Endogenous d-Serine Contributes to NMDA-Receptor–Mediated Light-Evoked Responses in the Vertebrate Retina

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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 98: 122–130, 2007. First published May 16, 2006; doi:10.1152/jn.00057.2006. We have combined electrophysiology and chemical separation and measurement techniques with capillary electrophoresis (CE) to evaluate the role of endogenous d-serine as an NMDA receptor (NMDAR) coagonist in the salamander retina. Electrophysiological experiments were carried out using whole cell recordings from retinal ganglion cells and extracellular recordings of the proximal negative response (PNR), while bath applying two d-serine degrading enzymes, including d-amino acid oxidase (DAAO) and d-serine deaminase (DsdA). The addition of either enzyme resulted in a significant and rapid decline in the light-evoked responses observed in ganglion cell and PNR recordings. The addition of exogenous d-serine in the presence of the enzymes restored the light-evoked responses to the control or supracontrol amplitudes. Heat-inactivated enzymes had no effect on the light responses and blocking NMDARs with AP7 eliminated the control amplitudes. Heat-inactivated enzymes showed high selectivity for d-serine without significant effects on glycine. Our results strongly support the concept that endogenous d-serine plays an essential role as a coagonist for NMDARs, allowing them to contribute to the light-evoked responses of retinal ganglion cells. Furthermore under our experimental conditions, these coagonist sites are not saturated so that modulation of NMDAR sensitivity can be achieved with further modulation of d-serine.

INTRODUCTION

For the past decade and a half, there has been a shift in thinking associated with d-amino acids. Previously thought to be of little consequence in higher species, it has become clear that some d-amino acids are physiologically relevant in vertebrates. The most convincing case for a functional role of a d-amino acid is that of d-serine, which is now thought to serve as an essential coagonist for N-methyl-d-aspartate receptors (NMDARs) in many regions of the CNS. The unique activation properties of NMDARs require the simultaneous binding of glutamate together with a coagonist that binds at the “glycine binding site.” Although it was originally assumed that glycine was the native coagonist for this essential function, recent studies suggest that in many regions of the nervous system d-serine may be the dominant endogenous coagonist (Mothet et al. 2000; Ren et al. 2006). D-Serine is synthesized in the nervous system from l-serine by the enzyme serine racemase, which was initially localized to astrocytes (Snyder and Kim 2000). In astrocyte cultures, release of d-serine is mediated by activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors intrinsic to glial cells (Mothet et al. 2005). Thus d-serine regulation of NMDARs has opened up a new modality for glial participation in the control of neuronal excitability. A recent study, however, has raised the possibility that serine racemase and d-serine may also be found in neurons, so the idea of an exclusive role for d-serine as a mediator for glial–neuronal control has become less certain (Kartvelishvily et al. 2006).

As a component of the CNS, the vertebrate retina has been a focus of pioneering studies of glial function and glial–neuronal interactions (Newman and Zahs 1998). In the retina, NMDARs are found on ganglion cells, the output cells of the retina, and some amacrine cells (Dixon and Copenhagen 1992; Mittman et al. 1990). Immunostaining methods have localized d-serine and serine racemase to glial cells in the retina, including Müller cells and astrocytes. Functionally, when the enzyme d-amino acid oxidase (DAAO) was added to the bathing medium, whole cell currents of retinal ganglion cells (RGCs) evoked by focal application of NMDA were attenuated. Additionally, extracellularly recorded NMDAR-mediated currents were reduced, suggesting that endogenous levels of d-serine may serve a coagonist function in the retina (Stevens et al. 2003).

