D-serine: A study of its function and regulation in the retina

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Chapter 1: General Introduction

It has often been said that the eyes are the window to the soul. While the veracity of this adage may be as uncertain as its true origins, the eyes have definitely been a window to the brain. As an outgrowth of central nervous system tissue, the retina has long been used by researchers to gain an understanding of not just the nature of vision, but also of the structure and function of the nervous system in general. Over a century ago, the retina proved an ideal tissue for Ramon y Cajal to study the intricacies of cell morphology and connections that would help lead him to follow the Neuron Doctrine—the idea that the nervous system is made up of billions of discrete cells. Since then, innumerable discoveries about the nature of the retina and of the way the nervous system functions have resulted from retinal studies.

Two decades after Ramon y Cajal received the Nobel Prize in medicine for his ground breaking morphological studies, Adrian and Matthews used an intricate dissection of the eye and optic nerve of the conger eel and frog to show that the fibers of the optic nerve fire all or nothing responses when light is presented to the eye (Adrian and Matthews 1927). Later, H. Keffer Hartline, expanding on the technique, teased out individual optic nerve fibers and recorded the first responses representing individual ganglion cells (Hartline and Graham 1932). His subsequent studies built the foundations for much of our basic understanding of ganglion cell light responses. He determined that each fiber had a specific response to light stimuli: some responded when a light was turned on, some when it went off, and others fired at both the onset and offset of the light (Hartline 1938). Each of these responses, he discovered, came from spatially defined

receptive fields (Hartline 1940). His methods, while fruitful in his hands, proved to be too laborious to produce many followers. It was the adoption of the microelectrode by Ragnar Granit, placed on the surface of the retina, that would stimulate decades of intense retinal research (Granit and Svaetichin 1939). Granit and Hartline both were awarded the Nobel Prize in 1967 for their work in making the first electrical recordings of light responses from retinal ganglion cells. They shared the award with George Wald who took another monumental step in describing how photopigments are involved in capturing light energy (Wald 1951).

These early experimenters took advantage of the essential characteristics that make the retina a great model system for neuroscience research. The ability to isolate an entire network of cells while still retaining its essential functionality and to be able to use its natural stimulus, light, to study that network combine to create the unique quality of the retina. In addition, the compactness and relatively simplicity of the retina has allowed for a thorough mapping of its structure that includes identification of its cell types as well as the discovery of the transmitters that facilitate communication between cells.

While the retina uses all of the major neurotransmitters found elsewhere in the nervous system; including GABA, glycine, and acetylcholine; the vertical light pathway is mediated by glutamate. The capture of light energy by the photopigments in the photoreceptors results in the closure of ion channels which hyperpolarizes the cells and decreases the amount of glutamate they release into the outer plexiform layer. This decrease in glutamate has opposite effects on the bipolar cells of the two parallel pathways into which the retina is segregated—the ON and the OFF. The ON bipolar

cells possess a unique metabotropic glutamate receptor, mGluR6, which, when bound by glutamate, acts through a second-messenger system to close an ion channel. As a result, a decrease in the amount of glutamate released from the photoreceptor results in the opening of the ion channel and a subsequent depolarization of the ON bipolar cell. The ionotropic glutamate receptors found on the OFF bipolar cells open when bound by glutamate. So the decrease in glutamate available means more closed channels and a hyperpolarization of the OFF bipolar cell. The bipolar cells then form ionotropic glutamatergic synapses in the inner plexiform layer with ganglion cells, whose axons project the output of the retina, through the optic nerve, to the brain. This main vertical, glutamate pathway is modified through the release of numerous excitatory and inhibitory neurotransmitters and neuromodulators, including from horizontal cells in the outer plexiform layer and amacrine cells in the inner plexiform layer.

The glutamate signal in the inner plexiform layer excites α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) and N-methyl-D-aspartate (NMDA) type ionotropic glutamate receptors on retinal ganglion cells. The AMPA receptor, the most common receptor in the nervous system, is a fast activating and fast inactivating cation channel which is highly permeable to sodium and potassium, but less so to the divalent ion calcium. The NMDA receptor has a much more complicated activation pattern which can be affected by a number of different ions, peptides, and amino acids and, importantly, its channel is highly permeable to calcium.

The NMDA Receptor

The unique properties of the NMDA receptor have been linked to a variety of complex physiological phenomenon including synaptic plasticity and memory storage (Bear 1996), excitotoxicity (Olney et al. 1991), and the regulation of neuronal development (Scheetz and Constantine-Paton 1994). Fundamental to all of these processes is the calcium permeability of the NMDA receptor channel. The influx of calcium can drive intercellular signaling cascades that result in structural changes to cellular architecture including synapse strength, or in the case of the excess calcium accumulation present in excitotoxicity, to apoptotic pathways. Neural development is a particularly interesting example in which the role of NMDA receptors has been heavily studied. In a number of brain regions, NMDA receptor numbers peak early, during periods of synaptogenesis, and decrease in adulthood (McDonald and Johnston 1990). One such region is the visual cortex, where at a time crucial in the development of left-right eye segregations of thalamo-cortical projections (LeVay et al. 1978) neurons in the target layers of the cortex are more sensitive to NMDA receptor antagonists than those in those same layers in adults (Fox et al. 1992).

At least some of these developmental changes may be due to the subunit composition of the NMDA receptors. Each NMDA receptor is made up of four subunits two NR1 subunits, of which there are multiple slice variations, and usually two NR2 subunits with the NR2A and NR2B being the most common and studied. There is a small expression of an NR3 subunit family as well which peaks around 1-2 weeks post-natal in rodents and decreases to a low, but persistent level in adulthood (Wong et al.

2002). The low expression levels and the lack of specific agonists and antagonists have so far limited the studies of the NR3 subunit (Nakanishi et al. 2009). In the case of the NR2 subunits, however, there is much research that connects the expression of NR2A versus NR2B with development. Early on, the NR1/NR2B configurations, which together produce the largest NMDA receptor currents (Kutsuwada et al. 1992), are the dominant receptor type throughout the brain. Later they are expressed at lower levels and are largely restricted to the cortex (Watanabe et al. 1992). The NR1/NR2A receptors are less sensitive to glutamate activation (Monyer et al. 1994) but do produce large peak currents (Meguro et al. 1992). These NR2A containing receptors develop later and predominate in the adult (Watanabe et al. 1992).

The Coagonist Site

As mentioned previously, the activation properties of NMDA receptors are unique and can be affected by a large number of modulatory peptides, phosphorylation, and blocked by divalent cations—especially magnesium. But perhaps the most unusual determinant for activation is the requirement for a second agonist in addition to glutamate. In early studies of the NMDA receptor, it was observed that the level of NMDA receptor responses in cultured neurons often related to the speed of the perfusion. In 1987, by analyzing the media and studying the amino acids present within, Johnson and Ascher determined that this potentiation seen under certain conditions was due to the presence of glycine and suggested that glycine, in addition to its role as an inhibitory neurotransmitter, played an allosteric role in activating NMDA receptors (Johnson and Ascher 1987). The following year the role of glycine was shown to go beyond that of

mere allosteric modulation of the receptor and to actually be a required agonist, co-equal with glutamate, for its activation (Kleckner and Dingledine 1988). Within a short time, it was discovered that several other amino acids could also function as coagonists including the unlikely D-enantiomer of serine (Snell et al. 1988) (Kleckner and Dingledine 1988) (Fadda et al. 1988). But given that glycine was already found in the brain and was known to function as a neurotransmitter and that D-amino acids were not thought to play any role in higher organisms, D-serine's ability to act as a ligand at the NMDA receptor was seen as more a curiosity than anything else.

D-serine

Because of the biphasic effects proposed for glycine, acting both as an inhibitory transmitter at strychnine-sensitive glycine receptors and as an excitatory transmitter at the NMDA receptor, some sought to look for a more selective agonist within the nervous system. When Hashimoto et al. used HPLC to quantify amino acid enantiomers from rat brain microdialysate, they discovered high levels of D-serine (23 % of total brain serine) and proposed that it may be used as an endogenous NMDA receptor coagonist (Hashimoto et al. 1992b). A subsequent binding study showed a high correlation between D-serine and NMDA receptor binding in the brain (Hashimoto et al. 1993b). A few years later, the lab of Solomon Snyder produced a ground breaking study of D-serine (Schell et al. 1995). Using a stereospecific antibody, they localized D-serine to areas rich in NMDA receptor distribution, including the frontal lobes and hippocampus in rats. In addition, they proposed a mechanism of release from cortical astrocytes after showing that stimulating non-NMDA type glutamate receptors led to the release of preloaded D-

[^{3}H]-serine from astrocyte cultures. Their model suggested that presynaptic glutamate release would activate receptors on both postsynaptic neurons and the glia that ensheathe the synapse. The activation of the glial receptors would then lead to release of the NMDA receptor coagonist, D-serine. Subsequent studies have shown D-serine release to be calcium and SNARE dependent (Mothet et al. 2005) and have provided morphological evidence for vesicular storage of D-serine and the possible release from glia (Martineau et al. 2008).

Perhaps the most important discovery in the case for D-serine as an endogenous ligand was the discovery of its synthesizing enzyme serine racemase (Wolosker et al. 1999d). Serine racemase, which converts L-serine to D-serine, was initially localized to cerebral astrocytes (Wolosker et al. 1999a) in a pattern similar to that of D-serine (Schell et al. 1995). More recent studies suggest that it may also be found in some neurons (Kartvelishvily et al. 2006). The fact that D-serine has no known function in metabolic pathways suggests that it may be created for the specific purpose of activating NMDA receptors. A phylogenic study examining both serine racemase and the NMDA receptor suggested that there may be a co-evolution of the two in vertebrates (Schell 2004). A direct role for D-serine was established when brain slices treated with the D-serine degrading enzyme, D-amino acid oxidase, showed reduced NMDA receptor currents, illustratiing for the first time in vivo that D-serine has a functional role at some NMDA receptors (Mothet et al. 2000).

In order to follow classic neurotransmitter theory, there must also be a means of ending the D-serine signal via uptake and/or degradation. In this area there is much work

left to do. The sodium-dependent ASCT2 type amino acid transporter has been shown to be present and to functionally transport D-serine and other neutral amino acids in and out of cells (O'Brien et al. 2005) (Dun et al. 2007). While the nature of this hetero-exchanger would allow for uptake of D-serine, it is also capable of being a method of release, depending on the gradients of the transferable amino acids.

The nervous system does contain an endogenous degradation pathway. When D-amino acid oxidase (DAO) was first discovered in the kidney by Sir Hans Krebs (Krebs 1935) it was assumed to be used to eliminate stereochemical mistakes or to rid the body of D-amino acids that arose from the diet or bacteria of the gut. Interestingly, much early research was done into the expression of DAO activity in the human brain. Among other things, it was discovered that DAO in the cerebellum of 13-14 month old children was only 15% of that in adults. Additionally, while there existed these relatively high levels of activity in the cerebellum, no activity was found in the cerebral hemispheres, even in adults 55-82 years old (Neims et al. 1966). These results foreshadowed findings decades later that DAO expression seems to be inversely related to D-serine levels and its subsequent activity at NMDA receptors in the brain. The cerebellum, in particular, experiences a developmental shift in expression of DAO and D-serine. In rodents, D-serine levels in Bergmann glia are high early in development and peak around P12-14. At which point DAO is suddenly expressed and D-serine levels rapidly fall by 97% in the adult (Schell et al. 1997).

Retinal NMDA receptors

While NMDA receptor expression has been reported in multiple cell types in some species, including catfish horizontal cells (O'Dell and Christensen 1989) and more recently rat photoreceptors (Fletcher et al. 2000) it seems unlikely that they play a major role in early retinal processing. On the other hand, NMDA receptors have been identified on all ganglion cells (Hartveit et al. 1994) (Grunert et al. 2002) (Zhang and Diamond 2006) and on a subset of amacrine cells (Fletcher et al. 2000) (Kalloniatis et al. 2004) (Hartveit et al. 1994), where they may play a crucial role in mediating GABAergic feedback (Ferreira et al. 1994) (Huba and Hofmann 1991). Like their counterparts in the brain, ganglion cell NMDA receptors are involved in both neurodevelopment (Simon et al. 1992) and excitotoxicity (Vorwerk et al. 2000). The other critical role for NMDA receptors in the brain, that of a coincidence detector in synaptic plasticity, does not seem to have a correlate in the retina.

Much remains unsolved concerning the functions of NMDA receptors in the retina. What is known is that they account for a substantial portion of the responses of retinal ganglion cells (Slaughter and Miller 1983), (Miller and Slaughter 1986), (Lukasiewicz and Roeder 1995). One theory about how they shape light responses is that they work in concert with the fast-activating AMPA receptors that are also present on ganglion cells. Diamond and Copenhagen suggested that the effect of the magnesium block, which occurs in the pore of the NMDA receptor at large negative potentials and is subsequently released as the cell depolarizes, is to maintain a relatively constant inward current over a wide range of membrane potentials (Diamond and Copenhagen 1993). In

spite of the voltage-dependent magnesium block, it has also been shown that NMDA receptors contribute to the resting noise and conductance properties of retinal ganglion cells (Gottesman and Miller 2003a).

