. Because each cell had unique response properties, the responses in different pharmacological treatments were normalized to the control of each cell. This allowed for across-cell comparisons of changes that resulted from the treatments. All data were normalized, presented graphically, fit and analysed statistically via Origin v.7.5 (Northampton, MA, USA). All results are expressed as mean $\pm$ SEM. Dose-response data were fit with the following DoseResp equation in Origin:

$$y=A1+\frac{A2-A1}{1+10^{(\log x_0-x)^p}}$$

where A1 is the bottom asymptote (fixed at 100 in Fig. 2B), A2 is the upper asymptote, $\log x_0$ is the ED₅₀ value, and p is the Hill coefficient (not evaluated because of system complexity). A paired, one-tailed Student's t -test was used to compare values between treatment groups. A P -value of less than 0.05 was considered significant for all statistical analyses.

Results

D-Serine potentiates NMDAR-mediated currents through its actions at the coagonist binding site. In previous studies, we have shown that NMDAR currents can be enhanced by the addition of exogenous D-serine (Stevens *et al.*, 2003; Gustafson *et al.*, 2007). Although it was assumed, as has been reported (McBain *et al.*, 1989), that this was the result of D-serine acting on the coagonist site of the NMDAR, this assumption has not been experimentally validated in the retina. We verified this by examining the ability of D-serine to overcome the block of 7-chlorokynurenic acid (7CKA, 25 μ M), a competitive antagonist at the coagonist site (Kleckner & Dingledine, 1989), before looking further into the effectiveness of D-serine and glycine. NBQX (10 μ M), picrotoxin (50 μ M), strychnine (10 μ M) and TTX (0.5 μ M) were added to an Mg²⁺-free Ringer solution to isolate the NMDAR contribution to light-evoked synaptic activity in retinal ganglion cells (this solution is labeled as 'control' in Fig. 1B and C). This pharmacological combination blocked or significantly reduced the OFF response through the actions of NBQX on the AMPA/KA receptors between photoreceptors and OFF bipolar cells (example trace shown in Fig. 1A). In contrast, the ON pathway remained functional because the mGluR6 receptors that mediate the photoreceptor to ON bipolar light response were unaffected by the toxins in the control. Figure 1B shows a series of recordings of the ON response from a retinal ganglion cell continuously bathed in the control Ringer solution. The addition of D-serine to the bathing medium led to an increase in the light-evoked current, illustrating the presence of unoccupied coagonist sites under the control conditions. The light-evoked responses were virtually eliminated following the addition of 7CKA, demonstrating that in the control mixture, the response was mediated almost exclusively by NMDARs. 7CKA conferred a near-complete block of the light response, which did not change appreciably when 10 μ M D-serine was added to the 7CKA bathing solution (not shown due to largely overlapping traces). However, when 100 μ M D-serine was added to the 7CKA environment, substantial recovery of the light-evoked response was evident. This ability of D-serine to overcome the competitive blocking action of 7CKA provides strong evidence that D-serine was acting at the coagonist site of NMDARs of retinal ganglion cells.

The cumulative results from ganglion cells that were studied using the protocol described in Fig. 1B are summarized in Fig. 1C. The light-evoked currents measured in retinal ganglion cells increased following the application of 100 μ M D-serine ($33.2 \pm 5.7\%$; $n = 10$, $P = 0.001$), demonstrating that the NMDAR coagonist sites were not saturated under these experimental conditions. The application of 7CKA virtually eliminated the light-evoked response (decreased to $5.3 \pm 1.0\%$ of the control level; $n = 11$, $P < 0.0001$). Very little recovery of the light response was observed with 10 μ M D-serine (to $11.3 \pm 2.4\%$ of control levels), but was always prominent when 100 μ M D-serine was added to the 7CKA environment, reaching a level of $58.4 \pm 7.5\%$ of that in the control. The variability of the responses in this antagonist/agonist mixture was large, with a range of 33.5–80.4% of the control ($n = 7$, $P = 0.0002$ when results in 25 μ M CKA were compared with those in CKA and 100 μ M D-serine). These observations demonstrate that, at a concentration of 100 μ M, D-serine begins to rival the competitive antagonism of 25 μ M 7CKA at the NMDAR coagonist binding site.