The light-evoked responses of RGCs have prominent NMDAR contributions (Massie and Miller 1990). The chemical identity of the coagonist for NMDAR activation, whether it is d-serine or glycine, has not been clearly established. Preliminary findings suggest that d-serine may play a role as an endogenous coagonist for NMDARs in the retina (Stevens et al. 2003), but evidence favoring this point of view is incomplete and no studies have yet examined d-serine’s role in light-evoked responses of RGCs. D-Serine has been detected in the retina, but levels of this amino acid are low (Miller 2004; O’Brien et al. 2005) perhaps because of the presence of DAAO in photoreceptors (St Jules et al. 1992) and Müller cells (Beard et al. 1988). In addition, the retina is a site in which glycine plays a prominent role as an inhibitory neurotransmitter released by a subset of amacrine cells (Marc 1989; Miller et al. 1977). Thus glycine may be comparatively high in the inner retina, the very region where NMDARs are located.
Complications related to previous work in the retina have come from recent studies related to the actions of DAAO. The enzyme is not selective for \( \text{D-} \)serine. It is more metabolically active against \( \text{D-} \)alanine and, more important, has been reported to deplete glycine levels (Denu and Fitzpatrick 1992). In addition, many commercially available DAAO preparations have been found to contain some level of \( \text{D-} \)aspartase activity, which may lead to the degradation of NMDA when it is used as an exogenous coagonist for NMDARs (Shleper et al. 2005). Thus a previous study in the retina, in which DAAO was used to demonstrate reduced sensitivity to exogenously applied NMDA, could have an alternative explanation (Stevens et al. 2003) because of possible contamination in the enzyme preparation with \( \text{D-} \)aspartase activity. In this study, we have addressed the limitations of prior work based on DAAO by carrying out experiments using a more selective \( \text{D-} \)serine degrading enzyme, \( \text{D-} \)serine deaminase (DsdA). Additionally, we have characterized the selectivity of both DsdA and DAAO against glycine and \( \text{D-} \)serine using capillary electrophoresis to measure the specificity of action of both enzymes.

We report that light-evoked responses in the retina are attenuated after the application of either of two different \( \text{D-} \)serine degrading enzymes—including, for the first time, the successful use of the highly selective enzyme DsdA in an intact tissue preparation. Furthermore, this enzymatically based attenuation of the light response of RGCs is dependent on activation of NMDARs because no change is seen when they are applied in the presence of the NMDAR antagonist 2-amino-7-phosphonooctanoic acid (AP7). These findings strongly support a role for endogenous \( \text{D-} \)serine as a coagonist of NMDAR contributions to light-evoked activity in the inner retina. Our results also suggest that under the experimental conditions of this study \( \text{D-} \)serine is the dominant and favored coagonist of NMDA receptors.

**METHODS**

**Electrophysiology**

Tiger salamanders (*Ambystoma tigrinum*) were purchased from a dealer (Charles D. Sullivan, Nashville, TN) and maintained in circulating cold-water tanks (4°C) with a 12-h room light/dark cycle. Animal maintenance and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Animals were killed by decapitation followed by double pithing.

Proximal negative response (PNR) experiments were performed in the amphibian eyecup preparation after removing the cornea and lens and draining the vitreous (Miller and Dacheux 1976). Beveled-glass microelectrodes, with tip resistances of a few megohms, were filled with Ringer, mounted on a micromanipulator, inserted into the retina to a depth of 60–100 \( \mu \)m, and adjusted to the maximum amplitude of the PNR (Burkhardt 1970). Amplification was achieved by a Grass P16 amplifier connected to an A/D converter (Digidata 1200) and displayed using a computer controlled by pCLAMP software (9.0, Molecular Devices, Sunnyvale, CA), sampling at 1 kHz. A small spot of light (110 \( \mu \)m in diameter) from a 12-V tungsten–iodide light source was positioned directly over the electrode. During recordings, focal light stimulation alternated with the presentation of a diffuse light stimulus that covered the entire retina. Stimulus intensity was controlled with neutral-density filters. The eyecup was continually superfused, at a rate of 1–2 ml/min, with a cooled (19°C), oxygenated, Mg\(^{2+}\)-free Ringer solution. To maximize the NMDAR contribution to the PNR, a control cocktail solution containing (in \( \mu \)M) 10 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; Tocris, Ellisville, MO), 10 strychnine, 50 picrotoxin, and 50 mecamylamine was applied. In these studies all additional pharmacological agents were added to this cocktail Ringer solution.