D-serine in the Retina

The retina might first seem like an unlikely place for D-serine to function as an endogenous ligand and modulator for the NMDA receptor. Glycine is used as an inhibitory neurotransmitter by a subset of amacrine cells (Miller et al. 1977) (Marc 1989) and the extracellular concentrations of glycine have been shown to be quite high (Slaughter and Miller 1991) (O'Brien et al. 2004). Additionally, unlike in the frontal cortex where much of the D-serine discoveries have taken place, in at least some species, both Müller cells and cone photoreceptors of the retina as well as cells of the pigment epithelium express D-amino acid oxidase (Beard et al. 1988a; St Jules et al. 1992). But initial studies in the retina demonstrated that NMDA receptor responses can be enhanced with application of exogenously applied D-serine, suggesting that NMDA receptor coagonist sites are not saturated by the high levels of glycine (Stevens et al. 2003). Potentially, this could be explained by the presence of glycine transporters in the retina. Initial studies of glycine transport in the retina reported that the GlyT1 type transporter was used solely by glycinergic amacrine cells as a means to accumulate transmitter (Pow 1998a). Later studies have suggested that glycine transporters, both the GlyT1 and the GlyT2, may be more widespread (Du et al. 2002) (Lee et al. 2005a) (Perez-Leon et al. 2004), (Salceda 2006), (Jiang et al. 2007). In addition, serine racemase and D-serine were shown to be present in the retinas of several species, with initial studies showing

strong labeling in Müller cells (Stevens et al. 2003) and more recent studies suggesting the possibility of expression in ganglion cells that may be developmentally related as well (Dun et al. 2008). Even with the high levels of glycine and the expression of DAO, D-serine seems positioned to play a dynamic role in the retina.

My experiments and contributions

The following chapters consist of four manuscripts for publication. Taken together, they present a compelling argument that D-serine functions as the dominant NMDA receptor coagonist in the retina of both tiger salamanders and of mice during light evoked activity. In addition, they examine several of the proteins and enzymes associated with D-serine and its role as a NMDA receptor coagonist.

Chapter two is a manuscript that was published in 2007 in the Journal of Neurophysiology. While I was the primary author on the paper, a share of the experiments was done by Eric Stevens. His contributions are seen in figure 2.1A,B and in figure 2.5C,D. Chapter three is a manuscript that is in press at Neuroreport. My contributions to this paper can be seen in results and write-up figure 3.3. Chapter four is a paper that was submitted to the European Journal of Neuroscience and is currently being revised. While I was the primary author on this paper, my experimental contributions were limited to the PNFP recordings in figure 4.3. The final chapter is a paper that has yet to be submitted. I am the primary author on this paper and performed all of the electrophysiological data presented in figures 5.2, 5.3, and 5.4 as well as the preliminary capillary electrophoresis (CE) experiments. Subsequent CE experiments were performed by Steve Sullivan.

The chapter order is more or less a chronological report of my experimental outcomes and techniques. There is a significant shift between chapters four and five when I redirected my research from working on the tiger salamander retina to working in a mutant mouse model. While the basic knowledge I acquired in electrophysiology transferred from the amphibian to the mouse, it was almost like starting from scratch—needing to build a whole new skill set from fabricating electrodes, to dissections, to figuring out how to patch. About the only skill that transferred was the skill of dealing with the days and days of frustration associated with whole-cell recording.

Chapter two is an investigation into the role that D-serine plays in light-evoked responses of the inner retina of the tiger salamander. Electrophysiological recordings are used to show that exogenous D-serine potentiates extracellular responses in the inner retina and that the degradation of D-serine by two different enzymes results in a decrease in the light evoked-currents of retinal ganglion cells. The decrease in response caused by the enzyme D-serine deaminase turns out not to be significantly different from the decrease seen when the NMDA receptor antagonist AP7 is applied, which suggests that D-serine is the major or perhaps sole coagonist in the conditions studied. Chapter three examines the role of serine racemase (SR) activity in determining D-serine levels and function in the salamander retina. A SR inhibitor, phenazine ethosulfate, decreases D-serine levels in retinal homogenates measured by CE and also decreases currents in extracellular and ganglion whole-cell recordings. In chapter four, we determined that the GlyT1 glycine transporter functions to keep glycine levels below the saturation point for the NMDA receptor coagonist site but another glycine transporter, the GlyT2, does not.

The final chapter, five, examines a mutant mouse model that lacks DAO function. CE is used to show that D-serine levels are increased in mutant mice when compared to wild-types. The physiological consequences of the mutation are determined to include a saturation of the coagonist site and an increase in the percentage of the light response that is mediated by NMDA receptors.

Chapter 2

Endogenous D-serine contributes to NMDA receptor-mediated light-evoked responses in the vertebrate retina

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 suppressive influence of the enzymes as well as the response enhancement normally associated with exogenous D-serine application. CE was used to separate amino acid racemates and study the selectivity of DAAO and DsdA against D-serine and glycine. Both 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 modulaton 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 NMDARs in many regions of the central nervous system. The unique activation properties of NMDARs require the simultaneous binding of glutamate together with a coagonist which 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 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 more clouded (Kartvelishvily et al. 2006).

As a component of the central nervous system, 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 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 retinal ganglion cells have prominent NMDAR contributions (Massey 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 retinal ganglion cells. D-serine has been detected in the retina, but levels of this amino acid are low (O'Brien et al. 2005; Miller 2004) perhaps because of the presence of DAAO in photoreceptors (St Jules et al. 1992) and Müller cells (Beard et al. 1988b). 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 (Miller et al. 1977; Marc 1989). 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 D-serine. It is more metabolically active against D-alanine and more importantly 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 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) due to possible contamination in the enzyme preparation with 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 D-serine degrading enzyme, DsdA. Additionally, we have characterized the selectivity of both DsdA and DAAO against glycine and 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 following the application of either of two different 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 retinal ganglion cells is dependent on activation of NMDARs—since no change is seen when they are applied in the presence of the NMDAR antagonist AP7. These findings strongly support a role for endogenous 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 D-serine is the dominant and favored coagonist of NMDA receptors.

Materials and Methods

Electrophysiology: Tiger salamanders (*Ambystoma tigrinum*) were purchased from a dealer (Charles D. Sullivan Co., Nashville, TN) and maintained in circulated cold-water tanks (4°C) with a 12 hour 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.

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 MΩ, were filled with Ringer, mounted on a micromanipulator, inserted into the retina to a depth of 60-100 μm 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 μm in diameter) from a 12V 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 (19ºC), oxygenated, Mg^{2+}-free Ringer solution. To maximize the NMDAR contribution to the PNR, a control cocktail solution containing (in μM) 10 NBQX (Tocris, Ellisville, NJ), 10 strychnine, 50 picrotoxinin, and 50 mecamylamine was

applied. In these studies all additional pharmacological agents were added to this cocktail Ringer solution.

Whole cell recordings were obtained from identified ganglion cells in a flatmount 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 (19ºC), oxygenated Ringer at a rate of 1-2 ml/min. Ganglion cells were observed using a water immersion objective. Patch electrodes (5-10 MΩs) were pulled using a P-97 Flaming Brown Pipette Puller (Sutter Instruments, Novato, CA) and filled with an intracellular solution containing, in mM: KCH_3SO_4, 98.0; $NaCH_3SO_4$, 3.5; $MgSO_4$, 3.0; $CaCl_2$, 1/0; EGTA, 11.0; HEPES, 5.0; D-glucose, 2.0; glutathione, 1.0; MgATP, 1.0; NaGTP, 0.5; pH 7.4. Voltage-clamp studies were carried out after adjusting the standing current to zero (average membrane potential = -68.9± 0.9 mV, n=22), using an Axoclamp 700A amplifier with a 10 kHz low pass Bessel filter at a sampling frequency of 4-10 kHz, digitized with a Digidata 1320 and recorded in PClamp 9.0 (all Molecular Devices, Sunnydale, CA). Series resistance was uncompensated.

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 μm. The duration of light stimulation was typically two or four seconds and was projected onto the preparation through a 20X 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 adition 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 contained (in mM) 110 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES, 5 D-glucose, pH 7.8. Except as indicated, all agents were purchased from Sigma Chemical (St. Louis, MO). D,L-AP7 (100 µM) was used to block NMDAR currents (Tocris, Ellisville, MO). Tissue D-serine degradation was carried out using 100 µg/ml of D-amino acid oxidase (Worthington, Lakewood, NJ) or 10 µg/ml of D-serine deaminase added to the perfusion medium. Bacterial DsdA, which has been shown to be much more efficient in the removal of D-serine compared to DAAO, was isolated and purified by the methods previously outlined (Shleper et al. 2005).

Capillary Electrophoresis: Ringer samples containing 10 µM of both D-serine and glycine were incubated at room temperature with DAAO (100 µg/ml) or DsdA (10 µg/ml) in low volume (1.6 µL) centrifuge tubes for times ranging from one minute to four hours. 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 60ºC for 15 minutes with 4-fluoro-7-nitrobenz-2-oxa- 1,3-diazole (NBD-F, Molecular Probes, Eugene, OR) before being analyzed by CE using a hydroxypropyl-ß-cyclodextrin (HP-ß-CD) separation buffer. All data were collected and analyzed using Karat 32 software (Beckman Coulter, Fullerton, CA).

Statistics: CE and electrophysiological data were analyzed in Origin, 7.0 or 7.5 (Northhampton, MA). All results are expressed as the mean ± SEM and a student t-test

was used to compare values; a p-value less than 0.05 was considered significant for all statistical analyses.

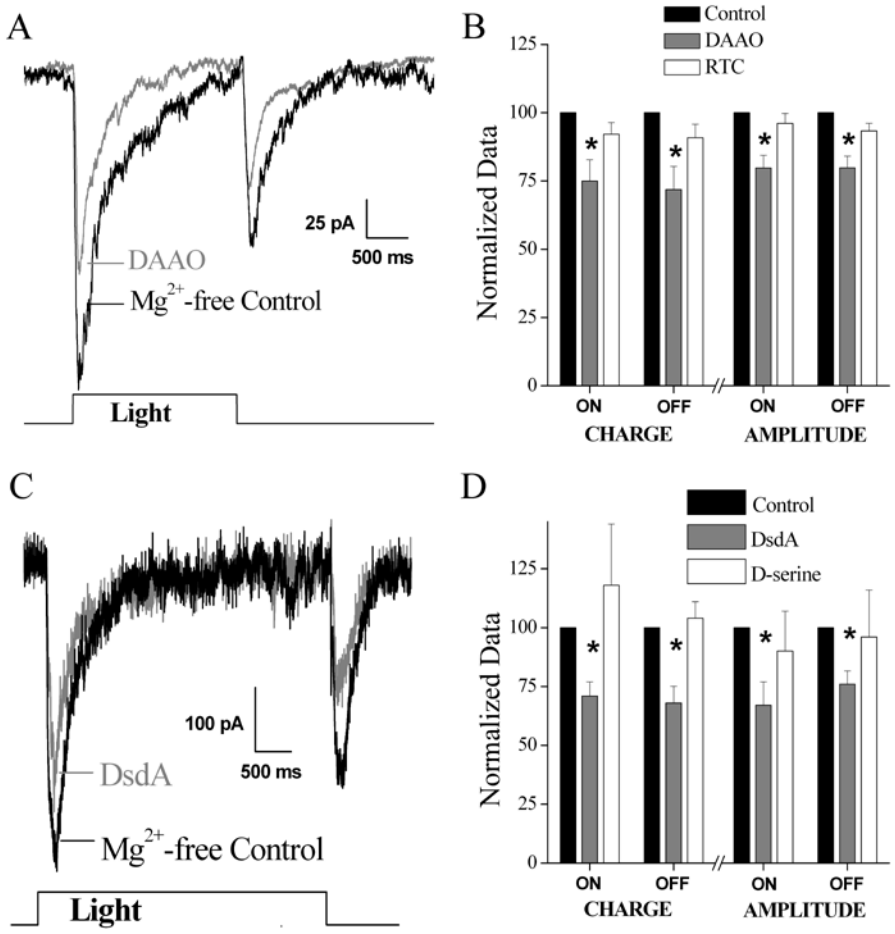
Results

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

Whole-cell recordings of retinal ganglion cells 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 following the peak as illustrated in Fig 2.1A. In this example, a 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 2.1B 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 to control levels ($p < 0.05$).

We carried out a series of additional experiments to evaluate the actions of the more selective enzyme DsdA (Fig 2.1C,D). DsdA attenuated both the ON and OFF responses of retinal ganglion cells. The peak amplitude of the ON response declined

Figure 2.1



Whole-cell recordings of light-evoked responses from retinal ganglion cells are attenuated following enzymatic degradation of endogenous D-serine. A, under voltage clamp conditions, a RGC shows a large inward current to light onset followed by a smaller current at light offset. The addition of 100 µg/ml DAAO to the medium leads to a reduction in the response. B, cumulative data shows that these effects are significant with responses under DAAO treatment showing decreases 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 to controls, n = 6. Full effect of enzyme occurred within 10 minutes of application and washout in Mg^{2+}-free Ringer (RTC) returned values to control levels within 15 minutes. C, an example of voltage clamp traces from a RGC showing the attenuation of light-evoked responses following DsdA application. D, bath application of DsdA decreases the total charge by 28.8 ± 5.9% (ON) and 32.0 ± 7.1% (OFF) and decreases the peak amplitude by 33.0 ± 10.0% (ON) and by 24.8 ± 5.6% (OFF). The addition of D-serine (200 µM) to the enzyme solution returned the responses to levels similar to those of control.

by 33.0 ± 10.0% while 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 2.1D). 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 that of the controls. Heat inactivation of the enzymes, by exposing the enzyme solutions to a boiling water bath for 5 minutes, eliminated the suppressive effects of DAAO and DsdA on the light-evoked currents (not illustrated).

