Published in final edited form as:

Eur J Neurosci. 2009 December ; 30(12): 2308–2317. doi:10.1111/j.1460-9568.2009.07020.x.

The glycine transporter GlyT1 controls *N*-methyl-D-aspartic acid receptor coagonist occupancy in the mouse retina

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Abstract

We examined the role of GlyT1, the high-affinity glycine transporter, in the mouse retina with an emphasis on the role of glycine as a coagonist of *N*-methyl-D-aspartic acid (NMDA) receptors. We pursued this objective by studying heterozygote mice deficient in the GlyT1 transporter (GlyT1^{-/+}) and compared those results with wild-type (WT) littermate controls (GlyT1^{+/+}). Capillary electrophoresis was used to separate and quantitatively measure glycine release from isolated retina preparations; pharmacologically blocking GlyT1 with N-[3-([1,1-biphenyl]-4-ylxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine in the WT retina generated a significantly larger accumulation of glycine into the bathing environment when compared with the GlyT1^{-/+} retinas. The relative occupancy state of the NMDA receptor coagonist sites was tested using whole-cell recordings from ganglion cells while bath applying D-serine or D-serine + NMDA. The interpretation of these studies was simplified by blocking post-synaptic inhibition with picrotoxin and strychnine. NMDA receptor coagonist sites were more saturated and less enhanced by D-serine in the GlyT1^{-/+} mice compared with the WT controls. Immunoblots of NMDA receptor subunits (NR1, NR2A and NR2B) in WT and GlyT1^{-/+} animals showed that the NR1 subunits were identical. These observations are discussed in view of contemporary issues about NMDA receptor coagonist function in the vertebrate retina and the role of glycine vs. D-serine as the endogenous coagonist.

Keywords

coagonist binding sites; GlyT1 glycine transporters; *N*-methyl-D-aspartic acid receptors; retina

Introduction

N-methyl-D-aspartic acid (NMDA) receptors (NMDARs) are distributed throughout the central nervous system and have diverse functions in mediating and modulating excitatory actions at glutamatergic synapses. These receptors are also found in the retina, where their predominant distribution is among ganglion and amacrine cells (Slaughter & Miller, 1983; Dixon & Copenhagen, 1992; Diamond & Copenhagen, 1993; Fletcher *et al.*, 2000).

NMDARs in the retina are of interest not only because of their physiological role in mediating light-evoked synaptic currents but also because of their excitotoxic properties, which may provide a risk factor for diseases of the retina such as glaucoma, ischemia or diabetic retinopathy (Lipton, 2001). Excitotoxic actions of glutamate on retinal ganglion cells have been demonstrated, with neuroprotection observed when NMDARs were blocked (Vorwerk *et al.*, 1996).

Among the many unique features of NMDARs is their requirement for a second agonist other than glutamate, which must be bound to its recognition site on the NR1 subunit for channel gating by glutamate. When the need for this coagonist was first described (Johnson & Ascher, 1987), it was assigned to glycine. However, shortly after the discovery of this coagonist requirement, Kleckner & Dingledine (1988) showed that many amino acids could substitute for glycine, including enantiomers such as D-serine. At the time of this observation, the presence of D-amino acids was considered to be an aberration in vertebrate tissues, perhaps reflecting a dietary source or bacterial contamination. However, Hashimoto *et al.* (1992) succeeded in showing that D-serine was present at significant levels in the brain and that its distribution was similar to that of NMDARs (Hashimoto *et al.*, 1993). Subsequently, Wolosker *et al.* (1999) succeeded in isolating and characterizing serine racemase, the enzyme that synthesizes D-serine from L-serine. Initially this enzyme was localized to astrocytes but more recent evidence suggests that it may also be present in neurons (Kartvelishvili *et al.*, 2006).

D-serine and serine racemase have been identified in the vertebrate retina (Stevens *et al.*, 2003; Williams *et al.*, 2006) and initially localized to Müller cells and astrocytes. Previous studies have suggested that D-serine plays a prominent role as an NMDAR coagonist (Stevens *et al.*, 2003; Gustafson *et al.*, 2007). However, glycine is also present in the retina, but is tightly regulated through GlyT1, which has been localized to a group of amacrine cells in the mammalian and avian retinas (Pow & Hendrickson, 1999) and Müller cells (Lee *et al.*, 2005) in the amphibian retina. What role then does GlyT1 have on the availability of glycine as a coagonist for NMDARs in the mammalian retina? In the current study, we approached this question by using a knockout mouse that has a deficiency in GlyT1 (GlyT1^{-/+}) (Tsai *et al.*, 2004). The homozygotes (GlyT1^{-/-}) die at birth precluding their use for these studies. For this reason, we used the heterozygotes and their GlyT1^{+/+} littermates to evaluate the importance of high-affinity glycine transport and the role that this system plays in regulating the coagonist sites of NMDARs of retinal ganglion cells. A preliminary report of these findings was published previously (Reed *et al.*, 2007).

Materials and methods

All experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Anaesthesia and methods were the same for all protocols. Mice were killed by an overdose of Nembutal (150 mg/kg i.p.; Ovation Pharmaceuticals, Deerfield, IL, USA), followed by pneumothorax, after which the eyes were quickly removed and the retina was surgically excised and mounted on filter paper. We obtained heterozygote GlyT1^{-/+} mice (GlyT1 knockout 129S6/SvEvTac) from the laboratory of J.T.C. and bred them in-house at the University of Minnesota.

Polymerase chain reaction genotyping

Genotypes of all of the animals used in this study were confirmed by polymerase chain reaction as previously described (Tsai *et al.*, 2004). Briefly, tail snips were digested overnight in Proteinase K and the DNA was purified using a Dneasy Kit protocol (Qiagen, Valencia, CA, USA). DNA fragments were amplified by polymerase chain reaction with

three primers. Primer 1 (5'-GCCTTGGGAAAAGCGCCTCC-3') corresponds to the PGK-neo cassette inserted into the mutant locus, primer 2 (5'-CCCCTACTTCATCATGCTGATC-3') corresponds to the wild-type (WT) allele and primer 3 (5'-TCATACCATAGCCCACGCCT-3') corresponds to both the mutant and WT alleles. The polymerase chain reaction primers were supplied by the University of Minnesota Biomedical Genomics Center. Hybridization between primers 2 and 3 (WT) yields a single 1.3 kb amplicon, whereas primers 1 and 3 (GlyT1^{-/+}) yield an additional 1.0 kb amplicon on DNA gel electrophoresis.