D-Serine is a more effective coagonist than glycine at retinal NMDARs

NMDARs are activated by the simultaneous binding of both glutamate and either D-serine or glycine. In previous studies we have shown that the coagonist sites of retinal NMDARs are not saturated in isolated retina and eyecup preparations (Stevens *et al.*, 2003; Gustafson *et al.*, 2007). To investigate the effectiveness of exogenously applied glycine and D-serine in saturating NMDAR coagonist sites in the retina, we carried out dose–response evaluations using WCRs from retinal ganglion cells. Figure 2A illustrates the light-evoked inward currents recorded under voltage-clamp, WCR conditions in response to a 2-s light stimulus. The ganglion cells were held so that there was zero current at rest. TTX and strychnine were added to the Mg^{2+} -free Ringer solution to block spiking, limit network inputs from spiking amacrine cells, and block inhibitory glycine inputs that would complicate the results when exogenous glycine was added. In this example, the addition of glycine at 1 mM and of D-serine at 10 μ M to the control enhanced the light-evoked response by generating similar changes in the peak amplitude. At these concentrations, D-serine produced a slightly larger response than the 100 times higher concentration of glycine when comparing the total charge entering the cell during the light stimulus. This difference primarily reflected the slower decay of the response in the presence of D-serine.

The sensitivity of retinal ganglion cell light responses to bath applied glycine and D-serine are compared in Fig. 2B. Because no significant difference was apparent between the ON and OFF responses, the measurements of total charge of the ON and OFF responses at each coagonist concentration were combined and evaluated as a percentage of the response measured in the control solution. The two displaced dose–response curves for D-serine and glycine illustrate that, in the intact retina, exogenously applied D-serine was about 30 times more effective in potentiating light-evoked currents in retinal ganglion cells as compared with glycine (D-serine $ED_{50} = 3.48 \pm 1.05 \mu$ M, glycine $ED_{50} = 118 \pm 48.1 \mu$ M, $n = 7$).

As a companion procedure for studying the sensitivity of NMDARs to glycine vs. D-serine in response to light, we used a more direct method of activating NMDARs, through pressure-ejecting NMDA onto ganglion cells using an NMDA-filled (1 mM) glass micropipette positioned immediately above the soma of the recorded ganglion cell. With this arrangement, a brief pressure pulse to the electrode generated a consistent inward current from each ganglion cell studied. This method permitted the activation of a large NMDAR-mediated inward current, while minimizing the cellular network that is obligatorily activated by light stimulation. Figure 2C shows a series of traces in which the addition of 100 μ M D-serine to the bathing medium resulted in a large increase in the response to puffed NMDA, while the increase in response to the same concentration of glycine was substantially smaller. The response to NMDA application was entirely blocked by the NMDAR

antagonist DCK (30 μ M). The dose–response curves shown in Fig. 2D ($n = 8$) revealed a similar difference in efficacy between D-serine and glycine potentiation as was seen with light stimulation. Using these two different strategies, one based on light stimulation and the other based on activation of NMDARs through direct NMDA application, it was clear that NMDARs in retinal ganglion cells were substantially more sensitive to bath-applied D-serine compared with glycine. Furthermore, these comparative results suggest that the addition of glycine or D-serine to the control does not alter the retinal network in such a way as to reveal any difference in the sensitivity to glycine vs. D-serine as an NMDAR coagonist. Thus, the difference in the efficacy between glycine and D-serine on the NMDAR responses of retinal ganglion cells can be largely explained by events occurring between the point of application and the microenvironment of the ganglion cell NMDARs.