Whole cell recordings (WCRs) were obtained from identified ganglion cells in a flat-mount retina preparation of the tiger salamander (Stevens et al. 2003). Isolated retinas were placed in a perfusion chamber, ganglion side up. The chamber was continuously perfused with cooled (19°C), oxygenated Ringer at a rate of 1–2 ml/min. Ganglion cells were observed using a water-immersion objective. Patch electrodes (5–10 MΩ) were pulled using a P-97 Flaming Brown pipette puller (Sutter Instrument, Novato, CA) and filled with an intracellular solution containing (in mM): KCl, 140; NaCl, 2.5; CaCl\(_2\), 1.8; MgSO\(_4\), 5; HEPES, 10 (pH 7.8). Voltage-clamp studies were carried out after adjusting the standing current to zero (average membrane potential = −68.9 ± 0.9 mV, \( n = 22 \)).

Light stimulation was provided by a computer-controlled LCD projector system using a tungsten–halogen light source (Burkhardt et al. 1998), unless indicated otherwise, the diameter of focal light stimuli was 250 \( \mu \)m. The duration of light stimulation was typically 2 or 4 s and was projected onto the preparation through a ×20 microscope objective (Olympus, Melville, NY) and focused on the plane of the ganglion cell layer. Responses to the onset and offset of the stimulus were evaluated as peak amplitude in addition to the total charge, which was determined by integrating the light-evoked currents. DAAO and DsdA were delivered in a nominally Mg\(^{2+}\)-free Ringer solution.

Extracellular Ringer solution (in mM) 110 NaCl, 2.5 KCl, 1.8 CaCl\(_2\), 10 HEPES, and 5 \( \text{d-} \)glucose (pH 7.8). Except as indicated, all agents were purchased from Sigma Chemical (St. Louis, MO). 1,10-AP7 (100 \( \mu \)M) was used to block NMDAR currents (Tocris). Tissue \( \text{D-} \)serine degradation was carried out using 100 \( \mu \)g/ml of DAAO (Worthington, Lakewood, NJ) or 10 \( \mu \)g/ml of DsdA added to the perfusion medium. Bacterial DsdA, which has been shown to be much more efficient than DAAO in the removal of \( \text{D-} \)serine, was isolated and purified by the methods previously outlined (Shleper et al. 2005).

**Capillary electrophoresis**

Ringer samples containing 10 \( \mu \)M of both \( \text{D-} \)serine and glycine were incubated at room temperature with DAAO (100 \( \mu \)g/ml) or DsdA (10 \( \mu \)g/ml) in low-volume (1.6 \( \mu \)l) centrifuge tubes for times ranging from 1 min to 4 h. Samples were then measured for their amino acid levels using a commercial capillary electrophoresis (CE) instrument with laser-induced fluorescence (LIF) detection (Beckman Coulter P/ACE MDQ), as previously described (O’Brien et al. 2005). The samples were fluorescently derivatized at 60°C for 15 min with 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F; Molecular Probes, Eugene, OR) before being analyzed by CE using a hydroxypropyl-β-cyclodextrin (HP-β-CD) separation buffer. All data were collected and analyzed after subtracting background noise using Karat 32 software (Beckman Coulter, Fullerton, CA).

**Statistics**

CE and electrophysiological data were analyzed in Origin, 7.0 or 7.5 (Northampton, MA). All results are expressed as the means ± SE and a Student’s \( t \)-test was used to compare values; a \( P \) value <0.05 was considered significant for all statistical analyses.
RESULTS

Light-evoked whole cell responses are attenuated after degradation of d-serine

Whole cell recordings of RGCs were carried out in the voltage-clamp mode to study the properties of synaptically mediated, light-evoked responses. These recordings were most commonly obtained from ON–OFF ganglion cells, whose light-evoked responses typically consisted of transient peaks at light on and off, with a somewhat slower response decay after the peak as illustrated in Fig. 1A. In this example, Mg$^{2+}$-free Ringer was used to minimize the magnesium block of NMDA receptors, which is typically present at the normal resting membrane potential of ganglion cells. The addition of DAAO to the bathing solution resulted in a reduction of the inward current response to the light stimulus. Figure 1B shows the cumulative data from six experiments and illustrates that the ON response peak amplitude was reduced by 21.8 ± 4.6% of the control. The total charge, determined by integrating the response, was 25.0 ± 7.8% less than control. The OFF responses were also reduced with a peak amplitude reduction of 21.8 ± 4.2% and a reduction in total charge to 28.1 ± 8.4% of control values (n = 6). The enzymatic reductions for ON and OFF responses were significant when compared with control levels (P < 0.05).