D-serine degradation attenuates extracellularly recorded NMDAR currents

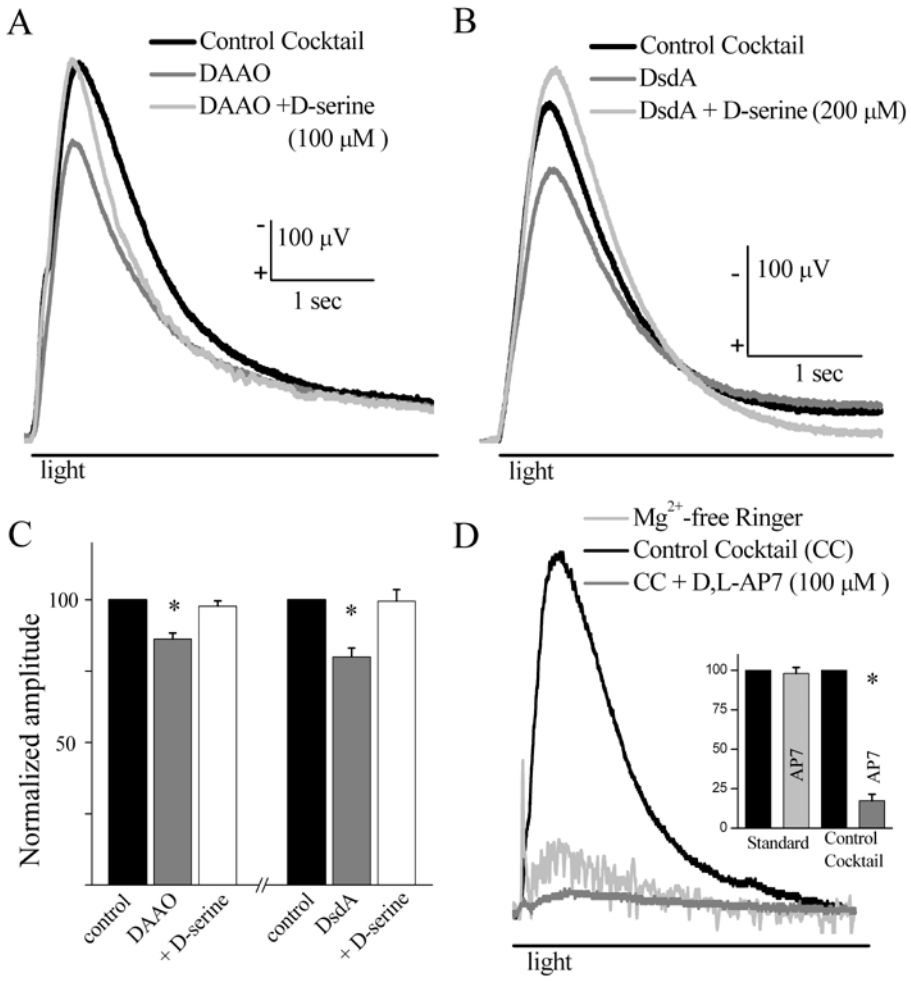
Beveled glass microelectrodes were used to measure the Proximal Negative Response (PNR) in the tiger salamander eyecup. The PNR is a response of the inner retina that reflects activity of third-order retinal neurons, including amacrine and ganglion cells (Burkhardt 1970). In standard, Mg^{2+}-free Ringer, 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 2.2D, inset: standard+AP7 vs standard). But 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 2.2D, light gray vs. 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 2.2D). 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 2.2A)(Stevens et al. 2003). To establish that this result was due to the activity of the enzyme, the enzyme was exposed to a boiling water bath for five minutes prior to 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 2.2B 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 (lower 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 to control responses (Fig. 2.2C; 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 due to a non-specific action, exogenous D-

Figure 2.2



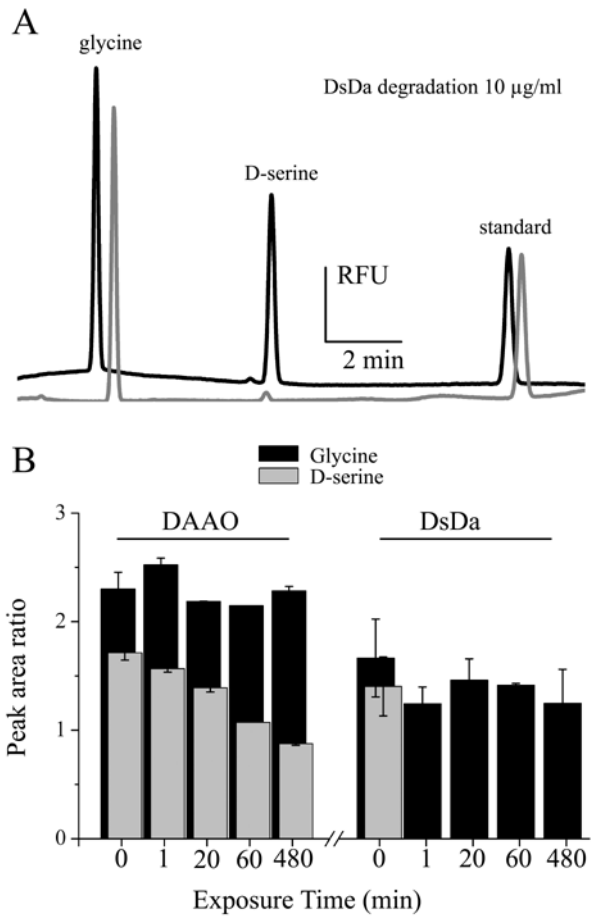
PNR amplitude is decreased following the application of D-serine degrading enzymes. A, a representative set of traces shows the reduction of the PNR amplitude following application of DAAO (100 μg/ml) and a subsequent increase following the addition of D-serine to the enzyme solution. B, addition of 10 μg/ml of DsDa similarly affects the PNR amplitude. C, cumulative results for PNR degradation experiments. The application of DAAO led to a significant decrease in PNR amplitude of 14.7 ± 2.1% (n = 7); similarly, DsDa decreased the amplitude by 20.0 ± 3.1% (n = 5). Addition of exogenous D-serine resulted in amplitudes that were indistinguishable from control cocktail levels. D, a set of traces showing the effects of our control cocktail. In Mg^{2+}-free Ringer, the PNR is small (light gray trace) and shows little NMDAR contribution (inset, black control vs. light gray AP7). In our control cocktail, the PNR increases greatly in amplitude (black trace) and the application of AP7 nearly eliminates the response (dark gray trace and inset: control cocktail black vs. dark gray).

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 larger than that of the control (upper, light gray traces, Fig. 2.2A, B, C). 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 higher D-serine level in the DsdA enzyme perfusate to the more powerful degradation action of DsdA against D-serine. The ability of D-serine to overcome the reduction of the PNR induced by DAAO and DsdA, coupled with the ineffectiveness of the heat-inactivated enzymes, provides strong evidence that the enzymes act by reducing extracellular endogenous levels of D-serine and thereby reduce the endogenous coagonist of NMDARs.

Confirmation of enzyme specificity

We explored 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 1992). This determination was essential, as any reduction in glycine by these enzymes would invalidate our conclusions about the role of D-serine vs 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

Figure 2.3



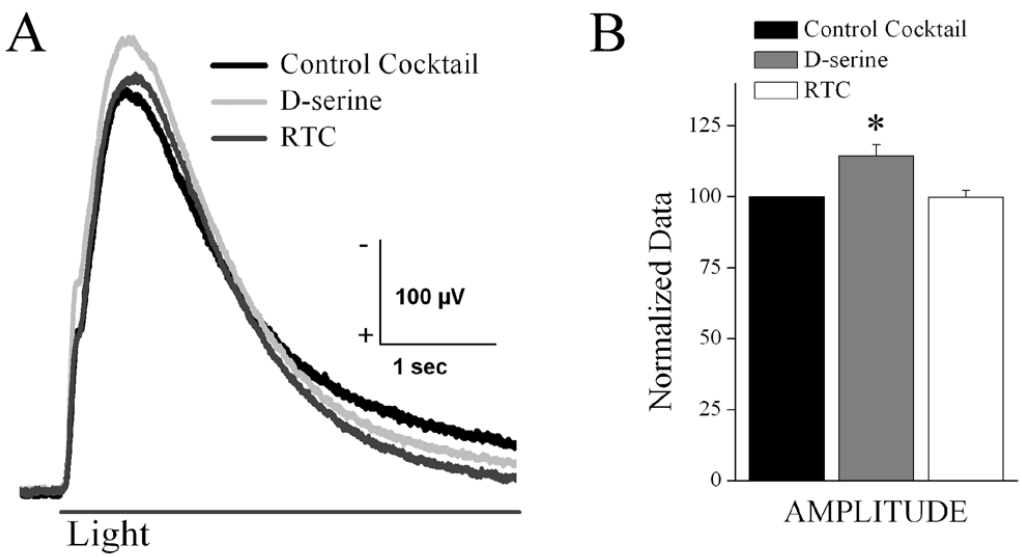
Capillary electrophoresis examination of DAAO and DsDa enzymatic activity. A, electropherograms of NBD-F labeled amino acids levels in a solution containing 10 µM glycine and D-serine with (gray) and without exposure (20 min) to DsDa (10 µg/ml) show its specificity and efficiency of action for D-serine, without affecting glycine. B, cumulative data of the peak area ratio (the integrated peak values divided by an amino acid standard) of glycine and D-serine show a slow and significant reduction of D-serine levels of solutions exposed to D-AAO, while DsDa action proves to be rapid, with no levels detected even with the shortest incubation times. Glycine levels are not affected by either enzyme, even after four hours of incubation.

significantly reduced D-serine concentrations while not significantly affecting glycine levels during incubation times from 1 minute to 4 hours (Fig 2.3B). 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. 2.3A, 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. 2.3B left). Thus, these studies clearly support the idea that enzymatic reductions in NMDA receptor contributions reflect the action of these enzymes on endogenous D-serine rather than glycine.

PNR amplitude is increased following 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 2.4A 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. 2.4B; $n = 6$, $p < 0.05$). 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 figure 2.2B).

Figure 2.4

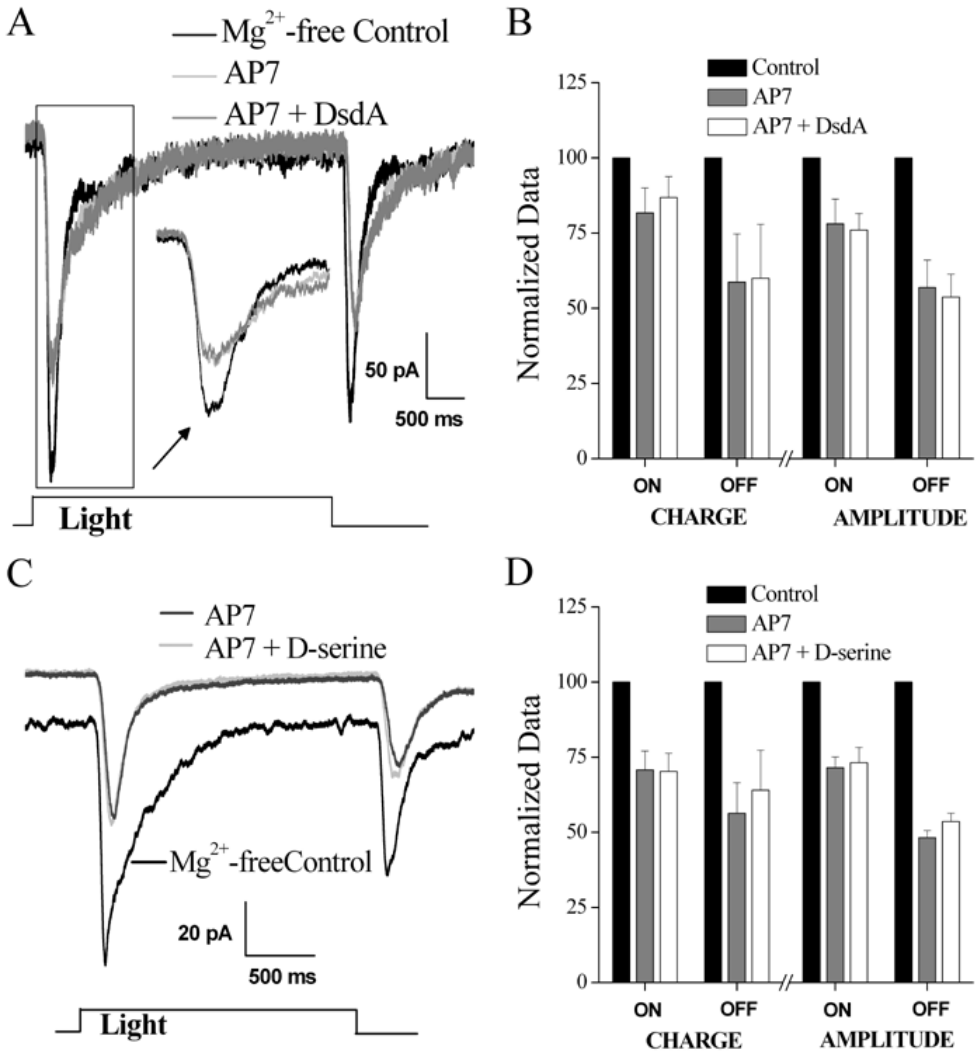


Application of exogenous D-serine increases the amplitude of the PNR. A, an example set of traces show an increase in amplitude of the PNR response following application of 100 µM of exogenous D-serine (light gray trace). A return to the control cocktail (RTC) decreased the amplitude to near the control level (dark gray). B, cumulative results show a significant increase of 14.4 ± 9.5% in the PNR amplitude when compared to controls, n = 6.