Blocking glycine transport potentiates the NMDAR-mediated currents of the inner retina

Two types of high-affinity glycine transporters have been identified in the brain, the GlyT1 and GlyT2 transporters. The GlyT1 is considered to play a major role in setting the extracellular level of glycine (Supplisson & Bergman, 1997) and has been localized to amacrine cells in mammalian and chick retinas (Pow & Hendrickson, 1999) and Müller cells in the amphibian (Lee *et al.*, 2005). Figure 3A demonstrates the potentiation of NMDAR-mediated, light-evoked currents from a retinal ganglion cell following the application of NFPS, a selective GlyT1 antagonist which was shown to be effective in blocking GlyT1 currents in oocytes at a threshold of 10 nM with no effect on GlyT2 (Aubrey & Vandenberg, 2001). In this example, the light-evoked ON and OFF currents were recorded from a cell whose activity was dominated by the OFF response. Time-expanded insets for the ON and OFF responses are included for clarity. When NFPS (10 μ M) was added to the control solution, both ON and OFF responses were enhanced in amplitude and prolonged in duration (Fig. 3A; NFPS, blue trace; control, black trace). In this cell, the small ON response became biphasic in the presence of NFPS. Although we have observed this phenomenon on several occasions when potentiating ganglion cell currents, it was not a characteristic feature of the response to NFPS and we therefore did not carry out additional studies that might help explain the appearance of this biphasic ON response. The addition of D-serine to the NFPS environment, at a concentration sufficient to saturate the coagonist sites (100 μ M), did not further enhance the response (green trace), demonstrating that the coagonist sites were saturated by blocking the GlyT1 transporter. The NMDAR antagonist AP7 (red trace) reduced the response to a level similar to that achieved when coapplied with NFPS (purple trace).

Figure 3B illustrates the effects of blocking the GlyT1 transporter on the extracellularly recorded PNFP. As an extracellular signal, the PNFP provides a major advantage over WCRs for studying long-term actions of pharmacological agents in a manner similar to the field potentials used for analysis of long-term potentiation (Teyler & DiScenna, 1987). Previous work has demonstrated that large NMDAR currents can be observed in the PNFP, and in a control cocktail (black trace; see Methods), the response to the onset of a focal light stimulus is almost entirely mediated by NMDARs (Gustafson *et al.*, 2007). The addition of NFPS (blue trace) to the control cocktail resulted in an increase in PNFP amplitude. In the presence of NFPS, the addition of D-serine (green trace) did not further augment the light-evoked response. When AP7 was added to the bathing medium, a significant reduction in the light response demonstrated the large contribution provided by NMDARs to the PNFP (red trace).

Figure 3C summarizes the results of our studies of NFPS by displaying the pharmacological conditions relative to those of the controls and divides the results between WCR from retinal ganglion cells (left) and studies of the PNFP (right). The total charge entering the cell for both the ON and the OFF components of the light response in the presence of NFPS was

significantly increased when compared with the control values (ON $125.0 \pm 9.4\%$, OFF $126.3 \pm 13.9\%$ of control levels; $n = 7$; $P = 0.019$ ON, 0.039 OFF). Similarly, in the presence of NFPS, the peak amplitude of the PNFP was $124.3 \pm 3.7\%$ of that measured in the control cocktail ($n = 6$, $P = 0.0023$). The subsequent addition of D-serine to the NFPS-containing bathing solutions did not significantly affect the responses. When AP7 was added to the NFPS environment, the evoked charge for ON and OFF ganglion cell response components and the peak of the PNFP were reduced, confirming the presence of NMDAR-mediated currents (WCRs were reduced to ON $73.5 \pm 3.7\%$; OFF $76.9 \pm 2.5\%$, and the PNFP by $30.4 \pm 7.5\%$ of control levels). No additional reduction in the ON and OFF components of ganglion cells was observed when NFPS was added following NMDAR blockade with AP7. This suggests that the enhancement of the response by NFPS occurred through its actions on the NMDAR-mediated currents. We conclude that under the conditions of our experiments, the GlyT1 transport system operates to keep glycine levels significantly below the saturation point for the NMDAR coagonist sites. When this uptake system was blocked, the external levels of glycine were elevated so that full occupancy of the coagonist sites was achieved, given that the addition of D-serine did not further enhance the responses.