Electrophysiology

Whole-cell current and voltage-clamp recordings were obtained using patch recording techniques in an isolated, superfused retina preparation. Bath-applied pharmacological agents were used to study the relative level of coagonist binding in identified retinal ganglion cells. We examined the effects of exogenous D-serine on the amplitude of currents evoked by light stimulation as well as the exogenous application of NMDA. Impulse generation was blocked with tetrodotoxin citrate (TTX) and, unless indicated otherwise, we also blocked GABA and glycine responses with picrotoxinin and strychnine, respectively. For each electrophysiology experiment, eyes were removed, hemisected and the retinas were gently teased free of the eyecup and pigment epithelium. The isolated retina was then placed ganglion cell side up on a nitrocellulose membrane (8 μ m, 13 mm, SCWP; Millipore, Billerica, MA, USA) whose center was punched out leaving a 2 mm concentric hole. The retina was mounted in a modified chamber (Newman & Bartosch, 1999) and secured with a platinum wire that was part of a nylon harp structure to secure the retina and filter paper within the chamber. The second retina was removed and placed in Ames medium for later use. The isolated retina preparation was continuously superfused with Ames medium (Ames & Nesbitt, 1981) (Sigma, St Louis, MO, USA) but without serum. Perfusate solutions were maintained in gas-bubbled reservoirs above the recording cage and gravity fed over the retina with a flow rate of approximately 3 mL/min. The solution was continuously bubbled with a 95/5% O₂/CO₂ mixture. Prior to dissection, the pH had been adjusted to 7.4 with NaOH. Osmolality was approximately 284 mOsmol/L for both the extracellular and intracellular electrode filling solutions. All experiments were conducted at room temperature (22°C). Generally, cells with large somas in the most proximal portion of the ganglion cell layer were targeted for recording.

Whole-cell recording electrodes

Patch recording electrodes were pulled from borosilicate glass (Freidrich & Dimmock; 1.2 mm OD; 0.8 mm ID) and polished in a Narishige microforge. The filling solution for the electrodes was as follows (in mM): 128 KCH₃SO₄, 5 NaCH₃SO₄, 2 MgCl₂, 5 EGTA, 5 HEPES, 1 glutathione, 2 ATP-Mg²⁺ and 0.2 GTP Na₃, pH was adjusted to 7.4. The filling solution was filtered with a 0.2 μ m syringe filter at the time of electrode filling. Filled electrodes had tip resistances in the range of 7–10 M Ω . Recordings were made with an Axon Multiclamp 700A computer-controlled amplifier connected to an Axon Instruments Digidata 1320 16 bit A-D converter (Molecular Devices, Sunnyvale, CA, USA) and a Windows PC. To reduce noise, offline filtering was performed at 0.5–2 kHz with an eight-pole Bessel software filter. Data were analyzed offline with pCLAMP/CLAMPFIT (Molecular Devices) and ORIGIN LAB (Northampton, MA, USA) commercial software.

Solutions and drug application

Except for the following and where indicated elsewhere in the text, all of the chemicals that we used were obtained from Sigma Chemical (St Louis, MO, USA). We obtained the following chemicals from Tocris (Ellisville, MO, USA): TTX, DL-2-amino-7-phosphonoheptanoic acid (D,L-AP7), NMDA, 2,3-dioxo-6-nitro-1,2,3,4-

tetrahydrobenzo[f]quinoxaline-7-sulfonamide and N-[3-([1,1-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine (NFPS). Glycine oxidase was kindly provided by Herman Wolosker (Technion-Israel Institute, Haifa, Israel).

Visual displays and responses

Light stimuli were generated with customized software (VisionEgg; Straw, 2008) running on a Windows PC. A beam of light from a 100 W halogen lamp was collimated, projected through a computer-controlled LCD panel (Burkhardt *et al.*, 1998) and then deflected into the Faraday cage with mirrors that directed the light into an optical port of an Olympus BX51WI microscope: a 40× water immersion lens was used to focus the light stimulus onto the retina. In this way, the light stimulus and retinal topology could be viewed simultaneously. A CCD camera (134B, Watec, Orangeburg, NY, USA) was mounted on an additional optical port for capturing images of dye-filled cells. For this purpose, the output of the camera was connected to a ConvertX (PX-M402U, Plextor, Fremont, CA, USA) image converter box, which fed into a computer through a USB terminal. Screen captures of dye-filled cells were taken from the screen image; for each cell, several images (four to nine) were obtained at different focal depths, manually adjusted to capture discrete image planes of the cell. These images were then convolved into a single image using IMARIS 3D software (Imaris Bitplane, St Paul, MN, USA). In this way, the axon, cell body and portions of the proximal dendrites were included in the final image (Fig. 1).

Capillary electrophoresis

We used capillary electrophoresis to separate and measure the glycine accumulation in the bathing medium of an isolated GlyT1^{-/+} or WT littermate mouse retina. Capillary electrophoresis separations were performed on a commercial capillary electrophoresis instrument (Beckman-Coulter MDQ, Fullerton, CA, USA) with laser-induced fluorescence detection. Retinas were isolated and incubated in separate 100 μ L wells of a teflon chamber filled with bicarbonate Ringer and oxygenated with a continuous flow of 95% O₂ and 5% CO₂ gas, while gently shaking the entire chamber (Rotomix, Thermolyne, Dubuque, IA, USA). Retinas were incubated for 50 min.

Both retinas of a single animal were removed and transferred to separate wells of the multiwell chamber; one retina served as the control and the other was incubated with 10 μ M of the GlyT1 inhibitor, NFPS. Following incubation, the medium from each retina was extracted and partitioned into three equal volumes of 25 μ L for capillary electrophoresis analysis. One of the three fractions received 2 μ L of 1 mM glycine to aid in determining and identifying the glycine peak; the second fraction was incubated for 30 min at 37°C with 2.5 μ L of 8 mg/mL glycine oxidase to degrade any glycine in the sample and confirm the glycine peak identity; and the third fraction received no additional additives and was used for the quantitative measurement of the glycine level in the bathing environment (see Fig. 2). For all samples, we added 5 μ M of α -amino adipic acid to serve as an internal standard for normalization of the data. Amino acids were fluorescently derivatized at 60°C for 15 min with 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (Molecular Probes, Eugene, OR, USA).