We carried out a series of additional experiments to evaluate the actions of the more selective enzyme DsdA (Fig. 1, C and D). DsdA attenuated both the ON and OFF responses of RGCs. The peak amplitude of the ON response declined by 33.0 ± 10.0%, whereas the total charge was reduced by 28.8 ± 5.9%. The peak amplitude of the OFF response was reduced by 24.8 ± 5.6% and the total charge was diminished by 32.0 ± 7.1% (n = 6, P < 0.05). In these experiments, we also determined whether the addition of exogenous d-serine to the enzyme bathing solution could restore the responses to control levels (Fig. 1D). These observations illustrated that, in many cases, the addition of d-serine in the presence of the enzyme increased response amplitudes to levels higher than those of the controls. Heat inactivation of the enzymes, by exposing the enzyme solutions to a boiling water bath for 5 min, eliminated the suppressive effects of DAAO and DsdA on the light-evoked currents (not illustrated).

D-Serine degradation attenuates extracellularly recorded NMDAR currents

Beveled-glass microelectrodes were used to measure the proximal negative response (PNR) in the tiger salamander eyecup. The PNR is a response of the inner retina that reflects activity of third-order retinal neurons, including amacrine and ganglion cells (Burkhardt 1970). In standard, Mg$^{2+}$-free Ringer, the NMDAR component of the PNR was minimal. Under these conditions, blocking NMDA receptors with the antagonist AP7 did not significantly reduce the PNR; a decrease of 2.0 ± 3.8% of the peak amplitude was observed under these conditions (n = 7; Fig. 2D, inset: standard + AP7 vs. standard). However, when a cocktail of antagonists was added to the bathing medium (picrotoxinin, strychnine, mecamylamine, and NBQX), the PNR was enhanced and the relative contribution from NMDARs was pronounced. The addition of the cocktail itself increased the PNR amplitude, as measured at the peak of the ON response (Fig. 2D, light gray vs. 

**FIG. 1.** Whole cell recordings of light-evoked responses from retinal ganglion cells (RGCs) were attenuated after enzymatic degradation of endogenous d-serine. A: under voltage-clamp conditions, the RGC showed a large inward current to light onset followed by a smaller current at light offset. Addition of 100 μg/ml D-amino acid oxidase (DAAO) to the medium led to a reduction in the response. B: cumulative data show that these effects are significant: DAAO treatment showed a decrease in total charge of 25.0 ± 7.8% (ON) and 28.1 ± 8.4% (OFF) and in amplitude of 21.8 ± 4.6% (ON) and 21.8 ± 4.2% (OFF) when compared with controls (n = 6). Full effect of enzyme occurred within 10 min of application and washout in Mg$^{2+}$-free Ringer (RTC) returned values to control levels within 15 min. C: example of voltage-clamp traces from a RGC showing the attenuation of light-evoked responses after d-serine deaminase (DsdA) application. D: bath application of DsdA decreased the total charge by 28.8 ± 5.9% (ON) and 32.0 ± 7.1% (OFF) and decreased the peak amplitude by 33.0 ± 10.0% (ON) and by 24.8 ± 5.6% (OFF). Addition of d-serine (200 μM) to the enzyme solution returned the responses to levels similar to those of control.
black trace) and converted it to a light response that was largely composed of NMDAR-mediated current. When AP7 (100 μM) was added to the cocktail, the ON response of the PNR was reduced by 82.6 ± 4.1% (n = 7, Fig. 2D). By using the cocktail as the control bathing solution, we were able to examine an NMDAR-dominated light-evoked response of the inner retina with the convenience of extracellular recordings, which provided a more stable recording environment.

We confirmed previous studies of the PNR (carried out using a slightly different cocktail) by demonstrating that enzymatic degradation of d-serine, through the addition of exogenous DAAO (100 μg/ml), reduced the magnitude of the NMDAR-dominated PNR (Fig. 2A) (Stevens et al. 2003). To establish that this result arose from the activity of the enzyme, the enzyme was exposed to a boiling water bath for 5 min before application, after which application to the retina was devoid of any detectable influence on the PNR amplitude (data not shown).