A second member of the glycine transport family, GlyT2, has been found throughout the brain (Liu *et al.*, 1993) and has recently been reported in the retina (Salceda, 2006; Jiang *et al.*, 2007). Pentenoate derivatives were one of the earliest compound classes that provided antagonist discrimination for the GlyT2 transporter over that of GlyT1 (Isaac *et al.*, 2001). Although these compounds had IC_{50} values as low as $1.03 \mu M$ against the GlyT2 transporter, they had only modest (about 10-fold) selectivity for GlyT2 over GlyT1. A substantial improvement on the selectivity for GlyT2 over GlyT1 was achieved when butanoic derivatives were developed by NPS Pharmaceuticals (Toronto, Canada). The compound we obtained from NPS Pharmaceuticals and used in our studies was ALX-5670. Measured in *in-vitro* studies of transfected cells, this butanoic derivative had an IC_{50} value for GlyT2 of 16 nM , with no effect observed on GlyT1 up to the $10\,000 \text{ nM}$ tested (I. Methvin, personal communication, NPS Pharmaceuticals). We used this selective GlyT2 antagonist to study the role GlyT2 transporters play in regulating glycine concentrations at NMDARs in the retina. Figure 3D illustrates the results of adding ALX-5670 ($100 \mu M$) to the control solution while recording whole-cell light responses from a retinal ganglion cell. The voltage-clamp response was unaffected by blocking the GlyT2 transporters with ALX-5670. The subsequent addition of D-serine resulted in an enhancement of the light response. The increases in both the initial peak amplitude and the slower time course of response recovery in the presence of D-serine confirmed that the NMDARs of the ganglion cell were not saturated in the control environment.

Similar to our studies with WCRs from retinal ganglion cells, we found no effect of ALX-5670 on the PNFP, as can be seen in the overlapping responses in the black (control cocktail) and blue (ALX 5670) traces in Fig. 3E. Cumulative results of the peak response of the PNFP (right) and the total charge entering the cell at the onset and offset of the light stimulation showed no effect from the addition of the GlyT2 antagonist ALX-5670 (Fig. 3F: ON $104.2 \pm 8.9\%$, OFF $102.6 \pm 5.5\%$, PNFP $100.2 \pm 0.9\%$ of control response; $P = 0.66$ ON, 0.41 OFF, 0.82 PNFP). The enhancement of the responses by the addition of D-serine confirmed that the NMDAR coagonist sites were not saturated, just as in the responses seen in previous studies (Stevens *et al.*, 2003; Gustafson *et al.*, 2007) and in Figs 1 and 2. Together, the results of our NFPS and ALX-5670 experiments support a role for the GlyT1 glycine transporter but not for the GlyT2 transporter in regulating glycine levels in proximity to NMDARs of retinal ganglion cells.

Discussion

The results from these experiments provide new insight into the functional significance of glycine transporters in the retina. Previous studies have demonstrated the essential role that glycine transporters play in the accumulation of glycine for neurotransmission through glycinergic amacrine cells of the retina (Pow, 1998). These cells form a prominent component for lateral inhibitory actions in the retina (Miller *et al.*, 1977; Marc, 1989). However, the findings here illustrate a new and different function for the GlyT1 transporter, that of keeping external glycine levels sufficiently low that the coagonist sites of NMDARs found in ganglion cells have a relatively low state of occupancy. This potentially allows the NMDARs to be modulated through their coagonist sites. When the GlyT1 transporter was blocked with the highly selective GlyT1 antagonist NFPS (Aubrey & Vandenberg, 2001), NMDAR coagonist sites became saturated and the NMDAR currents were no longer enhanced by application of exogenous D-serine. Thus, glycine transport in the retina clearly serves two functions. On the one hand, it appears essential for normal cycling of glycine to support glycinergic inhibitory neurotransmission, and on the other, GlyT1 transport also keeps external glycine sufficiently low such that the occupancy state of NMDAR coagonist sites remains below saturation.