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

Whole-cell recordings from retinal ganglion cells were used to examine the dependence of endogenous D-serine manipulations on NMDAR activity. Fig 2.5A illustrates an example of this strategy. A control light-evoked response (5A, black trace) was obtained from an On-Off ganglion cell in Mg^{2+}-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 bathing 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 2.5B provides summary data from five experiments carried out identically to that illustrated in 2.5A; 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, while 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 2.5C illustrates the alternative experiment, in which a control light-evoked response was obtained from an On-Off ganglion cell in a Mg^{2+}-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

Figure 2.5



Blockade of NMDARs eliminated the effects of D-serine degradation and application. A, whole-cell, voltage clamp recording shows the reduction in light-evoked response following application of AP7 (black vs. light gray trace). Subsequent addition of DsdA has no effect of the response (dark gray trace). The inset shows an expanded timecourse of the ON response to illustrate the amount of overlap between the AP7 and AP7 + D-serine traces. B, cumulative results from five experiments show the decrease in total charge and peak amplitude of the ON and OFF responses to the light stimulus—total charge decreased (ON) 18.3 ± 8.3% (AP7) and 13.1 ± 7.0% (AP7 + DsdA) and (OFF) 41.2 ± 16.0% (AP7) and 40.0 ± 18.0% (AP7 + DsdA); peak amplitude decreased (ON)

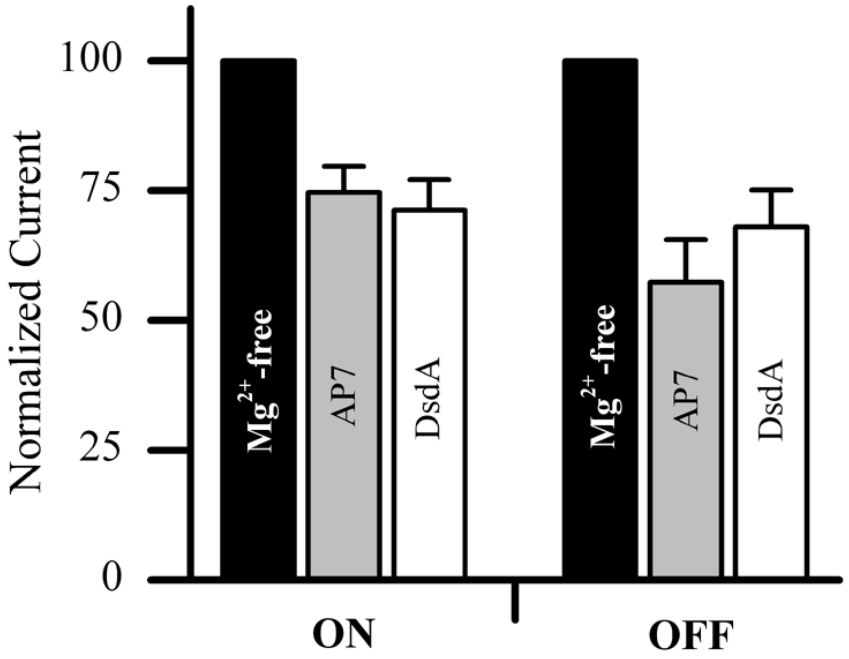
21.9 ± 8.3% vs. 23.9 ± 5.5% and (OFF) 43.1 ± 9.1% vs. 46.2 ± 7.6%. C, voltage clamp responses of a RGC show a decrease in peak amplitude, total charge and standing current following addition of AP7 (black vs. light gray traces). The addition of exogenous D-serine (100 μM) to the AP7 Ringer solution does not alter the response (dark gray trace). D, normalized data show the total charge and amplitude of responses from five experiments. Total charge decreased (ON) 29.2 ± 6.3% (AP7) and 29.8 ± 6.0% (AP7 + D-serine) and (OFF) 43.7 ± 10.2% (AP7) and 36.0 ± 13.3% (AP7 + D-serine); peak amplitude decreased (ON) 28.5 ± 3.5% vs. 26.8 ± 5.1% and (OFF) 51.7 ± 2.3% vs. 46.4 ± 2.8%.

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 2.5D summarizes the five experiments carried out identically to that illustrated in 2.5C. In summary, when NMDARs are blocked with AP7, neither D-serine nor DsdA modify the response to light. This indicates that the action of D-serine and DsdA is mediated solely through NMDARs.

The AP7 and DsdA reduction in light-evoked responses are indistinguishable

Figure 2.6 summarizes and compares the effects of AP7 and DsdA from the data presented previously. In all cells studied with AP7 (see fig 2.5C & 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% (ON) and 42.6 ± 8.2% for the OFF response. These values were not significantly different than the reductions observed following DsdA application (ON reduction of 28.8 ± 5.9% and OFF reduction of 32.0 ± 7.1%). A student t-test comparing the reductions found in AP7 to those with DsdA, revealed p-values = 0.673 for the ON and 0.356 for the OFF.

Figure 2.6



AP7 and DsdA lead to similar decreases in light-evoked current responses. C, cumulative data compare the RGC responses to bath applied AP7 (totals from figure 5C & D) and DsdA (from figure 1). The relative decreases in total charge for the ON responses were 25.3 ± 5.0% (AP7) and 28.8 ± 5.9% (DsdA). For the OFF responses, the decrease from control was 42.6 ± 8.2% (AP7) and 32.0 ± 7.1% (DsdA).

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 whole-cell recordings 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 while the heat-inactivated form was without effect. We suggest that the greater sensitivity of DsdA towards 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).

Since DsdA is more effective than DAAO in degrading D-serine, it is natural to ask whether DsdA is present in the brain and retina. While the presence of DAAO in vertebrates has long been known, this enzyme is more selective for D-alanine and D-proline compared to 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-hydroxypyruvate) 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, while it appears that DsdA is an unlikely player in mammalian nervous tissue, it has not to our knowledge been evaluated been evaluated with this more direct, functional technique in amphibians.

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 co-application of exogenous D-serine, while 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. Since 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. While 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 different experimental circumstances than 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.

While 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 (Miller et al. 1977; Marc 1989). 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 1998b), as 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. 2005b).

In the brain, the co-distribution 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 hence in regulating NMDAR sensitivity. We suggest that a similar relationship may exist in the retina. When the selective GlyT1 transport blocker NFPS (N[3-(4-fluorophenyl)-3-(4-phenylphenoxy)-propyl]sacrcosine) was applied to the salamander retina, the coagonist sites reflected in the NMDAR component of ganglion cell light responses were saturated (E.R. 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 retinal ganglion cells. Presumably this applies as well to those amacrine cells which have NMDARs, but 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 following 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 retinal ganglion cell 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 (Lukasiewicz and Roeder 1995). While 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, while 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 (Vyklicky, Jr. et al. 1990; Lester et al. 1993). 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), but 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 which first proposed a relationship for endogenous D-serine and NMDAR sensitivity. Despite the reportedly low tissue levels of D-serine in the retina (O'Brien et al. 2005; Miller 2004), 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) have proposed that SR is also found in neurons and this work has placed doubts about whether the enzyme and hence 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 by Pow (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.

Chapter 3

Light-evoked NMDA receptor-mediated currents are Correlated with D-serine synthesis

Experiments were carried out in the retina of the tiger salamander (*Ambystoma tigrinum*) to evaluate the importance of D-serine synthesis on light-evoked NMDA receptor-mediated components of ganglion cells and contributions to the Proximal Negative Field Potential (PNFP). We blocked the synthesis of D-serine through brief exposures of the retina to phenazine ethosulfate (Phz-ES) and validated the changes in tissue levels of D-serine using capillary electrophoresis (CE) methods to separate and measure amino acid enantiomers. Ten minute exposures to Phz-ES decreased D-serine levels in the retina by about 50% and significantly reduced the NMDA receptor contribution to light responses of the inner retina. This is the first report of a linkage between D-serine synthesis and NMDA receptor activity in the vertebrate retina.

Introduction

D-serine plays a major role as an NMDA receptor coagonist in the vertebrate retina (Stevens et al. 2003) (Gustafson et al. 2007), where it is synthesized from L-serine by the enzyme serine racemase (SR) (Wolosker et al. 1999d). In the retina, SR is primarily localized to glial cells, including Müller cells and astrocytes (Stevens et al. 2003), though it appears to be transiently expressed during development in mouse retinal ganglion cells (Dun et al. 2008) and appears early during synaptogenesis of the retina (Diaz et al. 2007). In the present study, we pursued the relationship between D-serine synthesis and its effect on NMDA receptor activation using electrophysiological recordings of synaptic currents in the inner retina combined with measurements of D-serine using capillary electrophoresis (CE) techniques. For these experiments, we evaluated the SR blocking agent phenazine ethosulfate, which, during brief applications, reduced D-serine and concomitantly reduced light-evoked NMDA receptor currents in the inner retina. These results further establish a tight relationship between the synthesis of D-and its impact on NMDA receptors by serving as the primary coagonist of the retina.

Methods

Electrophysiology: All recordings were carried out using a superfused retina-eyecup preparation of the tiger salamander (*Ambystoma tigrinum*) described previously (Gustafson et al. 2007). Light stimulation was provided by a computer-controlled LCD projector system previously described (Burkhardt et al. 1998).

All chemicals and other agents used in these experiments were purchased from Sigma Chemical (St. Louis, MO) with the exception of hyaluronidase and collagenase (Worthington Chemical, Lakewood, NJ); tetrodotoxin (TTX; Alomone labs, Jerusalem, Israel); picrotoxin (Fluka, Neu-Ulm, Switzerland); KCH_3SO_4 (Pfaltz & Bauer); D,L-2-amino-phosphonoheptanoate (AP7); 5, 7-dichlorokynurenic acid (5, 7-DCK) and 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxalinedione (NBQX) from Tocris (Ellisville, MO).

Tiger salamanders were purchased from a dealer (Charles D. Sullivan Co., Nashville, TN) and maintained in circulated cold-water tanks (4°C) with a 12 hour 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 sacrificed by decapitation followed by double pithing.

Antibody Preparation: A polyclonal antibody was generated in rabbits (Sigma Genosys) against the N-terminal peptide (NH_2-AQYCISFADVE-COOH) of mouse SR (Swiss-prot accession number Q9AZX7). We affinity-purified the serum over CNBr-Sepharose beads (Sigma) conjugated to the same peptide. Two eluent fractions containing the highest protein content were pH neutralized, pooled, and used for these experiments.

Immunostaining: Retinal tissue was harvested from the neotenous tiger salamander, *Ambystoma tigrinum* (TS), and processed for immunostaining using methods previously described (Stevens et al. 2003). Confocal images were processed and rendered using Adobe Photoshop 7.0 software (Adobe Systems Inc.), with three-dimensional

reconstructions generated using Imaris software (Bitplane Inc., Saint Paul, MN).

D-serine measurements: D- and L-serine measurements were made through the use of capillary electrophoresis methods that have been previously described (O'Brien et al. 2005). Limited animal availability restricted our chemical measurements to 12 animals for two separate experiments.

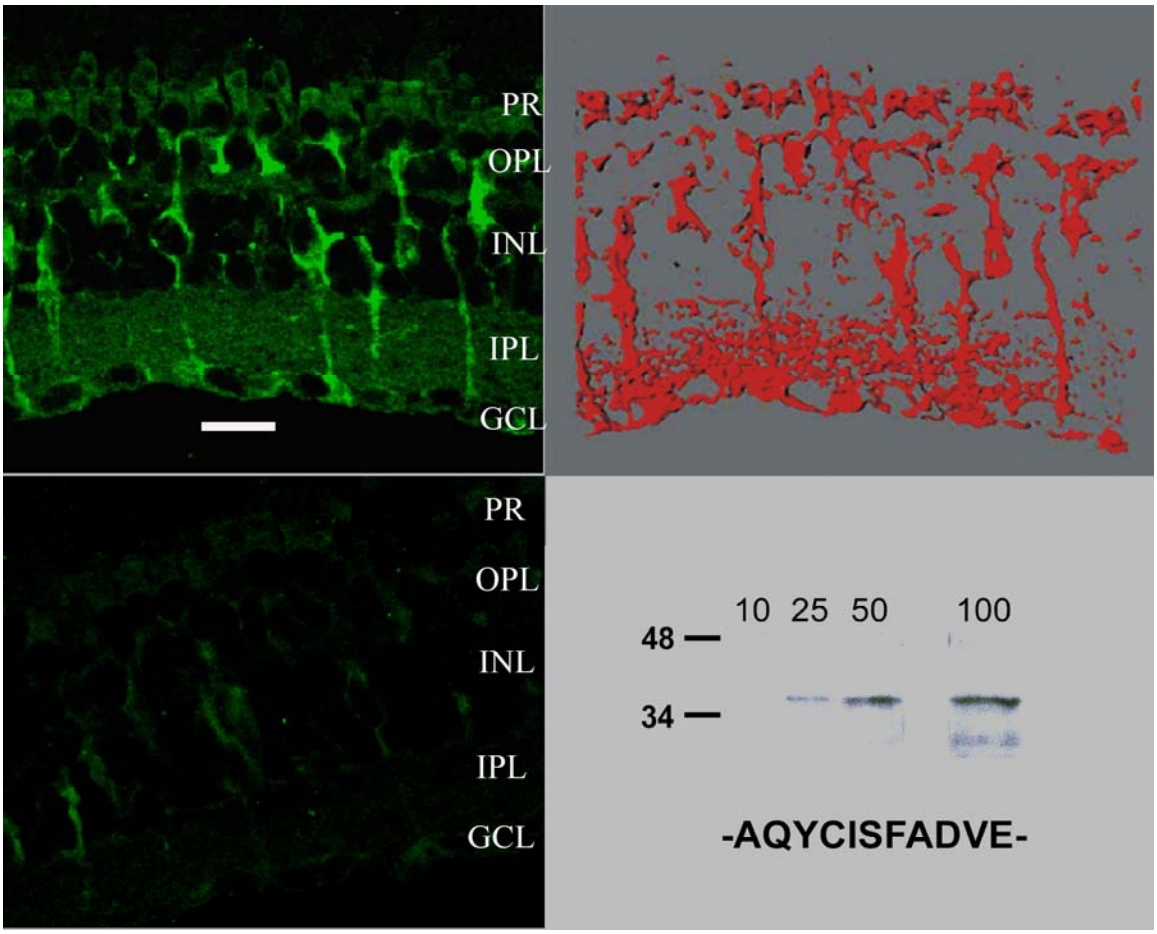
Retinal protein determination: All amino acid measurements were normalized to the total retinal protein content. The protein pellets from each sample were suspended in 120 μL of 2M NaOH and diluted 1:20 in distilled water. The protein concentration in the diluted sample was quantified using a Pierce (Rockford, Illinois) bicinchoninic acid assay kit; absorption was measured at 562nm.