Separations were performed at 15 kV (70 μ A). A 4 mW argon laser (488 nm) was directed at the migrating derivatized amino acids, which induced a fluorescent signal at 520 nm that was detected by a photomultiplier tube and digitally plotted as the magnitude of the fluorescence signal vs. time (electropherogram). Quantification of the data was achieved by integrating the glycine peak, divided by the integrated peak of the amino adipic acid internal standard. The conversion to concentration was performed by comparing the results with a series of glycine standards. The software used for display and peak integration was 32Karat provided by Beckman-Coulter.

Immunoblotting for N-methyl-D-aspartic acid receptor subunits

Retinas from GlyT^{-/+} mice and age-matched controls were homogenized by 10 s sonication in fresh ice-cold radio-immuno precipitation assay buffer (50 mM Tris buffer, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4) containing Complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After holding on ice for 5 min, extracts were centrifuged at 13 000 g. in a microcentrifuge for 12 min to pellet insoluble material. Supernatants were adjusted to 1 mg/mL protein with radio-immuno precipitation assay, aliquoted and frozen at -80°C until use. Proteins (20 µg/well) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Novex, Invitrogen Corp., Carlsbad, CA, USA) and transferred to polyvinylidene fluoride for immunoblotting. Membranes were cut into strips, each containing one lane of WT and one lane of GlyT1^{-/+} proteins, and incubated for 1 h in blocking buffer [50 mM Tris, 150 mM NaCl, 0.1% Tween-20, 3% bovine serum albumin (Sigma)]. Strips were incubated overnight at 4°C in one of the following primary antibodies: anti-NMDAR1 (1 : 2000, Cell Signaling #4204; Millipore, Billerica, MA, USA), anti-NMDAR2A (1 : 2000, Millipore 9354) and anti-NMDAR2B (1 : 4000, Millipore AB1557P). Blots were washed in Tris buffered saline-TWEEN and incubated together in peroxidase-coupled donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove PA, USA) at 1 : 4000 for 1 h, washed and visualized on film with a Lumi-Light ECL kit (Roche). To visualize loading controls, blots were stripped for 2 h (Restore Plus, Pierce, Rockford, IL, USA), washed and incubated with anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Chemicon MAB377, Millipore) at 1 : 50 000 for 20 min. GAPDH bands (38 kDa) were visualized with peroxidase-conjugated donkey anti-mouse secondary antibody (Jackson) and Lumi-Light enhanced chemiluminescence (Roche).

Results

This study was based on whole-cell recordings from 66 ganglion cells in WT (30) and GlyT1^{-/+} (36) mice. During the course of these experiments, we dye-filled a number of cells in the ganglion cell layer and verified that each cell had an identifiable axon, which emanated from the cell body towards the optic nerve layer of the retina (Fig. 1). Twenty-seven cells in this group were studied with focal vs. diffuse light stimulation to evaluate receptive field properties; in this population we identified transient On (1), transient Off (3), sustained On (13), sustained Off (3) and On-Off (7) subtypes. Thus, a range of physiological polarities and response types were included in our ganglion cell population. However, for the purposes of this study, all subtypes behaved similarly in terms of NMDAR coagonist binding state when we compared the WT vs. the GlyT1^{-/+} mice and, for that reason, the data from all cells have been aggregated into a single pool.

Blocking GlyT1 elevates glycine in wild-type mice but not in the GlyT1^{-/+} retinas

Figure 2A shows a partial electropherogram from a single separation study, demonstrating the glycine peak sandwiched between the larger peaks of taurine and glutamine, with the internal standard, α -amino adipic acid, at the end of the trace. The migration time of glycine was characterized by adding a known quantity of glycine to the experimental sample and also by demonstrating that the peak in the experimental sample was eliminated by the glycine-degrading enzyme glycine oxidase (Fig. 2B). Comparing the baseline glycine levels between WT and GlyT^{-/+} revealed an apparent trend towards greater glycine levels in GlyT^{-/+}, although this trend fell short of statistical significance ($t_8 = 1.35$, $P = 0.21$). However, blocking GlyT1 with NFPS significantly elevated glycine levels above untreated control retinas in the WT ($t_4 = 2.63$, $P = 0.058$) but not in GlyT^{-/+} ($t_4 = 1.17$, $P = 0.31$; Fig. 2C and D) mice. The final glycine concentration following NFPS treatment was virtually identical between genotypes ($t_8 = 0.25$, $P = 0.81$), suggesting that the reduced number of

retinal GlyT1s in the GlyT1^{-/+} animals accounts for the difference observed in extracellular glycine levels. Comparing the percent increase in glycine following NFPS treatment between the genotypes revealed that WT retinal glycine levels were augmented significantly more by NFPS than they were in GlyT1^{-/+} ($t_8 = 2.37$, $P = 0.046$) (Fig. 2D).

GlyT1^{-/+} and wild-type retinas show differences in light-evoked N-methyl-D-aspartic acid receptor coagonist occupancy levels

We tested the status of the NMDAR coagonist binding sites in single ganglion cells using D-serine added to the bathing medium to evaluate the comparative degree of NMDAR coagonist site occupancy (Gustafson *et al.*, 2007). We simplified the retinal network by blocking post-synaptic inhibitory inputs as well as impulse activity. For these experiments, the superfusate (Ames medium) contained 1 μ M TTX, 50 μ M picrotoxinin and 10 μ M strychnine. Unless otherwise indicated, all responses illustrated are the result of averaging 20 consecutive light-evoked responses. Figure 3A shows a current-clamp recording from a sustained On ganglion cell; the light-evoked control response showed transient and sustained components. In the presence of D-serine, the peak response increased, whereas the steady-state component remained relatively constant. When D,L-AP7 was added after returning to the control, the response was reduced to a small sustained component, indicating that NMDARs contributed substantially to the light response. To avoid the influence of shifting membrane potentials on the driving force for synaptic currents, we confined our experimental observations almost entirely to voltage-clamp studies. Figure 3B shows a voltage-clamp whole-cell recording (WCR) from a sustained On ganglion cell of a WT retina. The control response showed transient and sustained components, both of which increased during the application of 100 μ M D-serine. When D,L-AP7 was added, the response decreased below that of the control, indicating a contribution from NMDARs. In several studies, we added 100 μ M NBQX(2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f]quinoxaline-7-sulfonamide) (100 μ M) to the D,L-AP7 bathing solution to determine the pharmacological identity of the response components that were insensitive to D,L-AP7. The addition of NBQX (100 μ M) completely eliminated the light response, indicating that the D,L-AP7-insensitive light response can be attributed to AMPA/kainic acid receptors (not illustrated). Figure 3C shows a WCR from a GlyT1^{-/+} ganglion cell, in which the control response had a significant peak and plateau component, with a small overshoot at light off. When D-serine was added, the response was minimally increased, whereas D,L-AP7 significantly reduced the peak response, with a slight increase in the plateau component. Figure 3D presents a graphic summary of the relative change in the light-evoked responses of ganglion cells in the presence of D-serine. For the GlyT1^{-/+} mice, only 7/14 cells showed an increase in response to D-serine, whereas 9/9 cells in the WT retinas were enhanced through D-serine application; this latter population included seven On ganglion cells. Very little difference was evident in the outcome when comparing the peak amplitude and the response measurement obtained by integrating the current to give total charge.