The NMDAR currents of retinal ganglion cells measured in response to either light or the focal application of NMDA were augmented in the presence of exogenously applied coagonist, indicating that in our intact retinal preparations, the level of endogenous coagonist was not sufficient to saturate the NMDAR response. Although exogenous application of glycine and D-serine were capable of saturating the NMDAR currents, D-serine was approximately 30 times more effective at potentiating these currents than glycine. This difference could be the result of an inherent preference of NMDARs for D-serine. Although some studies have shown that NMDARs have a slight preference for D-serine (Matsui *et al.*, 1995), others have suggested that glycine is favored as a coagonist (Snell *et al.*, 1988; McBain *et al.*, 1989), no consistent rank order preference has been established. However, the 30-fold difference we observed for NMDAR currents in response to glycine and D-serine exceeds the differential sensitivity of NMDARs studied in expression systems and is likely, therefore, to reflect the difference in uptake between the two amino acids. In the case of glycine, GlyT1 transporters form a high-affinity uptake system that may keep external glycine in the submicromolar range (Supplisson & Bergman, 1997). In contrast, the only known transport of D-serine in the retina occurs through a less sensitive, Na-dependent mechanism (O'Brien *et al.*, 2005) that has been characterized as an ASCT2 transporter – a hetero-exchanger capable of both uptake and release depending on concentration gradients of D-serine and the other neutral amino acids for which the transporter is selective (including alanine, L-serine and cysteine). In fact, the location of the ASCT2 on cultured Müller cells, the cells which synthesize D-serine, prompted the suggestion that it may function as an important release mechanism for D-serine in the retina rather than as an uptake mechanism (Dun *et al.*, 2007). Studies of mouse brain synaptosomes reported the presence of ASCT2 with an IC₅₀ for uptake of D-serine approaching 1 mM. In addition, the brain also contains the Na-independent asc-1 transporter. It has been proposed as an important, high-affinity reuptake mechanism for D-serine (Rutter *et al.*, 2007), but has not been reported in the retina. Thus, when external glycine is applied, the high-affinity transport system is likely to reduce glycine levels and apparently does so in close proximity to the NMDA receptors. The positioning of NMDARs and GlyT1 transporters in the inner plexiform layer appears to have overlapping representation, although ultrastructural details are still lacking. In the brain, GlyT1 transporters have been described adjacent to NMDARs in ultrastructural studies (Smith *et al.*, 1992; Cubelos *et al.*, 2005). In contrast to glycine, when D-serine is added to the bathing medium, the lack of a high-affinity transport system allows D-serine to be less encumbered by uptake and more effective in reaching and

activating NMDAR coagonist binding sites. Because blocking the GlyT1 transporters leads to saturation of the NMDAR coagonist sites, it is not possible to evaluate the importance of uptake vs. NMDAR coagonist site sensitivity for D-serine vs. glycine. This difference in uptake was suggested to play a similar role in rat brainstem (Berger *et al.*, 1998). In fact, the similarities between the results we found in the retina and those measured in hypoglossal motoneurons in the Berger study are striking. In both studies, D-serine effectively increased responses in the low micromolar range, while a significantly higher dosage of glycine (100 μ M +) was required to potentiate the NMDAR currents.