Results

Experiments were done under voltage-clamp conditions while continuously superfusing the preparation with a normal Ringer. Following these initial observations, the bathing solution was changed to a nominally Mg^{2+}-free Ringer to which tetrodotoxin (TTX, 0.5 μM) and strychnine (10 μM) were added (the control Ringer). When steady-state conditions with the toxin control Ringer were established, voltage-clamp studies were initiated and the holding potential was adjusted to give zero holding current. The range of holding potentials which were used with these criteria ranged from -53 to -72 mV (average -64.9 ± 4.8 mV).

Serine Racemase is located in retinal Müller cells. Figure 3.1 (top left) shows a single section of the salamander retina immunostained with the SR antibody developed for this

Figure 3.1



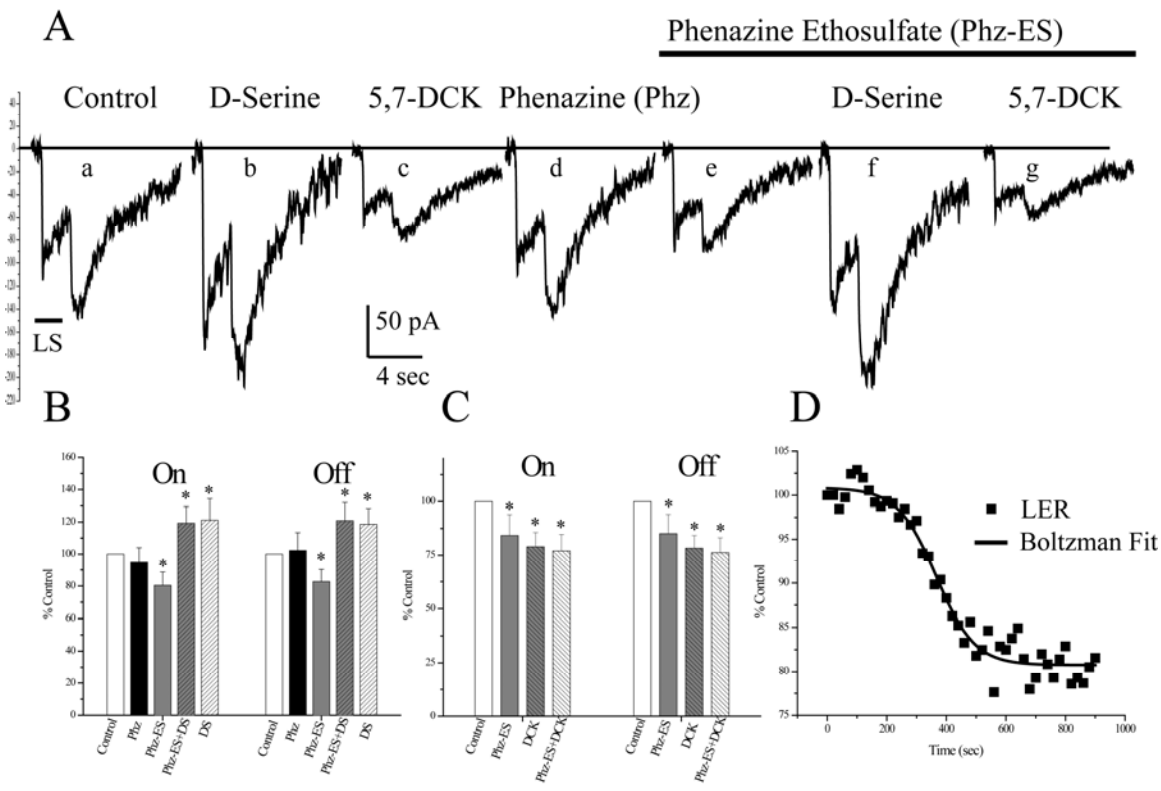
Serine Racemase distribution in the tiger salamander retina shows a prominent staining pattern in retinal Müller cells. An antibody to SR (green) labeled Müller cells in this cross section of salamander retina (upper left). Serine racemase label can also be appreciated in the photoreceptor layer. A 3D reconstruction of the staining from the SR antibody (red) from a z-series in this same section of retina (upper right). This label is severely curtailed in an adjacent retinal section when the antibody was preadsorbed with peptide (lower left). A Western blot of protein extracted from salamander at different concentrations labeled with the SR antibody displayed a single band at approximately 37 kD, the anticipated weight of the enzyme.

report. Prominent labeling is evident in the Müller cells, and the photoreceptor layer, including some photoreceptor terminals. A Western blot using this antibody against different concentrations of a protein homogenate of the salamander retina revealed a single band near the 37 kDa (lower right panel) size of SR (Wolosker et al. 1999d). The single image on the upper left was one of a stack of images from different depths that were used to generate a 3D reconstruction of the staining pattern (top right). Cross sections of the retina were also exposed to the SR antibody after it had been pretreated with the peptide (lower left), indicating that the staining pattern observed could be attributed to the distribution of SR.

Blocking serine racemase decreases NMDA currents in retinal ganglion cells.

Phenazine Ethosulfate (Phz-ES, Sigma Aldrich) inhibits the enzyme SR and reduces the synthesis of D-serine (Kim et al. 2005). We compared the effects of Phz-ES with the actions of its parent compound phenazine (Phz). Figure 3.2A illustrates the transient On and Off light-evoked inward currents (stimulus duration indicated by bar under first trace) recorded while the On-Off ganglion cell was bathed in the control Ringer. Addition of D-serine (100 μM) substantially enhanced both the On and Off responses to the light stimulus, indicating a contribution from NMDARs (Gustafson et al. 2007). When the selective and potent NMDAR coagonist site competitive antagonist, 5, 7-dichlorokynurenate (5, 7-DCK; 30 μM) was introduced, the On and Off responses were diminished compared to control values. After returning to a control Ringer (not illustrated), the cell was exposed to Phz (100 μM), which had no significant effect on the response amplitude. After the wash out of Phz, the addition of Phz-ES (10 μM) to the

Figure 3.2



Blocking SR decreased NMDA currents in retinal ganglion cells. (A) A WCVCR (V_{Hold} = -67 mV) from a RGC in an eyecup preparation showed the response to a 2 sec light stimulus (LS) in control Ringer, which was enhanced by the addition of the D-serine (100 μM), and blocked by 5,7-DCK (30 μM). When the SR antagonist, Phz-ES (10 μM, thick bar) was added, the response was diminished in amplitude similar to that observed in 5,7- DCK. This reduced response could still be potentiated by the addition of D-serine. Phz (100 μM) did not affect the SR enzyme and did not alter the control response to a significant degree. (B) Summary data from 7 cells demonstrating reduced On and Off responses when Phz-ES was added to the bathing medium and an unaltered potentiation by D-serine as well as the lack of change in the presence of Phz. (C) Summary data from 7 cells demonstrating a small, non-significant additional reduction in inward current when 5,7-DCK was added to Phz-ES. (D) Time course of the block by the Phz-ES fit with a Boltzman. WCR (■) and PNFP (●) measurements were averaged from responses at 20 second intervals (error bars not shown). Transit time of enzyme from reservoir subtracted prior to plotting. LER: light-evoked response; (* p<0.01 compared to control).

control bathing medium, rapidly diminished the light-evoked responses. In the presence of continuous Phz-ES, the addition of exogenous D-serine resulted in a response enhancement similar to that observed when D-serine was added to the control Ringer. The last trace in the sequence illustrates the responses observed when the bathing medium consisted of 5, 7-DCK added to the Phz-ES Ringer. In this case, the decline in response amplitude was slightly greater but similar to that observed when 5, 7-DCK was added to the control Ringer.

Figure 3.2B summarizes the actions of Phz, Phz-ES and the actions of D-serine added to the Phz-ES bathing medium (Phz-ES+DS) and contrasts that with D-serine added to the control bathing environment (DS). The comparisons are expressed as a percentage of the control response for light-evoked charge entering the cell during the On and Off responses. No significant change in response amplitude was observed when Phz was added to the bathing medium, but the application of Phz-ES resulted in a significant decrease in the light evoked currents ($p < 0.05$). When D-serine was added to either the control Ringer or the Phz-ES bathing solution, a significant and equivalent enhancement of the responses was evident.

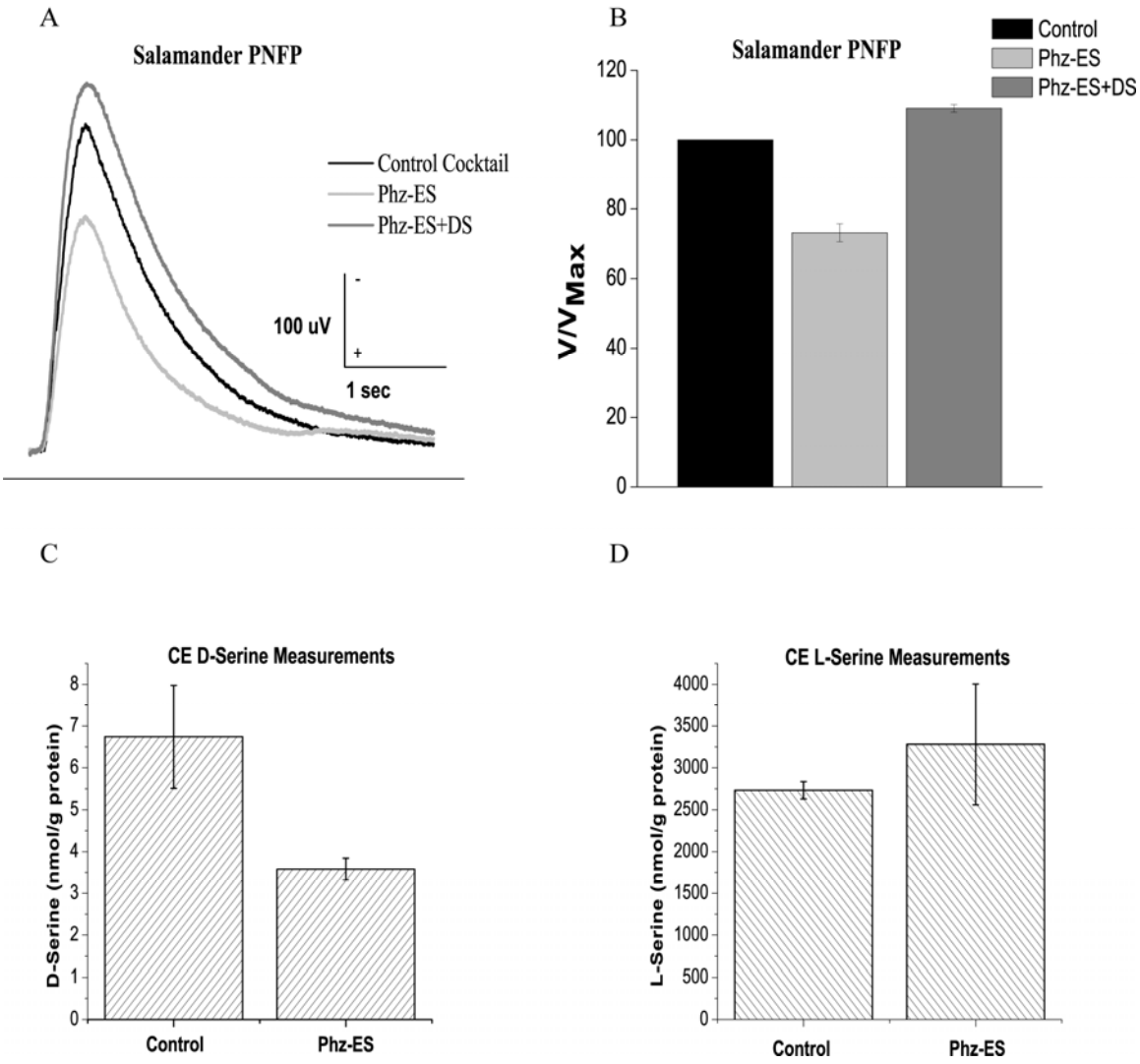
Figure 3.2C shows the actions of Phz-ES on light responses and interactions with the NMDA receptor antagonist 5, 7-DCK. Bath applied Phz-ES significantly decreased both On and Off responses. In the presence of Phz-ES, the addition of 5,7-DCK (30 μM) decreased the response slightly, similar to that observed when 5,7-DCK was added to the control Ringer.

Figure 3.2D illustrates the time course of the effects of Phz-ES on WCRs (■) and the PNFP (●). Through trial and error, we determined that the curve relating the actions of Phz-ES on the light responses was better fit by a Boltzman relationship rather than one or more exponential functions, suggesting that a mechanism other than simple diffusion of Phz-ES is required to describe its mode of action.