D-serine more effectively potentiates the N-methyl-D-aspartic acid-evoked responses of ganglion cells in wild-type mice

A second method used for evaluating the state of NMDAR coagonist occupancy included a comparison of bath-applied NMDA with and without concurrent application of D-serine. These experiments involved WCRs from ganglion cells in the voltage-clamp mode in the absence of light stimulation. Bath application of NMDA can be expected to reach both synaptic and non-synaptic pools of NMDARs and, for that reason, might conceivably reveal different results, particularly if a large non-synaptic pool of NMDARs exists, which have a different level of coagonist site occupancy. We carried out this study by applying the sequence of (i) 30 s of NMDA (500 μ M); (ii) several minutes of control; (iii) 30 s of NMDA + D-serine (100 μ M); (iv) several minutes of control and (v) 30 s of NMDA. For these

experiments, each cell recording was obtained from a virgin retina that had no prior exposure to pharmacological agents. The magnitude of the response to exogenous NMDA varied considerably and did not reveal any consistent difference between the WT and GlyT1^{-/+} mice. We were unable to determine whether this reflected differences in the size or type of the ganglion cell. Figure 4A illustrates a WCR obtained from a GlyT1^{-/+} ganglion cell bathed in TTX, in which a 30 s application of NMDA (upper trace) resulted in a slow inward current of 142 pA; after recovery and washout with control Ringer, the middle trace shows the results of NMDA + D-serine, with some enhancement of the peak response (219 pA at peak), although the response itself had different phasic components. The bottom trace shows recovery of the response to 500 μ M NMDA after a washout period of several minutes (peak amplitude 124 pA). The duration bar in the upper trace of this and the other panels indicates a 30 s application of the agents. Figure 4B shows the same test sequence in a WT mouse in TTX (control response 27 pA) with D-serine enhancement (40 pA) and a return to NMDA after washout (31 pA). The D-serine increase was observed in 4/5 of the GlyT1^{-/+} mice, with one animal showing no change in the response to NMDA + D-serine. In contrast, 5/5 of the WT ganglion cells showed a measurable increase induced by D-serine. Because some amacrine cells are likely to be activated by these procedures and secondarily provide a synaptic input into ganglion cells, we also performed some experiments in which the influence of GABA_A and glycine inputs was reduced through the application of picrotoxinin (50 μ M) and strychnine (10 μ M), in addition to TTX. Figure 4C illustrates the actions of NMDA in a GlyT1^{-/+} mouse ganglion cell in which NMDA was added to the bathing environment, which contained picrotoxinin and strychnine at the indicated levels. The control response had a peak value of -81 pA, whereas the NMDA + D-serine response was larger (-121 pA) with a washout and repeat NMDA response of -63 pA. Figure 4D shows the actions of NMDA on a WT mouse retina. The control response was -81 pA, whereas NMDA evoked a response of -244 pA and returning to a control NMDA application gave a peak response of -63 pA. Figure 4D summarizes the differences in the GlyT1^{-/+} and WT cells when D-serine was added to the NMDA bathing medium in TTX. The peak amplitude responses are expressed as a percent of the normalized control values. In TTX alone, the WT showed a slight increase over the GlyT1^{-/+} mice that was significant ($t_7 = 3.40$, $P = 0.014$).

Figure 4E demonstrates that, when the experiments were performed in the presence of picrotoxinin/strychnine, the enhancement was greater for both the WT and GlyT1^{-/+} mice than those exposed to NMDA and D-serine in the presence of TTX. However, in this case, the increase in the presence of D-serine also remained greater for the WT ganglion cells. It is important to emphasize that D-serine potentiated the NMDA-evoked currents of ganglion cells in only some of the GlyT1^{-/+} (2/6) cells but it potentiated the NMDA-evoked currents in all of the cells from WT retinas (5/5); the difference between the two groups was significant ($t_8 = 2.31$, $P = 0.049$).

Immunoblotting

We used Western blotting to test whether changes in NMDAR subunit expression could account for the functional saturation of NMDAR that we observed in GlyT^{+/-} retinas. For example, a significant loss of expression of the NR1 subunit, which contains the coagonist binding site, could lead to apparent saturation of NMDARs even with unchanged glycine concentrations. Figure 5 shows that expression of the three NMDAR subunits tested (NR1, NR2A and NR2B) does not change substantially between WT and GlyT^{+/-} mice. From densitometry measurements, the expression levels of NMDAR subunits in GlyT^{+/-} as a percentage of the corresponding WT expression were: NR1, 130.9%; NR2A, 76.50% and NR2B, 88.3%. It should be noted that these are measurements of total subunit expression in the retina and thus do not distinguish among membrane-bound, cytoplasmic, synaptic or extrasynaptic proteins.