Although initial studies of the retina suggested that glycine transport was confined to the actions of GlyT1 in amacrine cells of mammals and chicks and in Müller cells of amphibians (Pow & Hendrickson, 1999; Du *et al.*, 2002; Lee *et al.*, 2005), more recent reports suggest a possible role for GlyT2 as well as an expanded role for GlyT1 (Perez-Leon *et al.*, 2004; Salceda, 2006; Jiang *et al.*, 2007). In the present study, we used specific inhibitors to these two transporters in order to examine their role in regulating glycine function at the coagonist sites of NMDARs. Our results suggest that the action of the GlyT1 transporter, but not that of the GlyT2, is responsible for limiting glycine activity at the NMDAR coagonist sites. Application of NFPS, a selective antagonist of GlyT1, was sufficient to increase the light-evoked currents of retinal ganglion cells as well as the extracellularly recorded PNFP. Presumably, this was due to an increase in the glycine concentration available at the coagonist sites of the NMDARs. This increased concentration was sufficient to saturate the coagonist sites, given that the subsequent addition of D-serine did not further augment the responses. NFPS had no measurable effect on whole-cell currents of retinal ganglion cells when coapplied with the NMDAR antagonist AP7, suggesting that NFPS was not affecting the responses through another mechanism. The GlyT2-specific blocker ALX-5670 did not affect the light-evoked responses measured in this study. Although this does not rule out the presence of GlyT2s in the retina, it does suggest that they do not play a role in the excitatory, glutamatergic pathways we studied. In addition, the lack of effect argues that ALX-5670 does not interact with GlyT1s, even at the relatively high dose used here, as an inhibition of GlyT1s leads to an increase in light-evoked responses.

The present study suggests that, in the absence of glycine uptake via GlyT1, glycine plays a significant role as an NMDAR coagonist. This does not mean, however, that glycine is occupying the coagonist sites under normal conditions. In fact, these results signify the only direct evidence that we have seen of endogenous glycine acting on NMDARs of the amphibian retina. We have previously demonstrated that when endogenous D-serine levels were enzymatically degraded, the light-evoked NMDA currents were reduced to levels similar to those observed when NMDARs were blocked with antagonists (Gustafson *et al.*, 2007). In view of these findings, the present study suggests that GlyT1 activity keeps glycine levels near NMDARs at sufficiently low concentrations so as to allow D-serine to play a major role as an NMDAR coagonist. In contrast, Kalbaugh *et al.* (2009) have used retinal slices of the mouse and rat to suggest that light-evoked glycine release serves a dynamic, modulatory coagonist function while D-serine plays a more static, background role. The prospect for both dynamic and static regulation of the NMDAR coagonist sites raises exciting new possibilities for the regulation of NMDARs and their role in retinal function.

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Abbreviations

7CKA	7-chlorokynurenic acid
ALX-5670	(2S,3R)-2-amino-3-[-(9-phenyl-9H-fluoren-9-yl)oxy]butanoic acid
AP7	D,L-2-amino-phosphonoheptanoate
DCK	5, 7-dichlorokynurenic acid
NBQX	2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxalinedione
NFPS	(R)-(N-[3-(4'-fluorophenyl)-3-(4'-phenyl-phenoxy)propyl])sarcosine
NMDAR	N-methyl-D-aspartate receptor
PNFP	proximal negative field potential
TTX	tetrodotoxin
WCR	whole-cell recording

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Fig. 1.

D-Serine potentiated NMDAR-mediated currents through its actions at the glycine binding site, using an isolated perfused retina preparation. (A) A voltage-clamp WCR of a retinal ganglion cell showing the increased ON response and the loss of the OFF response following the addition of a toxin mixture (TTX and strychnine) combined with the AMPA receptor antagonist NBQX. (B) A voltage-clamp WCR (holding -61 mV) from a retinal ganglion cell in an isolated retina in which the light response was evoked by a 2-s light stimulus (thick bar) in a control Ringer solution which isolated NMDA-mediated currents and inhibited OFF responses (see text for details). This control response was potentiated by D-serine ($100 \mu\text{M}$, dark gray trace) and blocked by the NMDAR coagonist site antagonist 7CKA ($25 \mu\text{M}$, light gray trace). This block was overcome by the addition of D-serine to the 7CKA solution ($100 \mu\text{M}$, gray trace). (C) Summary data plotting the total charge which entered the cell during the light stimulus as a function of the control response (* $P < 0.01$ compared with control, # $P < 0.01$ comparing the response in 7CKA alone with recovery in $100 \mu\text{M}$ D-serine + 7CKA). Error bars represent $\pm$ SEM).