Actions of phenazine on the proximal negative field potential (PNFP). Fig 3.3A illustrates a recording of the PNFP from the salamander retina, evoked by a 120 μm spot of light. A long light exposure was used to evoke both On and Off responses, but only the On response is illustrated. When phenazine (100 μM) was added to the bathing medium for 10 minutes, the response to light was superimposed on the control response (not illustrated). After returning to the control environment for 10 minutes, the introduction of Phz-ES (10 μM) decreased the response amplitude. In the presence of Phz-ES, the addition of D-serine (100 μM) increased the light response. In this example, the retina was exposed to the Phz-ES for 10 minutes, followed by a return to the control cocktail. Fig 3.3B shows the results of 7 different experiments and illustrates a consistent decline in PNFP amplitude resulting from Phz-ES, and an increase in PNFP amplitude when D-serine was added to the Phz-ES bathing medium. When Phz-ES exposures were longer than 10 minutes, we did not see a return of the responses to control values and for that reason, we carried out our chemical determinations using a 10 minute exposure time line for Phz-ES.

Phenazine ethosulfate decreases D-serine in the retina. We analyzed the effects of Phz-ES on D- and L-serine levels in the salamander retina. Because the D-serine tissue

Figure 3.3



Extracellular recording of the proximal negative field potential (PNFP). Application of phenazine-ethosulfate (Phz-ES) decreased the amplitude of the PNFP. A: an example set of traces shows a decrease in PNFP amplitude following bath application of Phz-ES (light gray trace) when compared to the control cocktail response (black trace). The addition of exogenous D-serine to the Phz-ES bathing media (gray trace) increased the response beyond that of the original control. **B**: cumulative results showed a significant decrease of 26.8 ± 2.6 % in the PNFP in the presence of Phz-ES and a significant increase of 9.1 ± 1.1 % following addition of D-serine (both compared to control, n = 6). **C:** shows the change in measured levels of D-serine for the homogenized retina exposed for 10 minutes to phenazine ethosulfate, which produced an approximate 50% decline in D-serine levels. **D:** shows that L-serine levels measured from the same retinas were not significantly changed

levels are low, we pooled twelve retinas for each of two experiments, with one retina from each animal serving in either the control or the Phz-ES bathing solutions. Fig 3.3C shows the D-serine changes that resulted from two repetitions of this procedure. During a 10 minute exposure the D-serine levels decreased by approximately 50%. L-serine levels (3.3D), also measured in these experiments, were not significantly changed.

In summary, findings with whole-cell recordings from retinal ganglion cells, the PNFP and chemical determinations converge to support the idea that Phz-ES decreased tissue levels of D-serine, which, in turn, decreased the light response of ganglion cells without compromising the sensitivity of ganglion cell NMDARs to exogenous D-serine. The time course of changes in D-serine and measured changes in NMDAR-mediated synaptic currents suggests a fairly tight coupling between synthesis, release and availability of D-serine as a coagonist for NMDARs.

Discussion

Although SR has been localized to the retina with immunostaining techniques, this is the first report of a direct relationship between D-serine synthesis and the light-evoked NMDAR-mediated responses of the inner retina. A measureable change in tissue levels of D-serine, generated by brief exposures to Phz-ES were correlated with a decrease in the light-evoked synaptic activity of ganglion cells that could be attributed to NMDARs. There is now compelling evidence that D-serine plays a major role as an NMDAR coagonist in the retina (Stevens et al. 2003) (Gustafson et al. 2007) (Williams et al. 2006), as it does in the brain (Mothet et al. 2000). The present experiments bear on

this interpretation, since they revealed a relatively tight coupling between D-serine synthesis and light-evoked currents generated by NMDARs. Indeed, the depressive actions of Phz-ES on NMDAR light-evoked currents were comparable to those observed when the light response was attenuated by blocking the NMDAR with 5,7-DCK. This is also consistent with other experiments (Gustafson et al. 2007) in the salamander which demonstrated that enzymatically degrading D-serine, using D-serine deaminase (DsdA) or D-amino acid oxidase (DAAO) reduced NMDAR currents to an amplitude similar to that observed when NMDARs were blocked with selective antagonists. Kalbaugh et al (Kalbaugh et al. 2009) have recently suggested that glycine serves a dynamic role as an NMDAR coagonist. In the present experiments, we cannot eliminate a small contribution of glycine as a coagonist, but it is clear that glycine does not rapidly substitute for D-serine, since the measured decrease in D-serine levels was not compensated by a return of the NMDAR-mediated component of the light response, at least over the time course of our experiments.

Source of D-serine in the retina. We developed an antibody against SR, using a peptide segment unique to the enzyme. This antibody provided a single band of staining in the Western blot with a MW of 37 kDa, the same as SR (Wolosker et al. 1999c) (Wolosker et al. 1999b). When this antibody was used for immunostaining in the salamander retina, the most obvious labeling was apparent in the Müller cells, the prominent radial glia in the retina. This result confirms previous studies of the retina using antibodies for D-serine and SR obtained from other sources (Stevens et al. 2003). In addition to the prominent staining found in Müller cells, it appeared that the inner segments of

photoreceptors, particularly those of cones, were labeled, although they appeared more lightly stained than Müller cells. But, like the Müller Cells, photoreceptor staining was eliminated with preadsorption of the antibody with the peptide fragment. It is worth noting that DAAO has been reported in amphibian cones (St Jules et al. 1992), so the possibility that D-serine is a functional constituent of photoreceptors requires further study.

While we generally focus on NMDARs as an inner retinal glutamate receptor of ganglion and amacrine cells (Massey and Miller 1990) (Slaughter and Miller 1983) (Dixon and Copenhagen 1992), evidence for NMDARs in horizontal cells of the fish retina has been known for many years (O'Dell and Christensen 1989). In a recent study in the carp retina Shen et al have demonstrated that when glycine was added to the bathing medium NMDAR contributions to horizontal cell responses were observed (Shen et al. 2006). The possibility that photoreceptors might utilize D-serine does not diminish the more obvious presence of SR in the inner retina, where it appears that Müller cells are the major cellular element which contains SR. For that reason, it seems reasonable to conclude that in the inner retina, where NMDAR function is prominent, the source of D-serine is likely to come from Müller cells. The immunostaining carried out in this study supports the idea that D-serine subserves one component of a glial-neuronal control pathway.

Conclusion

A rapid reduction in tissue levels of D-serine was observed following the

introduction of phenazine ethosulfate to inhibit D-serine synthesis by the enzyme serine racemase, using the isolated tiger salamander retina. The decrease in D-serine was correlated with reductions in light-evoked currents mediated by NMDA receptors, characterized through whole-cell recordings of retinal ganglion cells and extracellular recordings of the Proximal Negative Field Potential. These observations provide the first direct link between tissue levels of D-serine, D-serine synthesis and the critical role that D-serine plays as a coagonist for NMDA receptors in the inner retina.

Chapter 4

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

In this study, we have demonstrated that D-serine interacts with 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 have shown that exogenous D-serine was more than 30 times as effective at potentiating NMDAR currents when compared to glycine. In lieu of this, we 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. The analysis of this study 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, either by the release of D-serine or glycine. These results are discussed in view of contemporary findings which favor D-serine over glycine as the major coagonist of the NMDA receptors found in ganglion cells of the tiger salamander retina.

Introduction

NMDA 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 (Massey and Miller 1990; Dixon and Copenhagen 1992; Mittman et al. 1990; Slaughter and Miller 1983).

Among the unique properties of NMDARs is the requirement for a second agonist, in addition to glutamate, to initiate channel gating (Johnson and 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 and Dingledine 1988). The dogma that D-amino acids played no functional role in higher organisms initially made D-serine an unlikely candidate. But Hashimoto et al. (Hashimoto et al. 1992a), using an HPLC method for separating amino acid entantiomers, 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. 1993a) and a synthesizing enzyme, serine racemase (SR), was isolated and characterized (Wolosker et al. 1999a). Initially, SR was localized to astrocytes, but more recent studies

have suggested that it may also be present in neurons in the brain (Kartvelishvily et al. 2006).

In the retina, early studies of NMDAR coagonist function, using retina slice preparations, suggested that the coagonist sites were saturated (Gottesman and Miller 1992; Lukasiewicz and 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 SR 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 SR (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; Marc 1989).

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—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 view 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. Animals were sacrificed by decapitation followed by double pithing. Tiger salamanders were purchased from a dealer (Charles D. Sullivan Co., Nashville, TN) and maintained in circulated cold-water tanks (4°C) with a 12 hour room light/dark cycle.

Whole-cell recordings (WCRs): Two unique retinal preparations from Tiger salamanders (*Ambystoma tigrinum*) were utilized in these experiments: 1) an isolated flatmount retina (Stevens et al. 2003) and 2) an intact retinal eyecup preparation. The experiments utilizing the flatmount preparation (figures 4.1 and 4.2) began by removing the cornea and lens and draining the vitreous (Miller and Dacheux 1976). Following this, the internal limiting membrane was removed either mechanically or by removing atomized alumina particles adherent to the surface of the retina with jeweler 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 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). Eyecup preparations (figure 4.3) began with enucleation of the eye, and placing a crystal of dextran conjugated tetramethylrhodamine (10,000 MW; Invitrogen, Carlsbad, CA) 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 $CaCl_2$, 1.0 Mg Cl_2, 10 HEPES, 5 D-glucose, pH 7.8. After cooling, the cornea, lens, and vitreous were removed as above, and the internal limiting membrane treated with collagenase and hyaluronidase. The eyecup (in agarose) was then placed in a 15 ml plastic centrifuge tube half filled with normal Ringer, secured in a light tight container on ice, and bubbled with oxygen for 2- 5 hours while the dye diffused into the ganglion cells. The eyecups, still in agarose, were 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 1/4 to 1/3 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.

Whole cell recordings were obtained from identified ganglion cells. Patch electrodes (5- 15 MΩs) were pulled using a P-97 Brown Flaming Pipette Puller (Sutter Instruments, Novato, CA) and filled with an intracellular solution containing (in mM): KCH_3SO_4, 98.0; $NaCH_3SO_4$, 3.5; $MgSO_4$, 3.0; $CaCl_2$, 1.0; EGTA, 11.0; HEPES, 5.0; D-glucose, 2.0; glutathione, 1.0; MgATP, 1.0; NaGTP, 0.5; pH 7.4. To visualize cells upon completion of the experiment, 5, 6- Carboxyfluorescein (Invitrogen, Calsbad, CA) was

occasionally added to this pipette solution. Positive pressure was applied to the solution in the recording pipette to access the surface of the ganglion cell under the enzymatically loosened ILM. 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 ILM 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), or manually with the Dagan 3900 (Dagan Corporation, Minneapolis, MN), each configured to record with a 10kHz 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 response of the cell determined by stimulating with a 2 second 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 tetrodotoxin (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% to 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 MΩ), we did not consider it necessary to correct for voltage errors associated with uncompensated series resistance. Voltages reported in the manuscript 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 20X microscope objective (Olympus Melville, NY) and focused on the plane of the ganglion cell layer. A Fish-Schurmann 6143 heat filter (New Rochelle, NY) and neutral density wedges (Kodak, Rochester, NY) were used to attenuate the full intensity of the lamp to a background intensity of 9 Cd/m^2. Unless otherwise indicated, light stimuli were centered on the recording electrode and consisted of a 2 second, 250 μm diameter spot at a 1.0 log unit increase in contrast. The inter stimulus interval was 20 seconds. Direct application of NMDA was achieved by applying a brief pressure pulse with a Picospritzer II (Genreal Valve Corp., Fairfield, NJ) to a micropipette with a low resistance tip filled with 1 mM NMDA in normal Ringer with an inter stimulus interval of 1 minute. 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 second light stimulus (On response) and two seconds 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 retinal eyecup preparations, as described previously (Gustafson et al. 2007). Briefly, following removal

of the cornea and lens, the vitreous was drained and the eyecup placed in a perfusion chamber. The eyecup was continuously perfused with chilled (19°C), oxygenated Ringers. 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 (cc) was used: (in μM) 10 NBQX, 10 strychnine, 50 picrotoxinin, 50 mecamylamine in nominally magnesium-free Ringer. 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)propyl])sarcosine (NFPS) is a GlyT1 antagonist (Aubrey and 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. 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); tetrodotoxin (TTX; Alamone labs, Jerusalem, Israel); Picrotoxin (Fluka, Neu-Ulm, Switzerland); KCH3SO4 (Pfaltz & Bauer); D,L-2-amino-phosphonoheptanoate (AP7); 5,

7-dichlorokynurenic acid (5, 7-DCK); and 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxalinedione (NBQX) from Tocris (Ellisville, MO).