Discussion

Two different high-affinity transport systems for glycine have been identified in the brain, including GlyT1 and GlyT2, each of which requires external Na^+ to drive the uptake. Although it was originally thought that the expression of GlyT1s was confined to glial cells and GlyT2s were generally localized to neurons (Guastella *et al.*, 1992; Smith *et al.*, 1992), more recent evidence suggests that GlyT1s play an important role in glutamatergic pathways and are expressed in both pre- and post-synaptic neurons (Cubelos *et al.*, 2005). Amacrine cells play an important role in controlling extracellular glycine levels in the retina (Pow, 1998). However, as opposed to the brain, the uptake of glycine in the retina is mediated exclusively by GlyT1, which in the mammalian and avian retinas is distributed in amacrine cells (Pow & Hendrickson, 1999), whereas GlyT1 is expressed in Müller cells in the amphibian retina (Lee *et al.*, 2005). In the present study we used a mouse deficient in GlyT1 (Tsai *et al.*, 2004) and found that the glycine concentration in the chamber containing an isolated retina showed very little change when GlyT1 was blocked with a selective antagonist (NFPS) (Bergeron *et al.*, 1998; Aubrey & Vandenberg, 2001). In contrast, the same experiment carried out in a WT retina showed a low level of glycine released into the bathing medium and a comparatively larger increase in glycine when NFPS was applied. The measured levels of glycine that accumulated in the bathing chamber (low μM concentrations) were sufficient to activate the GlyT1 (Matsui *et al.*, 1995; Supplisson & Bergman, 1997). Thus, GlyT1 contributed to the observed levels of glycine in the chamber and accounted for the large difference in glycine release when NFPS was applied. However, if the concentration of glycine in the chamber bathing environment was uniformly distributed throughout the extracellular spaces of the retina, all of the NMDAR coagonist sites would be saturated (Supplisson & Bergman, 1997) and this was not what we observed in ganglion cell recordings from WT retinas. In contrast, we observed NMDAR coagonist site saturation in many ganglion cells from the GlyT1^{-/+} retinas, as if the glycine levels that we saw in the chamber volume were relevant for those in the region of the NMDARs.

Supplisson & Bergman (1997) have provided convincing evidence from studies in oocytes, which coexpressed NMDARs and GlyT1s, that the unstirred layer effect at the surface of the membrane allowed the GlyT1 to effectively reduce the local concentration of glycine to levels below the NMDAR coagonist site threshold. Studies in the central nervous system have demonstrated that GlyT1s are found near NMDARs (Smith *et al.*, 1992; Cubelos *et al.*, 2005), suggesting a functional linkage between these two mechanisms. Thus, small regions of the extracellular space surrounding the glycine transporters can result in a local environment that is relatively depleted in glycine compared with adjacent regions where glycine transporters are absent. Of course, glycine from more distant regions surrounding the glycine transporter, where the background concentration is higher, will continually diffuse into the region of the transporter, establishing an equilibrium condition between uptake, nearby release and diffusion. This concept explains why the mean extracellular levels that we found in the bathing chamber do not reveal how local high-affinity transport mechanisms can change the microenvironment concentration of glycine.

The findings of this study clearly establish that the NMDAR coagonist sites have higher occupancy levels when GlyT1s are deficient in their expression. This deficiency could come about in two different ways, including an abnormal spatial distribution of the transporters or a deficiency in density, although they are not mutually exclusive. Evidence suggests that the brains of GlyT1^{-/+} mice have a reduced expression of the GlyT1 with a reduction to about half of their WT littermates (Tsai *et al.*, 2004; Martina *et al.*, 2005). If we translate our interpretation of glycine accumulation in the chamber, with the demonstrated importance of the GlyT1 activity for keeping external glycine levels low, it is inescapable that glycine levels surrounding the NMDARs of retinal ganglion cells are tonically exposed to a higher

level of glycine when compared with their WT counterparts. For that reason, ganglion cell recordings from the GlyT1^{-/+} retinas are less enhanced by exogenous D-serine compared with the augmenting action of D-serine on ganglion cells from WT retinas.

Western blots of whole retina demonstrated the presence of NR1, NR2A and NR2B NMDAR subunits in our WT and GlyT1^{-/+} animals, consistent with other studies that have demonstrated a major representation of these subunits in the inner retina (Fletcher *et al.*, 2000). The apparent saturation of GlyT1^{-/+} NMDAR coagonist sites in our results could be explained by a substantial loss of NR1 subunits, which contain the 'glycine' binding site. Alternatively, differences in glycine/D-serine sensitivity have been reported for NMDAR containing NR2A vs. NR2B subunits (Mishina *et al.*, 1993; Matsui *et al.*, 1995), so changes in the expression of these two subunits could also affect NMDAR coagonist sensitivity. Our Western blot data indicate that NR1, NR2A and NR2B subunit expression differs little between WT and GlyT1 transporter-deficient retinas, suggesting that there is little developmental compensation in NMDAR subunit composition in response to the knockout condition. It should be noted that the Western blot technique measures total levels of subunits and does not distinguish between membrane-bound subunits and subunits found in other cellular compartments.

Previous work has demonstrated that NMDARs must be functionally present for D-serine to enhance the light-evoked responses of retinal ganglion cells; the same applies to D-serine enhancement to the proximal negative response, a light-evoked field potential of the inner retina (Burkhardt, 1970; Gustafson *et al.*, 2007). Blocking NMDARs with antagonists essentially eliminates the enhancing actions of exogenous D-serine. However, as D-serine has no affinity for the GlyT1 (Thomson, 1990; Grimwood *et al.*, 1992; Broer *et al.*, 1999; Ribeiro *et al.*, 2002), we can assume that the more saturated condition of NMDARs in the GlyT1^{-/+} retinas reflects an elevated level of glycine in the extracellular space adjacent to NMDARs. Because the expression of GlyT1 is reduced in the GlyT1^{-/+} mice (Tsai *et al.*, 2004; Martina *et al.*, 2005), it seems likely that an insufficient number of transporters are available to keep all regions surrounding the NMDARs at reduced levels of glycine. This could explain why some ganglion cells in the GlyT1^{-/+} retinas showed near saturation of the NMDAR coagonist sites, whereas others showed complete saturation, based on the exogenous application of D-serine.

Exogenous application of NMDA generated a significantly larger current in the presence of inhibitory antagonists (picrotoxinin/strychnine/TTX) than it did when applied in TTX alone. As the chloride reversal potential was established at approximately -88 mV, the holding potential of -65 mV in our experiments meant that chloridemediated inhibition would generate a current of opposite polarity to that evoked by the direction actions of NMDA on the ganglion cell. As NMDARs are also found on amacrine cells (Dowling & Boycott, 1965; Witkovsky & Dowling, 1969; Slaughter & Miller, 1983; Muller & Marc, 1990; Dixon & Copenhagen, 1992), their activation could enhance feedforward inhibition onto ganglion cells. Thus, blocking post-synaptic inhibition with picrotoxinin/strychnine will eliminate the algebraic interaction and enhance the light responses. However, as feedforward inhibition from amacrine cells onto ganglion cells is carried out through dendrodendritic connections, inhibitory synaptic inputs into the dendrites can shunt the flow of excitatory current generated at more distal dendritic locations (Miller, 1979); this inhibitory action will also reduce the flow of excitation through the dendrites into the soma. Thus, the larger currents evoked by NMDA in the presence of toxins is consistent with the elimination of shunting inhibition in the dendrites as well as the algebraic interaction of the currents in the soma.