Fig. 2.

D-Serine was a more effective agonist than glycine at retinal NMDA receptors. (A) A whole-cell voltage-clamp recording (holding potential -63 mV) of a ganglion cell in an isolated retina in response to a 2-s light stimulus in control Ringer solution (black trace) and with the addition of 10μ M D-serine (dark gray trace) and 1 mM glycine (gray trace). (B) Dose-response curves for D-serine (filled squares) and glycine (open circles) illustrating the total charge entering the cell in response to the light stimulus normalized to the control response. ON and OFF responses were combined. (C) A voltage-clamp WCR (holding -65 mV) from a retinal ganglion cell in an isolated retina preparation responding to NMDA (1 mM in control Ringer solution) pressure ejected from a glass micropipette positioned just above the retina. This response was potentiated when D-serine (100μ M, dark gray trace) or glycine (100μ M, gray trace) was added to the bathing medium. The response was completely blocked by the NMDA antagonist DCK (30μ M, light gray trace). (D) Summary data from eight cells plotting average responses to NMDA puff application ($\pm$ SEM) in different concentrations of D-serine or glycine as a percentage of the charge entering the cell in control Ringer solution.

Fig. 3.

Blocking glycine transport potentiated the NMDAR-mediated currents in perfused eyecup preparations. (A) A voltage-clamp WCR (holding potential -57 mV) from a retinal ganglion cell in an eyecup preparation responding to a 2-s light stimulus in control Ringer solution (black trace) was enhanced with the addition of the GlyT1 antagonist NFPS ($10 \mu\text{M}$; blue trace), and reduced by the addition of the NMDA antagonist AP7 ($100 \mu\text{M}$; red trace). The increased response in NFPS was blocked by AP7 (purple trace), and was not further enhanced by the addition of a saturating dose of D-serine ($100 \mu\text{M}$, green trace). Expanded traces of ON and OFF responses are included for clarity. (B) Extracellular recordings of the PNFP show a similar pattern of responses to a light stimulus: with an increase in amplitude in the PNFP following bath application of NFPS ($10 \mu\text{M}$), a decrease with AP7 ($100 \mu\text{M}$),

and no enhancement with exogenous D-serine (100 μ m). (C) Summary data from WCRs (eight cells) plotting the charge entering the cell during the light stimulus (ON) and for the 2 s following the light stimulus (OFF) as a function of the control response and from the PNFP ($n = 6$) plotting peak amplitude. (D–F) NMDA-mediated currents in retinal ganglion cells were not affected by GlyT2 inhibition. (D) A WCR (holding -68 mV) from a ganglion cell in an eyecup preparation evoked by a 2-s light stimulus (thick bar) in control Ringer solution (black trace) was unaffected by the addition of the GlyT2 antagonist ALX-5670 (100 μ m; blue trace). The addition of D-serine (100 μ m) to ALX-5670 (green trace) caused an increased inward current. (E) The addition of ALX-5670 (100 μ m, blue trace) did not affect the PNFP (control cocktail, black trace, largely overlapping), while D-serine potentiated the response (100 μ m, green trace). (F) Summary data from seven cells plotting the charge which entered the cell during the light stimulus (ON) and for the 2 s following the light stimulus (OFF) as a function of the control response and from six PNFP recordings plotting the peak amplitude (* $P < 0.05$, # $P < 0.01$ compared with control, error bars represent $\pm$ SEM).