Statistics and fitting: Electrophysiological data were analyzed in Clampfit 9.0 (Molecular Devices) to determine peak amplitude and charge and data was averaged, graphed, fit and the statistics were done in Origin v.7.5 (Northhampton, MA). All results are expressed as the mean ± SEM. Dose response data were fit with the following equation:

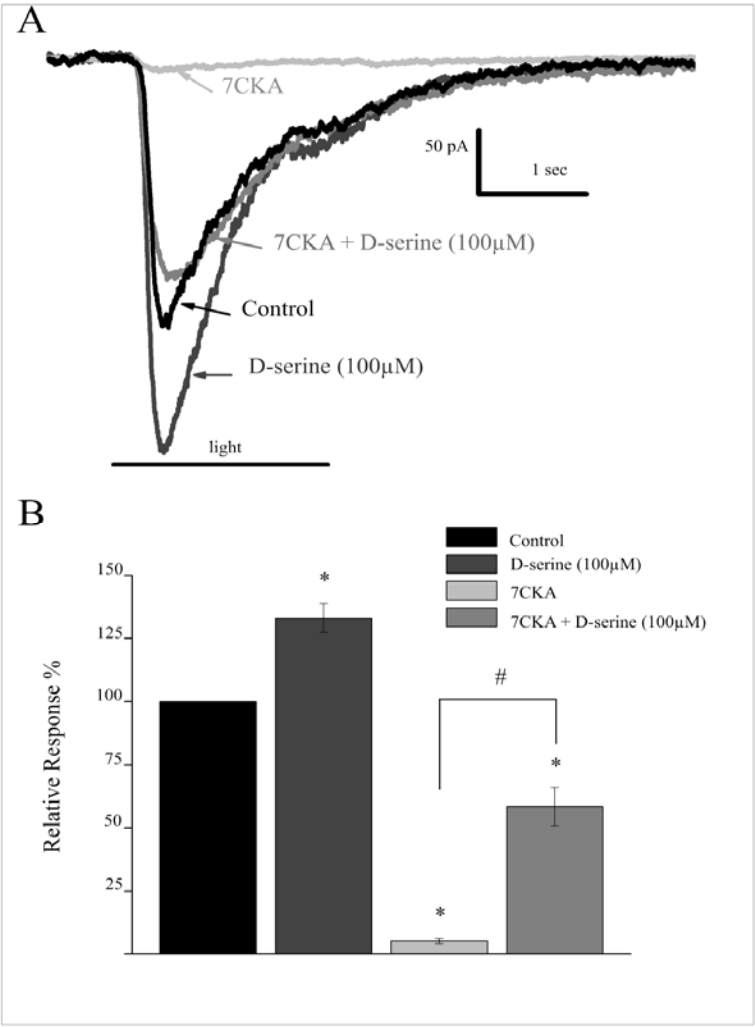
$$y = A1 + \frac{A2 - A1}{1 + 10^{(Log\,x0 - x)p}}$$

where A1 is the bottom asymptote (fixed at 100 in figure 4.2B), A2 is the upper asymptote, log x0 is the ED_{50} value, and p is the Hill coefficient (not evaluated because of system complexity). A paired, one-tailed student 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). While it was assumed that this was the result of D-serine acting on the coagonist site of the NMDAR, we verified this by examining the ability of D-serine to overcome the block of 7-chlorokynurenic acid (7CKA, 25 μM), a competitive antagonist at the coagonist site (Kleckner and Dingledine 1989) before looking further into the effectiveness of D-serine and glycine. NBQX (10 μM), picrotoxin (50 μM), strychnine (5 μM), and TTX (0.5 μM) were added to a Mg^{2+}-free Ringer to isolate the NMDAR contribution to light-evoked synaptic activity in retinal ganglion cells (this solution is labeled as "control" in figure 4.1). 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 (not shown). 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 4.1A shows a series of recordings of the ON response from a retinal ganglion cell continuously bathed in the control Ringer. 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

Figure 4.1



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 (holding -61 mV) from a RGC in an isolated retina in which the light response was evoked by a 2 sec light stimulus (thick bar) in a control Ringer which isolated NMDA mediated currents and inhibited Off responses (see text for details). This control response was potentiated by D-serine (100 µM, dark gray trace) and blocked by the NMDA receptor coagonist site antagonist, 7CKA (25 µM, light gray trace). This block was overcome by the addition of D-serine to the 7CKA solution (100 µM, gray trace). B: summary data from 9 cells plotting the total charge which entered the cell during the light stimulus as a function of the control response (* $p < 0.01$ compared to control, # $p < 0.01$ comparing the response in 7CKA alone to recovery with D-serine. Error bars represent ± SEM).

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 μM D-serine was added to the 7CKA bathing solution (not shown). However when 100 μM 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.

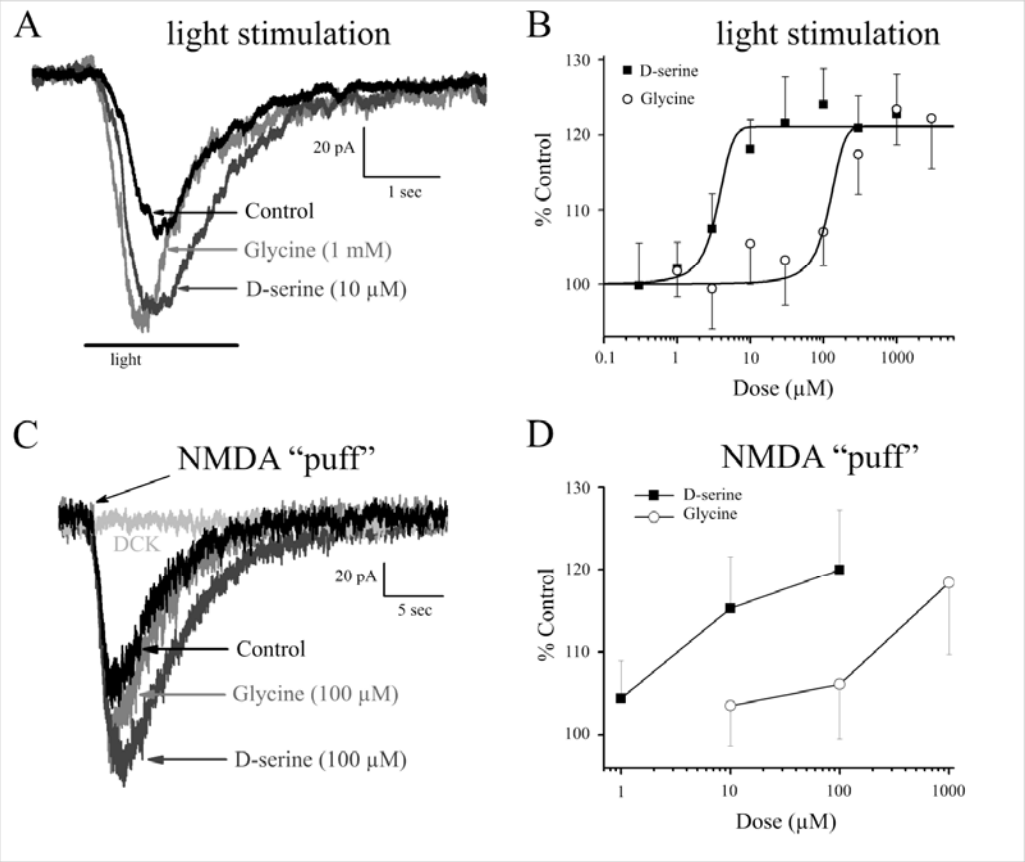
The results from 9 different ganglion cells that were studied using the protocol described in 4.1A are summarized in 4.1B. The light-evoked currents measured in retinal ganglion cells increased following the application of 100 μM D-serine (33.2 ± 5.7%), demonstrating that the NMDAR coagonist sites were not saturated under these experimental conditions. The application of 7CKA virtually eliminated the light-evoked response (a decrease of 94.7 ± 1.0%). Significant recovery of the light response was not observed with 10 μM D-serine (not shown), but was always prominent when 100 μM D-serine was added to the 7CKA environment, reaching a level of 58.4 ± 7.5% of that in the control ($p < 0.05$). These observations demonstrate that D-serine interacts competitively with 7CKA to enhance NMDAR currents at the 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 4.2A illustrates the light-evoked inward currents recorded under voltage-clamp, WCR conditions in response to a two second light stimulus. The RGCs were held so that there was zero current at rest. TTX and strychnine were added to the Mg^{2+}-free Ringer to block spiking, to limit network inputs from spiking amacrine cells, and to 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 μM 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 figure 4.2B. Since 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 when compared to glycine (D-serine ED_{50}= 3.48 ± 1.05 μM, glycine ED_{50}= 118 ± 48.1 μM, n = 7).

Figure 4.2



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 sec light stimulus in control Ringer (black trace) and with the addition of 10 µM 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 RGC in an isolated retina preparation responding to NMDA (1mM in control Ringer) pressure ejected from a glass micropipette positioned just above the retina. This response was potentiated when D-serine (100 µM, dark gray trace) or glycine (100 µM, gray trace) was added to the bathing medium. The response was completely blocked by the NMDA antagonist, DCK (30 µM, light gray trace). D: summary data from 8 cells plotting average responses to NMDA puff application (± SEM) in different concentrations of D-serine or glycine as a percentage of the charge entering the cell in control Ringer.

As a companion procedure for studying the sensitivity of NMDARs to glycine versus 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 (1mM) 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. In addition, it has been proposed that many NMDARs in the retina may be positioned extrasynaptically (Matsui et al. 1998) and puffing NMDA may activate a different fraction of receptors on RGCs than are normally activated by the light stimulation. Figure 4.2C shows a series of traces in which the addition of 100 μM 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 5,7-dichlorokynurenic acid (DCK, 30 μM). The dose-response curves shown in 4.2D (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 to 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 versus 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.

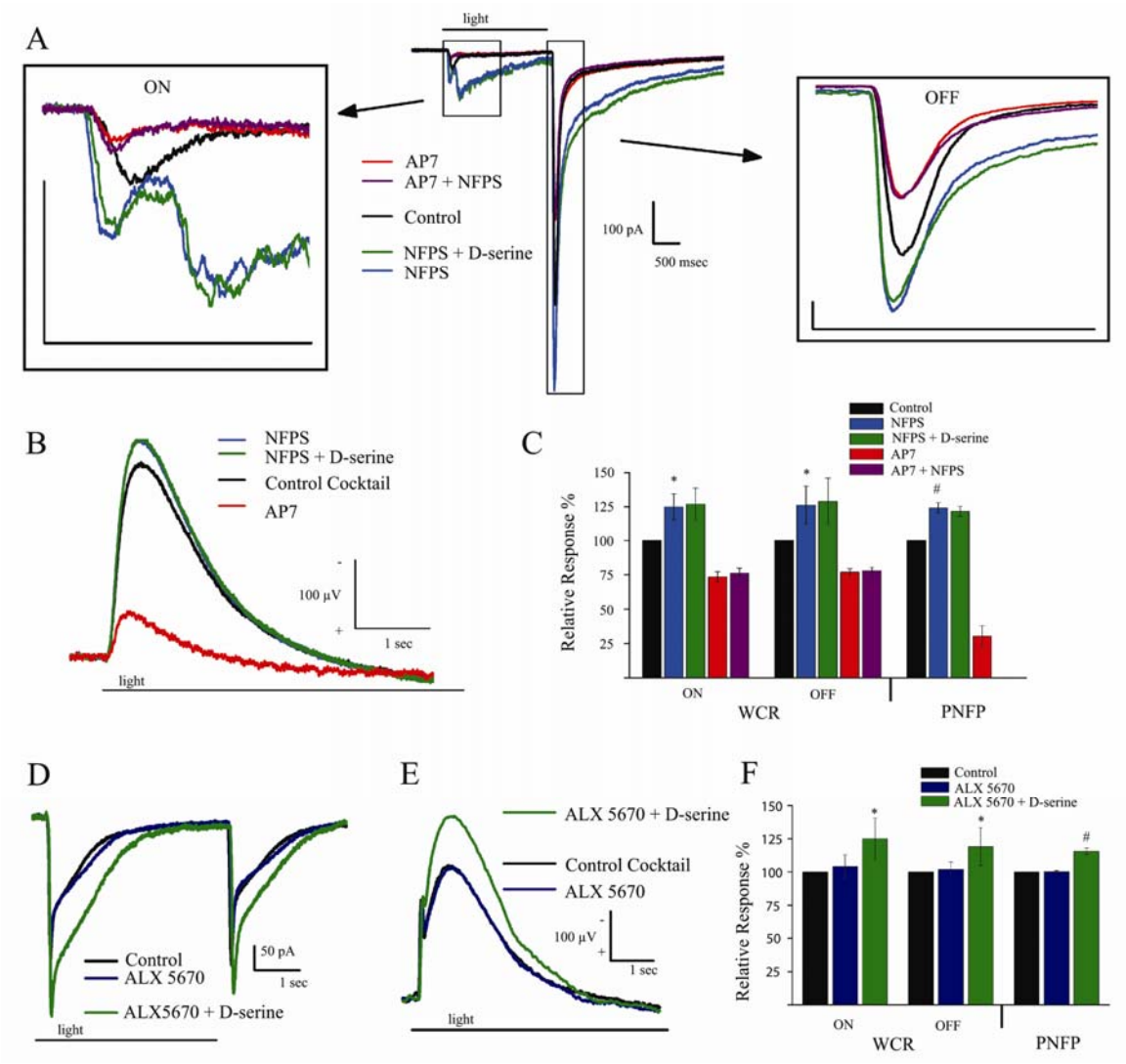
Blocking glycine transport potentiates the NMDAR-mediated currents of the inner retina. Two types of high affinity glycine transporters have been identified in the brain, including the GlyT1 and GlyT2 transporters. The GlyT1 is considered to play a major role in setting the extracellular level of glycine (Supplisson and Bergman 1997) and has been localized to amacrine cells in mammalian and chick retinas (Pow and Hendrickson 1999) and Müller cells in the amphibian (Lee et al. 2005a). Figure 4.3A demonstrates the potentiation of NMDAR-mediated, light-evoked currents from a retinal ganglion cell following the application of (R)-(N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)-propyl])sarcosine (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 and 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 μM) was added to the control solution, both ON and OFF responses were enhanced in amplitude and prolonged in duration (NFPS, blue trace; control, black trace). The addition of D-serine to the NFPS environment, at a concentration sufficient to saturate the coagonist sites (100 μM), 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 co-applied with NFPS (purple trace).