We studied the actions of DsdA (10 μg/ml) through bath application of the enzyme: DsdA decreased the NMDAR-dominated PNR. Figure 2B shows the results from a d-serine degradation experiment. In the presence of the control cocktail a large PNR response was recorded (black trace). After the addition of DsdA to the Ringer, the PNR response decreased (bottom gray trace). Both DAAO and DsdA reduced the PNR amplitude, with an average reduction of 14.7 ± 2.1% for DAAO and 20.0 ± 3.1% for DsdA when compared with control responses (Fig. 2C; DAAO n = 7, DsdA n = 5; P < 0.05). To confirm that the effect of the enzymes on the PNR was restricted to their degradation of endogenous d-serine and not the result of a nonspecific action, exogenous d-serine was added to the DAAO and DsdA solutions. The addition of d-serine to the enzyme solutions returned the PNR responses to control levels and, in some cases, led to an enhanced response greater than that of the control (Fig. 2, A–C, top light gray traces). In our experiments, 100 μM d-serine added to the DAAO solution was sufficient to bring the response back to or beyond control values. In contrast, however, it was necessary to use a higher concentration of d-serine (200 μM) to achieve the same results when using the DsdA enzyme. We attribute this need for a higher d-serine level in the DsdA enzyme degrading enzymes. A–C, inset: control vs. light gray, 2-amino-7-phosphonoheptanoic acid (AP7)]. In our control cocktail, the PNR appreciably increased in amplitude (black trace) and the application of AP7 nearly eliminated the response (dark gray trace and inset: control cocktail black vs. dark gray).

Confirmation of enzyme specificity

We examined the possibility that the suppressive effects of DAAO and DsdA on the NMDAR-mediated responses of the inner retina could be the result of an action of these enzymes on glycine rather than d-serine. A degradation of glycine by DAAO has been previously reported (Denu and Fitzpatrick...
This determination was essential because any reduction in glycine by these enzymes would invalidate our conclusions about the role of d-serine versus glycine as an NMDAR coagonist in the retina. We addressed this problem by using capillary electrophoresis (CE) to examine both the time course and specificity of action of the two enzymes on d-serine and glycine. When added to a Ringer solution containing 10 µM each of d-serine and glycine, DAAO and DsdA both significantly reduced d-serine concentrations while not significantly affecting glycine levels during incubation times from 1 min to 4 h (Fig. 3B). Although the effects on d-serine versus glycine were similar with the two enzymes, DsdA was more efficient and powerful in its action on d-serine. DsdA led to the complete elimination of the d-serine peak in the electropherogram at the shortest incubation time without significantly affecting the glycine peak (Fig. 3, A and B, right). In contrast, an identical study with DAAO revealed a much slower, time-dependent drop in d-serine levels, but with no significant influence on glycine (Fig. 3B, left). Thus these studies clearly support the idea that enzymatic reductions in NMDA-receptor

PNR amplitude is increased after application of exogenous d-serine

We used the enhanced PNR as a tool for evaluating the level of saturation of the NMDAR coagonist site under our experimental conditions. Figure 4A illustrates a PNR recording in which exogenous d-serine was added to the control cocktail mixture, resulting in an elevation of the PNR amplitude. The PNR enhancement by the addition of d-serine (100 µM) to the control solution led to an increase in this field potential of 14.4 ± 3.9% versus control; washout was sufficient to record a return to the control response (Fig. 4B; n = 6, P < 0.05). Interestingly, response enhancement was also observed after some applications of d-serine added to DAAO or DsdA, enhancing the amplitude beyond the original values of the controls (see Fig. 2B).