Although the present study strongly indicates that a deficiency of GlyT1s leads to an elevated extracellular level of glycine and a more saturated coagonist state of NMDARs, this

does not mean that glycine is the normal tissue coagonist of NMDARs. In fact, studies performed in the amphibian and rat retina have raised the possibility that D-serine is the primary, functional NMDAR coagonist (Stevens *et al.*, 2003). Additional studies in the retina have demonstrated that, when D-serine levels are reduced using enzymatic degradation applied to the whole retina, the light-evoked NMDAR contribution is similar to that observed when the NMDARs are blocked with an antagonist (Gustafson *et al.*, 2007). Thus, one possible view of GlyT1 found in amacrine and glial cells is that its high sensitivity and proximity to NMDARs of ganglion cells are sufficient to reduce glycine in the microenvironment of these receptor sites, such that D-serine is the major player as the NMDAR coagonist. It remains unclear why GlyT1 expression seems to be restricted to amacrine cells in the mammalian and avian retina (Pow & Hendrickson, 1999) or to Müller cells in the amphibian retina (Lee *et al.*, 2005). It is equally unclear at the moment whether glycine plays a role in more dynamic modulation of NMDARs, as glycine plays a role as an inhibitory neurotransmitter (Miller *et al.*, 1977; Marc, 1989) and presumably goes through light and dark-driven fluctuations in release and local concentration changes. However, because D-serine does not appear to be taken up by a high-affinity transport system (O'Brien *et al.*, 2005) but through a lower affinity amino solute carrier type 2-type amino acid transport pathway (Dun *et al.*, 2007), the extracellular levels of D-serine may more readily reach the coagonist threshold in strategically placed release sites near NMDARs. Additional studies of the D-serine release sites and the release mechanism will undoubtedly be needed to further clarify the role of D-serine vs. glycine as dynamic and static contributors to NMDAR function in ganglion cells of the retina.

Recent studies using rat and mouse retinal slices (Kalbaugh *et al.*, 2009) have suggested that D-serine and glycine both play a role as NMDAR coagonists, with glycine contributing to a light-evoked modulation and D-serine serving as a background, steady-state modulator of NMDAR coagonist occupancy. Unfortunately, the evaluation of the D-serine contribution in the study of Kalbaugh *et al.* (2009) was performed using the enzyme D-amino acid oxidase, which is much slower and less efficacious than the faster, more effective enzyme D-serine deaminase. The use of the latter enzyme in studies of the salamander retina resulted in a suppression of light-evoked NMDAR currents similar to that observed when NMDARs were blocked with antagonists (Gustafson *et al.*, 2007). Nevertheless, the concept of both dynamic and static regulation of NMDAR function through the influence of coagonists brings a new and exciting challenge to our understanding of NMDAR function in the retina as well as the other partitions of the central nervous system.

Acknowledgments

We appreciate many helpful discussions with Eric Gustafson and excellent editing and figure illustration assistance from Derek Miller.

Abbreviations

D,L-AP7	DL-2-amino-7-phosphonoheptanoic acid
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NFPS	N-[3-([1,1-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartic acid receptor
TTX	tetrodotoxin citrate

WCR whole-cell recording
 WT wild-type.

References

- Ames A III, Nesbett FB. In vitro retina as an experimental model of the central nervous system. *J. Neurochem* 1981;37:867–877. [PubMed: 7320727]
- Aubrey KR, Vandenberg RJ. N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS) is a selective persistent inhibitor of glycine transport. *Br. J. Pharmacol* 2001;134:1429–1436. [PubMed: 11724748]
- Bergeron R, Meyer TM, Coyle JT, Greene RW. Modulation of N-methyl-D-aspartate receptor function by glycine transport. *Proc. Natl Acad. Sci. U S A* 1998;95:15730–15734. [PubMed: 9861038]
- Broer A, Brookes N, Ganapathy V, Dimmer KS, Wagner CA, Lang F, Broer S. The astroglial ASCT2 amino acid transporter as a mediator of glutamine efflux. *J. Neurochem* 1999;73:2184–2194. [PubMed: 10537079]
- Burkhardt DA. Proximal negative response of frog retina. *J. Neurophysiol* 1970;33:405–420. [PubMed: 5439344]
- Burkhardt DA, Fahey PK, Sikora M. Responses of ganglion cells to contrast steps in the light-adapted retina of the tiger salamander. *Vis. Neurosci* 1998;15:219–229. [PubMed: 9605524]
- Cubelos B, Gimenez C, Zafra F. Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. *Cereb. Cortex* 2005;15:448–459. [PubMed: 15749988]
- Diamond JS, Copenhagen DR. The contribution of NMDA and non-NMDA receptors to the light-evoked input-output characteristics of retinal ganglion cells. *Neuron* 1993;11:725–738. [PubMed: 8104431]
- Dixon DB, Copenhagen DR. 2 Types of glutamate receptors differentially excite amacrine cells in the tiger salamander retina. *J. Physiol Lond* 1992;449:589–606. [PubMed: 1355793]
- Dowling JE, Boycott BB. Neural connections of the retina: fine structure of the inner plexiform layer. *Cold Spring Harb. Symp. Quant. Biol* 1965;30:393–402. [PubMed: 4956618]
- Dun Y, Mysona B, Itagaki S, Martin-Studdard A, Ganapathy V, Smith SB. Functional and molecular analysis of D-serine transport in retinal Muller cells. *Exp. Eye Res* 2007;84:191–199. [PubMed: 17094966]
- Fletcher EL, Hack I, Brandstatter JH, Wassle H. Synaptic localization of NMDA receptor subunits in the rat retina. *J. Comp. Neurol* 2000;420:98–112. [PubMed: 10745222]
- Grimwood S, Moseley AM, Carling RW, Leeson PD, Foster AC. Characterization of the binding of [3H]L-689,560, an antagonist for the glycine site on the N-methyl-D-aspartate receptor, to rat brain membranes. *Mol. Pharmacol* 1992;41:923–930. [PubMed: 1375318]
- Guastella J, Brecha N, Weigmann C, Lester HA, Davidson N. Cloning, expression, and localization of a rat brain high-affinity glycine transporter. *Proc. Natl Acad. Sci. U S A* 1992;89:7189–7193. [PubMed: 1353889]
- Gustafson EC, Stevens ER, Wolosker H, Miller RF. Endogenous D-Serine Contributes to NMDA-Receptor-Mediated Light-Evoked Responses in the Vertebrate Retina. *J. Neurophysiol* 2007;98:122–130. [PubMed: 17507508]
- Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T, Takahashi K. The presence of free D-serine in rat brain. *FEBS Lett* 1992;296:33–36. [PubMed: 1730289]
- Hashimoto A, Nishikawa T, Oka T, Takahashi K. Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. *J. Neurochem* 1993;60:783–786. [PubMed: 8419554]
- Johnson JW, Ascher P. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 1987;325:529–531. [PubMed: 2433595]
- Kalbaugh TL, Zhang J, Diamond JS. Coagonist release modulates NMDA receptor subtype contributions at synaptic inputs to retinal ganglion cells. *J. Neurosci* 2009;29:1469–1479. [PubMed: 19193893]