Manipulation of d-serine levels has no effect when NMDARs are blocked

Whole cell recordings from RGCs were used to examine the dependency of endogenous d-serine manipulations on NMDAR activity. Figure 5A illustrates an example of this strategy. A control light-evoked response (Fig. 5A, black trace) was obtained from an ON–OFF ganglion cell in Mg²⁺-free Ringer. This was followed by the introduction of AP7, to block NMDA receptors; when a steady-state response was observed in the AP7 environment, the trace shown in gray was obtained, illustrating a reduction in the on and off inward currents. This was followed by the addition of the DsdA enzyme to the AP7 bath, which was without any additional influence on the light response. The DsdA + AP7 trace was indistinguishable from the response evoked in AP7 alone. The inset is an expanded timescale from the on response to illustrate how closely the two traces (gray and light gray) overlap. Figure 5B provides summary data from five experiments carried out identically to that illustrated in Fig. 5A; the total charge reduction from NMDAR block was 18.3 ± 8.3% for the on response and 41.2 ± 16.0% for the off response, whereas the peak amplitude measurements showed a reduction of 21.9 ± 8.3% for the on and 56.9 ± 9.1% for the off response (P > 0.05). Figure 5C illustrates the alternative experiment, in which a control light-evoked response was obtained from an ON–OFF ganglion cell in Mg²⁺-free Ringer, after which the addition of AP7 reduced the light response and also had a significant effect in reducing the background inward current (or elevation of an outward current) accounting for the response displacement. Superimposed on the AP7 recording is a recording from the same cell obtained after adding 100 µM d-serine to the AP7 bathing solution. Because the two traces virtually superimpose, it is clear that the normally enhancing actions of d-serine on light-evoked currents of ganglion cells require the presence of functional NMDARs. Figure 5D summarizes the five experiments carried out identically to that illustrated in Fig. 5C. In summary, when NMDARs are blocked with AP7, neither d-serine nor DsdA modified the response to light. This indicates that the action of d-serine and DsdA is mediated solely through NMDARs.
The AP7 and DsdA reductions in light-evoked responses are indistinguishable

Figure 6 summarizes and compares the effects of AP7 and DsdA from the data presented previously. In all cells studied with AP7 (see Fig. 5, C and D), the reduction in relative total charge of ganglion cell light responses versus those observed in the Mg$^{2+}$-free control was 25.3 ± 5.0% for the ON response and 42.6 ± 8.2% for the OFF response. These values were not significantly different from the reductions observed after DsdA application (ON reduction of 28.8 ± 5.9% and OFF reduction of 32.0 ± 7.1%). A Student’s t-test, comparing the reductions found in AP7 to those with DsdA, revealed P values of 0.673 for the ON and 0.356 for the OFF.

DISCUSSION

The present study has established that D-serine is a functional endogenous NMDAR coagonist in the salamander retina and is essential for NMDARs to participate in generating synaptic currents in retinal ganglion cells. Bathing the retina in D-serine degrading enzymes (DAAO and DsdA) attenuated light-evoked responses observed in WCRs of ganglion cells and those of the field potentials reflected in the enhanced PNR. In this study, we have introduced the enzyme D-serine deaminase (DsdA) to an application involving intact tissue, whereas previous studies used this expressed and purified enzyme preparation to study excitotoxicity in tissue culture preparations (Shleper et al. 2005). We used capillary electrophoresis techniques to separate and quantitatively analyze the relevant amino acid enantiomers and demonstrated that DsdA was more effective than DAAO in reducing D-serine, although both enzymes showed high selectivity for D-serine over glycine. Similar to the effects of DAAO, DsdA decreased NMDAR currents in ganglion cells, whereas the heat-inactivated form was without effect. We suggest that the greater sensitivity of
DsdA toward d-serine and its higher rate of degradation make it the enzyme of choice over DAAO for rapidly and effectively reducing endogenous d-serine levels when using intact tissue preparations. Given the purity of the DsdA enzyme used in this study, it has the additional merit of being free from other conflicting contaminants, such as the d-aspartase that sometimes can be found in commercial preparations of DAAO (Shleper et al. 2005).

Because DsdA is more effective than DAAO in degrading d-serine, it is natural to ask whether DsdA is present in the brain and retina. Although the presence of DAAO in vertebrates has long been known, this enzyme is more selective for d-alanine and d-proline compared with d-serine. DsdA is found in bacteria, but it is also found in birds. A recent study comparing the two enzyme pathways has been reported based on the use of an HPLC-based technique for analyzing and comparing the two by-products of d-serine degradation by DAAO (3-hydroxypropionate) and DsdA (pyruvate). Using this method, the brains of rats and chickens were compared for d-serine metabolites. D-Serine degradation in bird brains could be attributed to the actions of both DAAO and DsdA, but in the rat brain d-serine was metabolically restricted to DAAO. However, although it appears that DsdA is an unlikely player in mammalian nervous tissue, it has not to our knowledge been evaluated with this more direct, functional technique in amm.

In addition to the link between endogenous d-serine and light-evoked NMDAR activation established in this study, we have addressed several major issues that have clouded our understanding about the role of d-serine in the retina. The current study has established that, at physiological pH conditions, the enzymatic action of DAAO shows high selectivity for d-serine while having little effect on glycine levels. Thus a previous report on DAAO degradation of glycine presumably reflected the high pH (10.3) used in that analysis (Denu and Fitzpatrick 1992). In contrast, our study of DAAO selectivity was done using normal Ringer, at physiological pH (7.8). Under these conditions we did not detect any glycine degradation even after several hours of exposure. We also established that when DAAO was applied to the retina, the associated decrease in NMDAR-mediated events was restored with coapplication of exogenous d-serine, whereas the application of the heat-inactivated enzyme did not influence NMDAR currents. The addition of exogenous d-serine to overcome the suppression of NMDAR currents as a result of enzyme application has provided compelling additional evidence that the active form of the enzyme reduced NMDAR currents through its action on the endogenous pool of d-serine. This result extends the observations reported by Stevens et al. (2003), in which the inactive form of the enzyme was not evaluated and together with identical results found with DsdA strongly support the idea that endogenous d-serine plays a major role as the NMDAR coagonist.

We determined that the addition of d-serine degrading enzymes consistently and reliably reduced the light-evoked response, as measured electrophysiologically in PNR recordings and through WCRs of retinal ganglion cells. The two enzymes resulted in very similar reductions in light responses that could be attributed to NMDAR activation. Because these enzymes have no observed effects on glycine, we conclude that endogenous d-serine plays a major role as an NMDAR coagonist in the salamander retina. Although it is important to emphasize that our results do not eliminate the possibility that glycine also serves a companion NMDAR coagonist role, we were unable to find a significant level of residual NMDAR function that was not eliminated through the enzymatic exposures used in this study. Therefore if glycine plays a significant role as an NMDAR coagonist in the retina, it must be operational under experimental circumstances different from those used in this study. Indeed, we believe that the results of this study have reversed the burden of proof for identifying the endogenous NMDAR coagonist in the retina by replacing a presumed coagonist, glycine, with d-serine for which strong compelling evidence is now available. However, because of the multitude of different functional states in which the retina is designed to detect visual signals, it is premature to eliminate glycine from any functional role in setting NMDAR sensitivity. Nevertheless, our results provide a compelling case for d-serine as the major coagonist of NMDARs in the retina.

Although we think it is unlikely that glycine plays a prominent role as an NMDAR coagonist in the retina, it occupies a place of prominence in retinal function and, for that reason alone, glycine merits some discussion. Glycine is a prominent, inhibitory amino acid in the retina, utilized by a subset of amacrine cells (Marc 1989; Miller et al. 1977). It is widely assumed that, in the brain, glycine uptake through the GlyT1 transporters, which are localized to astrocytes, serves to regulate the availability of glycine and determines its role as a coagonist of NMDARs (Berger et al. 1998). In contrast to the brain, the distribution of the GlyT1 transporters in the retina is more complex and may differ across species. In mammalian and chick retinas, it appears that GlyT1 transporters are absent in glia, but are found instead in a subset of glycinergic amacrine cells (Pow and Hendrickson 1999) where, surprisingly, the GlyT1 transporter plays a central role in glycine accumulation in these cells (Pow 1998) because very little net glycine synthesis is present. In contrast to the mammalian/chick retinas, recent studies in the bullfrog retina have presented evidence for GlyT1 transporters in Müller cells (Du et al. 2002; Lee et al. 2005).
In the brain, the codistribution of NMDARs and GlyT1 transporters has been examined at the ultrastructural level (Smith et al. 1992) and revealed a close correspondence and apposition of these two membrane embedded proteins. This correspondence has raised speculation that the GlyT1 transporters play a special role in setting the background levels of glycine and thus in regulating NMDAR sensitivity. We suggest that a similar relationship may exist in the retina. When the selective GlyT1 transporter blocker NFPS (N[3-(4-fluorophenyl)-3-(4-phenylphenoxy)-propyl]saccharosine) was applied to the salamander retina, the coagonist sites reflected in the NMDAR component of ganglion cell light responses were saturated (ER Stevens, unpublished observation), strongly suggesting that the GlyT1 transporter served to reduce the external levels of glycine so that NMDAR coagonist sites were not saturated. When these observations are put in the context of the current study, it endorses the following generalization about the relationship between D-serine and glycine as coagonists for NMDA receptors in the retina: under normal conditions, the GlyT1 transporters maintain external glycine levels substantially below the level at which glycine interacts significantly with the coagonist sites of NMDARs. The high affinity of the GlyT1 transporters for glycine uptake combined with the lower affinity of the NMDAR coagonist sites for glycine (Matsui et al. 1995) reduces external glycine such that D-serine dominates the binding of the coagonist sites and serves to control the relative contribution of NMDARs to the synaptic currents of RGCs. Presumably this applies as well to those amacrine cells that have NMDARs, although these cell types were not included in this analysis.