- Kartvelishvily E, Shleper M, Balan L, Dumin E, Wolosker H. Neuron-derived D-serine release provides a novel means to activate N-methyl-D-aspartate receptors. *J. Biol. Chem* 2006;281:14151–14162. [PubMed: 16551623]
- Kleckner NW, Dingledine R. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science* 1988;241:835–837. [PubMed: 2841759]
- Lee S, Zhong Y, Yang X. Expression of glycine receptor and transporter on bullfrog retinal Muller cells. *Neurosci. Lett* 2005;387:75–79. [PubMed: 16084022]
- Lipton SA. Retinal ganglion cells, glaucoma and neuroprotection. *Prog. Brain Res* 2001;131:712–718. [PubMed: 11420983]
- Marc RE. The role of glycine in the mammalian retina. *Prog. Retin. Res* 1989;8:67–107.
- Martina M, Turcotte ME, Halman S, Tsai G, Tiberi M, Coyle JT, Bergeron R. Reduced glycine transporter type 1 expression leads to major changes in glutamatergic neurotransmission of CA1 hippocampal neurones in mice. *J. Physiol* 2005;563:777–793. [PubMed: 15661817]
- Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T, Wada K. Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. *J. Neurochem* 1995;65:454–458. [PubMed: 7790891]
- Miller, RF. The neuronal basis of ganglion cell receptive field organization and the physiology of Amacrine cells. In: Schmitt, FO.; Worden, FG., editors. *The Neurosciences*. MIT Press; Cambridge: 1979. p. 227-245.
- Miller RF, Dacheux RF, Frumkes TE. Amacrine cells in *Necturus* retina: evidence for independent gamma-aminobutyric acid- and glycinerelating neurons. *Science* 1977;198:748–750. [PubMed: 910159]
- Mishina M, Mori H, Araki K, Kushiya E, Meguro H, Kutsuwada T, Kashiwabuchi N, Ikeda K, Nagasawa M, Yamazaki M. Molecular and functional diversity of the NMDA receptor channel. *Ann. N. Y. Acad. Sci* 1993;707:136–152. [PubMed: 9137549]
- Muller JF, Marc RE. GABA-ergic and glycinergic pathways in the inner plexiform layer of the goldfish retina. *J. Comp. Neurol* 1990;291:281–304. [PubMed: 2298935]
- Newman EA, Bartosch R. An eyecup preparation for the rat and mouse. *J. Neurosci. Methods* 1999;93:169–175. [PubMed: 10634502]
- O'Brien KB, Miller RF, Bowser MT. D-Serine uptake by isolated retinas is consistent with ASCT-mediated transport. *Neurosci. Lett* 2005;385:58–63. [PubMed: 15950382]
- Pow DV. Transport is the primary determinant of glycine content in retinal neurons. *J. Neurochem* 1998;70:2628–2636. [PubMed: 9603230]
- Pow DV, Hendrickson AE. Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinas. *Vis. Neurosci* 1999;16:231–239. [PubMed: 10367958]
- Reed, BT.; Tsai, G.; Coyle, JT.; Miller, RF. 2007 Abstract Viewer and Itinerary Planner. Society for Neuroscience; Washington, DC: 2007. The coagonist site of NMDA receptors is more saturated in the GlyT1 knockout mouse retina compared to that of the WT. Online 576.6. 2007. Ref Type: Abstract
- Ribeiro CS, Reis M, Panizzutti R, De Miranda J, Wolosker H. Glial transport of the neuromodulator D-serine. *Brain Res* 2002;929:202–209. [PubMed: 11864625]
- Slaughter MM, Miller RF. The role of excitatory amino acid transmitters in the mudpuppy retina: an analysis with kainic acid and N-methyl aspartate. *J. Neurosci* 1983;3:1701–1711. [PubMed: 6135763]
- Smith KE, Borden LA, Hartig PR, Branchek T, Weinshank RL. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. *Neuron* 1992;8:927–935. [PubMed: 1534013]
- Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, Miller RF. D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. *Proc. Natl. Acad. Sci. U S A* 2003;100:6789–6794. [PubMed: 12750462]
- Straw AD. Vision egg: an open-source library for realtime visual stimulus generation. *Front Neuroinformatics* 2008;2:4. [PubMed: 19050754]
- Supplisson S, Bergman C. Control of NMDA receptor activation by a glycine transporter co-expressed in *Xenopus* oocytes. *J. Neurosci* 1997;17:4580–4590. [PubMed: 9169519]

- Thomson AM. Glycine is a coagonist at the NMDA receptor/channel complex. *Prog. Neurobiol* 1990;35:53–74. [PubMed: 1699248]
- Tsai G, Ralph-Williams RJ, Martina M, Bergeron R, Berger-Sweeney J, Dunham KS, Jiang Z, Caine SB, Coyle JT. Gene knockout of glycine transporter 1: characterization of the behavioral phenotype. *Proc. Natl. Acad. Sci. U S A* 2004;101:8485–8490. [PubMed: 15159536]
- Vorwerk CK, Lipton SA, Zurakowski D, Hyman BT, Sabel BA, Dreyer EB. Chronic low-dose glutamate is toxic to retinal ganglion cells. Toxicity blocked by memantine. *Invest. Ophthalmol. Vis. Sci* 1996;37:1618–1624. [PubMed: 8675405]
- Williams SM, Diaz CM, Macnab LT, Sullivan RK, Pow DV. Immunocytochemical analysis of D-serine distribution in the mammalian brain reveals novel anatomical compartmentalizations in glia and neurons. *Glia* 2006;53:401–411. [PubMed: 16342169]
- Witkovsky P, Dowling JE. Synaptic relationships in the plexiform layers of carp retina. *Z. Zellforsch. Mikrosk. Anat* 1969;100:60–82. [PubMed: 5354186]
- Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proc. Natl. Acad. Sci. U S A* 1999;96:13409–13414. [PubMed: 10557334]

Fig. 1.