The results of this study have functional implications for the regulation of NMDAR sensitivity in the retina. The consistent potentiation of the PNR after the application of exogenous D-serine argues that, under our experimental conditions, the coagonist sites of the NMDARs are not saturated. Thus manipulation of D-serine levels above or below this background level should, respectively, enhance or diminish synaptic currents generated by NMDARs. In fact, early experiments examining the role of the coagonist site in RGC NMDARs found evidence that increases in stimulation intensity led to an increase in available coagonist concentration as measured against the competitive coagonist site inhibitor 5,7-dichlorokynurenic acid (Łukasiewicz and Roeder 1995). Although this effect was attributed to glycine release from amacrine cells, our results support a different interpretation based on the dominance of D-serine as the major and perhaps only coagonist for NMDA receptors. It is important to note that the coagonist sites of NMDAR can mediate two complementary but distinct functions. One is to serve as the coagonist with glutamate to open the ion channel, whereas the other function is manifest when D-serine is below saturation levels, in which case the degree of saturation at this site plays a role in the rate of NMDAR desensitization: saturation of the site decreases the rate of desensitization of NMDARs (Lester et al. 1993; Vytklicky et al. 1990). Thus both the magnitude and the time course of NMDAR currents are modulated by the degree of saturation at the coagonist site. Yet a third role for the coagonist site was more recently proposed in which saturation at this site stimulated internalization of NMDARs (Nong et al. 2003), although the functional significance of this effect remains unknown.

The present study has expanded the evidence that D-serine plays a role as a coagonist for NMDARs in the retina and removed some of the experimental ambiguity of previous studies that first proposed a relationship for endogenous D-serine and NMDAR sensitivity. Despite the reportedly low tissue levels of D-serine in the retina (Miller 2004; O’Brien et al. 2005), degradation of endogenous D-serine reduces NMDAR-mediated currents. Because NMDARs contribute to neuronal excitation in the inner retina (Massey and Miller 1990), it will be important for future studies to determine how the synthesis, storage, release, and uptake of D-serine are controlled and how these mechanisms fit into concepts that reflect glial-neuronal interactions.

The original studies of the D-serine synthesizing enzyme serine racemase (SR) localized the enzyme exclusively to astrocytes (Snyder and Kim 2000) and observations in the retina supported the localization of SR to Müller cells and astrocytes (Stevens et al. 2003). More recently, Kartvelishvily et al. (2006) proposed that SR is also found in neurons and this work has placed doubts about whether the enzyme and thus D-serine is exclusively a glial-derived modulator of NMDA receptors. It appears that this issue may be related to differences in the specificity of the antibodies used for localization of the racemase enzyme. In addition to the original study of Stevens et al. (2003), a more recent publication (Williams et al. 2006) has further supported the idea that SR is found in glial cells in the retina. Thus far, no study of the retina has implicated significant neuronal localization of SR, but clearly, more work will be needed to clarify whether SR and D-serine are exclusively within the operational domain of glial cells.

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