A single ganglion cell dye-filled with Alexa 488; the axon is marked with an arrow. Using a stack of images obtained by focusing through the plane of the ganglion cell, the soma, axon and dendrites could be followed. This ganglion cell was identified physiologically as a sustained On cell with its dendritic tree confined to the inner part of the inner plexiform layer.

Fig. 2.

Glycine release is different in WT vs. GlyT1^{-/-} retinas. (A) Capillary electrophoresis electropherogram that shows elution of the glycine peak between that of taurine (Tau) and glutamine (Gln) and the late peak of the internal standard [α -amino adipic acid (Aad)]. (B) Glycine peak identification. An expanded electropherogram shows the glycine peak region with an elevated glycine level (top trace) reflecting glycine added to the medium, the glycine level from the bathing chamber (middle trace) and the elimination of glycine (bottom trace) by adding glycine oxidase (GO) to the solution. (C) Expanded glycine peak regions of superimposed electropherograms. The two top traces show virtually no change in glycine levels when NFPS was added to the chamber (top trace, dotted line) vs. the release level of the control retina from the same animal (second trace, solid line). In contrast, glycine from a WT retina exposed to NFPS resulted in a substantial level of glycine enhancement (third trace, dashed line) compared with that of the companion control retina (bottom trace, solid line). (D) Comparison of WT and GlyT1^{-/-} retinas exposed to NFPS. Differences between WT and WT + NFPS are significant ($t_4 = 2.63$, $P = 0.058$), whereas the difference between GlyT1^{-/-} and GlyT1^{-/-} + NFPS is not significant ($t_4 = 1.17$, $P = 0.31$). The right two bar graphs compare the differences between the WT + NFPS and GlyT1^{-/-} retinas expressed as a percent increase over the control values ($t_8 = 2.37$, $P = 0.046$). Gly, glycine; RFU, relative fluorescence units.

Fig. 3.

D-serine potentiates the light-evoked responses of ganglion cells. (A) A current-clamp recording from a WT sustained On ganglion cell showed enhancement of the peak light response in D-serine and a significant reduction in the light response when D,L-AP7 was added to the bathing solution. (B) Voltage-clamp records from a sustained On ganglion cell of a WT retina; D-serine enhanced the light response, whereas the application of D,L-AP7 reduced the peak response and plateau components. (C) Voltage-clamp recording from an On cell of a GlyT1^{-/-} retina, which had a prominent peak and small plateau components. When D-serine was added, a very slight increase in the peak response was observed, with little change in the plateau response, whereas adding D,L-AP7 reduced the peak and plateau components. (D) Bar graph summary of D-serine enhancement using both peak amplitude and response integration values for the GlyT1^{-/-} and WT retinas ($\pm$ SE). Both measurement techniques gave similar results, with a clear difference evident when comparing the two animal groups ($t_7 = 3.90$, $P = 0.0059$). Each light-evoked response illustrated in A-C was averaged from 20 consecutive light stimuli. All recordings were performed in continuous perfusion with Ames medium to which 1 μ M TTX, 50 μ M picrotoxin and 10 μ M strychnine were added.

Fig. 4.

D-serine (DS) enhances the NMDA-evoked responses of retinal ganglion cells in WT mice more than it does in GlyT1^{-/+} mice. (A) Whole-cell recording from a GlyT1^{-/+} retina in response to 500 μ M NMDA (top trace) in the presence of TTX (peak amplitude -142 pA), followed by an enhanced response (middle trace, -219 pA) when D-serine was added to the NMDA. The bottom trace shows the result of repeating the NMDA application after washing the retina for several minutes (bottom trace, -124 pA). (B) Exposure of a ganglion cell from a WT retina in the presence of TTX, where the peak response went from -27 pA (control, top trace) to -40 pA (NMDA + D-serine, middle trace) followed by a second NMDA application (bottom trace, -31 pA). (C and D) NMDA-evoked responses in TTX (1

μM), picrotoxinin ($50 \mu\text{M}$) and strychnine ($10 \mu\text{M}$). (C) Responses from a GlyT1^{-/+} mouse retina. The control NMDA response evoked a peak inward current of -18 pA (upper trace), whereas NMDA + D-serine (middle trace) evoked an inward current of -121 pA ; the repeat control response was -63 pA . (D) Responses from a WT retinal ganglion cell; NMDA in the control + toxin environment (upper trace) evoked a slow inward current of -81 pA ; the NMDA + D-serine response (middle trace) was -244 pA , whereas the repeat control response (bottom trace) was -63 pA . (E) Bar graph summary comparing NMDA-evoked currents in WT and GlyT1^{-/+} as a percent increase over the normalized control response in the presence of TTX. In these studies, D-serine potentiated NMDA-evoked currents of ganglion cells in both the GlyT1^{-/+} mice (5/5) as well as the WT controls (4/5). The average enhancement of the NMDA-evoked current when D-serine was coapplied was greater for the WT when compared with the GlyT1^{-/+} heterozygotes ($t_7 = 3.40$, $P = 0.014$). (F) A comparison between the NMDA-evoked responses of ganglion cells in WT and GlyT1^{-/+} ganglion cell recordings that illustrates the greater enhancement of the NMDA response when the experiments were carried out in the presence of TTX, strychnine and picrotoxinin. For these studies, D-serine potentiated NMDA-evoked currents of ganglion cells in some of the GlyT1^{-/+} (2/6) but it potentiated the NMDA-evoked currents for all of the WT controls ($t_8 = 2.31$, $P = 0.049$). For both panels, error bars represent $\pm\text{SE}$. Physiological recordings were processed with offline filtering at 2 Hz with an eight-pole Bessel filter, with further noise reduction carried out in Origin using Fourier transform filtering.

Fig. 5.

Western blot comparing levels of NR1 (~ 116 kDa), NR2A and NR2B (~160 kDa) subunits in extracts from age-matched WT and GlyT^{+/-} mouse retinas. GAPDH (38 kDa) was used as a loading control. All lanes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the same gel; after transfer the membrane was cut into strips for primary antibody incubation. The figure was constructed by realigning digitally scanned strips in Adobe Photoshop using molecular weight markers; any subsequent level and contrast adjustments were performed simultaneously on all parts of the blot.