Figure 4.3B illustrates the effects of blocking the GlyT1 transporter on the extracellularly recorded Proximal Negative Field Potential (PNFP). The PNFP represents a major advantage over WCRs for studying long-term actions of pharmacological agents. Previous work has demonstrated that large NMDAR currents can be observed in the PNFP, and in a control cocktail (cc, black trace; see methods), the response to the onset of a focal light stimulus was largely mediated by NMDARs (Gustafson et al. 2007). The addition of NFPS (blue trace) to the cc 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 4.3C 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 OFF components of the light response in the presence of NFPS was significantly increased when compared to the control values (ON 25.0 ± 9.4%; OFF 26.3 ± 13.9%; $n = 7$, $p < 0.05$). Similarly, the peak amplitude of the PNFP was 24.3 ± 3.7% larger in the presence of NFPS ($n = 6$, $p < 0.01$). The subsequent addition of D-serine to the NFPS containing bathing solutions did not significantly affect

Figure 4.3



Blocking glycine transport potentiated the NMDA receptor mediated currents in perfused eyecup preparations. A: a voltage-clamp WCR (holding potential -57 mV) from a RGC in an eyecup preparation responding to a 2 sec light stimulus in control Ringer (black trace) was enhanced with the addition of the GlyT1 antagonist, NFPS (10 µM; blue trace), and reduced by the addition of the NMDA antagonist, AP7 (100 µM; 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 µM, 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 µM), a

decrease with AP7 (100 μM), and no enhancement with exogenous D-serine (100 μM). C: summary data from WCRs (8 cells) plotting the charge entering the cell during the light stimulus (On) and for the 2 sec 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 RGCs were not affected by GlyT2 inhibition. D: a WCR (holding -68 mV) from a RGC in an eyecup preparation evoked by a 2 sec light stimulus (thick bar) in control Ringer (black trace) was unaffected by the addition of the GlyT2 antagonist, ALX-5670 (100 μM; blue trace). The addition of D-serine (100 μM) to ALX-5670 (green trace) caused an increased inward current. E: the addition of ALX5670 (100 μM, blue trace) did not affect the PNFP (cc, black trace, largely overlapping), while D-serine potentiated the response (100 μM, green trace). F: summary data from 7 cells plotting the charge which entered the cell during the light stimulus (On) and for the 2 sec following the light stimulus (Off) as a function of the control response and from 6 PNFP recordings plotting the peak amplitude (*$p < 0.05$, # $p < 0.01$ compared to control, error bars represent ±SEM).

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 significantly reduced (WCRs were reduced by ON 26.5 ± 3.7%; OFF 23.1 ± 2.5%, and the PNFP by 69.6 ± 7.5% when compared to control levels, p-values < 0.05). There was, however, no additional reduction in the ON and OFF components of ganglion cells or the PNFP in the presence of AP7 alone, suggesting that the enhancement of the response by NFPS and its reduction by adding AP7 to the NFPS occurred through the actions of NFPS 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, since 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 as well (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). While these compounds had IC_{50} values as low as 1.03 μM against the GlyT2 transporter, they had only modest (~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). The compound we obtained from NPS Pharmaceuticals and used in our studies was

(2S,3R)-2-amino-3-[-(9-phenyl-9H-fluoren-9-yl)oxy] butanoic acid (ALX 5670). This butanoic derivative had an IC_{50} value for GlyT2 of 16 nM, with a preference over GlyT1 of >10,000 nM measured in in vitro studies of transfected cells (Methvin Isaac, 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 4.3D illustrates the results of adding ALX 5670 (100 μM) 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 RGCs, 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 figure 4.3E. 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 (Figure 4.3F: ON 104.2 ± 8.9%, OFF 102.6 ± 5.5%, PNFP 100.2 ± 0.9% of control response). The enhancement of the responses by the addition of D-serine confirmed that the NMDAR coagonist sites were not saturated (increase of ON 25.6 ± 15.5%; OFF 19.9 ± 14.2%, $p < 0.05$; PNFP 15.6 ± 2.4%, $p < 0.01$; $n = 6$ for each). 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 transportors 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 1998b). These cells form a prominent component for lateral inhibitory actions in the retina (Miller et al. 1977; Marc 1989). But, 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 and 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 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. While 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 was glycine. This difference could be the result of an inherent preference of NMDARs for D-serine. While some studies have shown a slight preference for D-serine (Matsui et al. 1995), others have suggested that glycine is favored (Snell et al. 1988; McBain et al. 1989), so that 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 must 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 and Bergman 1997). In contrast, the uptake 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 type transporter—a hetero-exchanger capable of both uptake and release depending on concentration gradients (Dun et al. 2007). 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 IPL 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 (Cubelos et al. 2005; Smith et

al. 1992). In contrast to glycine, when D-serine is added to the bathing medium, the low affinity transport system allows D-serine to be less encumbered by uptake and more effective in reaching and activating NMDAR coagonist binding sites. Since blocking the GlyT1 transporters leads to saturation of the NMDAR coagonist sites, it is not possible to evaluate the importance of uptake versus NMDAR coagonist site sensitivity for D-serine versus glycine.

While 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 and Hendrickson 1999; Du et al. 2002; Lee et al. 2005a), some 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 it is the action of the GlyT1 transporter, but not that of the GlyT2, that 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, since 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. While 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.

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. have used retinal slices of the mouse and rat to suggest that light-evoked glycine serves a dynamic, modulatory coagonist function while D-serine plays a more static, background role (Kalbaugh et al. 2009). 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.

Chapter 5

A mouse lacking D-amino acid oxidase activity shows increased D-serine levels and altered NMDA receptor activation in the retina

Activation of the NMDA receptor requires the binding of both glutamate and a coagonist. In many tissues, including the retina, D-serine has been shown to be an essential endogenous ligand at this site. While D-serine and its synthesizing enzyme, serine racemase, are both present in the retina, less is known about its degradation. Studies have shown D-amino acid oxidase (DAO) activity in the retina, but what effect it has on retinal D-serine has yet to be investigated. We obtained a mutant ddY/DAO^{-} mouse that lacks DAO activity to examine what effect the enzyme has on D-serine levels and function in the retina. Using capillary electrophoresis, our results indicate that D-serine levels are increased about four-fold in the mutant mice. Patch-clamp recordings from retinal ganglion cells show that this increase in D-serine is enough to saturate the coagonist site in the mutant mice, in opposition to the unsaturated state found in wild-types. In addition, the NMDA receptor component of the light response is much larger in the mutant mice suggesting that increased levels of D-serine may have an effect on glutamate receptor expression during development.

Introduction

The NMDA receptor (NMDAR) is an ionotropic glutamate receptor that along with the AMPA receptor accounts for the vast majority of excitatory neurotransmission in the CNS. While both receptor types require glutamate for channel opening, the NMDAR is unique in requiring a second coagonist, either D-serine or glycine (Johnson and Ascher 1987) (Kleckner and Dingledine 1988). Initially, the assumption was that glycine served as the endogenous coagonist, due in part to its prevalence in the CNS and its use as an inhibitory neurotransmitter. But the discovery that relatively high levels of D-serine were found in the brain (Hashimoto et al. 1992b) and the subsequent discovery of serine racemase (SR) (Wolosker et al. 1999d), an enzyme which converts L-serine to D-serine, suggested a role for endogenous D-serine.

In the retina, NMDARs are found on retinal ganglion cells (RGCs) and a subset of amacrine cells. They contribute to a portion of the light-evoked responses (Slaughter and Miller 1983), are responsible for a level of background noise in RGCs (Gottesman and Miller 2003b), and have been shown to play a critical role in excitotoxic retinal damage (Vorwerk et al. 2000). Initial studies of the NMDAR coagonist in the retina revealed the presence of D-serine and SR and suggested that the coagonist sites were not saturated, since addition of exogenous D-serine increased responses whole-cell responses from RGCs (Stevens et al. 2003). In salamander, D-serine seems to be essential for NMDAR responses in RGCs, since the degradation of D-serine by the enzyme d-serine deaminase (DsdA) results in a decrease in light-evoked current responses that is similar to those when NMDARs are antagonized with AP7 (Gustafson et al. 2007).

In addition to the synthesizing enzyme SR, a degradation pathway via D-amino acid oxidase (DAO) is present in the brain and retina (Neims et al. 1966) (Schell et al. 1997) (Beard et al. 1988a) (St Jules et al. 1992). A strain of mice that lacks DAO activity due to a point mutation (G181R) has been identified (Konno and Yasumura 1983). This ddy/DAO$^-$ mouse has been shown to have increased levels of D-serine in serum and brain samples when compared to its wild-type counterparts, with no obvious compensatory changes in D-serine associated proteins. Several changes have been reported in these animals including enhanced hippocampal LTP and improved special learning (Almond et al. 2006). These results suggest that D-serine levels may be controlled in part by DAO activity and that increased D-serine may affect NMDAR function. In this study, we have characterized the effects that this mutation has on the retina. We report that there is a lack of DAO activity in the mutant retina that results in an increase of tissue levels of D-serine in the ddy/DAO$^-$ mice. This increase has a significant effect on light-evoked responses of RGCs both through creating a saturating level of coagonist in the mutant mice and by increasing the percentage of the response that is mediated by NMDARs. In addition, we report that D-serine appears to be the major NMDAR coagonist in RGCs of both wild-type and mutant retinas.

Methods

All experiments were done in accordance with animal protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. We obtained ddy/DAO$^-$ and ddy/DAO$^+$ mice from the Konno lab and bred them in-house at the University of Minnesota. The mice were killed using an overdose of Nembutal (100 mg/kg).

Electrophysiology

Whole-cell voltage and current clamp recordings were made from retinal ganglion cells (RGCs) in an inverted retina-eyecup preparation. The eyes were removed to a Ringer solution and a dissection was performed under normal room light conditions. Following the removal of the front of the eye and lens, the eyecup was treated with an enzyme solution containing collagenase and hyaluronidase (120 & 465 units/ml respectively). The enzyme solution was washed and several small incisions were made to aid in the inversion of the eyes over a dome in a custom perfusion chamber (Newman and Bartosch 1999). The eyecups were secured using a harp that was made of platinum wire and nylon. They were continuously perfused with a 95/5% O_2/CO_2 bubbled Ringer solution containing, in mM, 111 NaCl, 32 $NaHCO_3$, 3 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 0.5 NaH_2PO_4, and 15 glucose at a rate ~2.5ml/min. The retina was viewed using infrared optics as a patch electrode (resistance of 4-10 MΩ) containing, in mM, 128 KCH3SO4, 5 NaCH3SO4, 2 MgCl2, 5 EGTA, 5 HEPES, 1 Glutathione, 2 ATP-Mg+2, 0.2 GTP(3Na), and an Alexa 568 or 488 dye was lowered to the surface of the retina. Gentle pressure was used to clear the surface of a selected cell to enable a patch to form, but with careful

attention to minimize disruption to the surrounding tissue. After establishing the whole-cell recording configuration, the spiking pattern of each cell was recorded. All cells used here displayed an ON response (21 transient ON, 3 sustained ON, and 14 ON/OFF). No difference was seen in ON versus OFF responses, but due to lower numbers of OFF responses, only ON responses are included in the results reported here. Current-clamp clamp recordings were made in normal Ringer solution. For voltage-clamp recordings a nominally free magnesium Ringer solution was used and TTX (1 μM) and strychnine (10 μM) were added to block action potentials and glycine receptors. Voltage-clamp studies were carried out after adjusting the standing current to zero (average membrane potential = –64.7 ± 0.9 mV, n = 38), using an Axoclamp 700A amplifier with a 10-kHz low-pass Bessel filter at a sampling frequency of 4–10 kHz, digitized with a Digidata 1320, and recorded in pClamp 9.0 (all Molecular Devices). Series resistance was uncompensated. Following recordings, fluorescence microscopy was used to visualize axons in order to identify each cell as a RGC. Reported data is not corrected for a liquid junction potential of -8.3 mV that was calculated in pClamp. All experiments were performed at room temperature (22º).

Light stimulation was provided by a computer-controlled LCD projector system using a tungsten-halogen light source (Burkhardt et al. 1998). The diameter of focal light stimuli was 200 μm. The intensity was adjusted to elicit a less than maximal response, but was roughly 1900 cd/m^2. The duration of light stimulation was two or four seconds with an inter-stimulus interval of 20 seconds. The image was projected onto the preparation through a 20X 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.

Immunohistochemistry

A polyclonal antibody against the N-terminal peptide (NH_2-AQYCISFADVE-COOH) of mouse SR (Sigma Genosys) was used in these experiments. Retinas were harvested and slices prepared as described previously (Morgans et al. 2005). Briefly, eyecups were immersion fixed in freshly in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 20-40 minutes. Following a wash in PBS (0.01 M, Ph 7.4), they were cryoprotected by consecutive immersion in 10%, 20% and 30% sucrose in PBS. The tissue was then embedded in OCT (Sakura Finetek, Torrence, CA), frozen, and cut at 15μm thickness on a cryostat. Sections were mounted onto Super-Frost glass slides. Sections were blocked for 30-60 min at RT in 3% normal horse serum, 0.5% Triton X-100, 0.025% PBS then incubated at 4ºC overnight in primary antibody (1:300). Following rinses in PBS, sections were incubated in secondary antibody at RT for 1 hr, CY3 (Jackson Immunochemicals, Mississauga, Canada) diluted 1:500 in PBS. The slides were washed again in PBS and then coverslipped with Crystal/Mount mounting medium (Biomeda, Foster City, CA). Images were acquired on a Zeiss LSM 510 confocal microscope with a 63×/1.40 oil-immersion objective. All confocal figures show single optical sections of < 1 μm thickness. For figures, brightness and contrast of images were optimized with Adobe Photoshop.

DAO activity assay

Eyecups were immersion-fixed in 2% paraformaldehyde for 30 minutes, washed in PBS and cryoprotected in a sucrose series. Chunks of medial cerebellum ($1mm^3$) were similarly fixed as positive controls. Fixed eyecups were embedded in 5% agar and transverse retinal sections (100μm) cut on a vibratome. Cerebellar sections were cut in the parasagittal plane. Sections were incubated 20h in the following reaction mixture (final volume, 500μl): A 0.05M Tris-HCl, pH 7.6 containing 0.1% horseradish peroxidase (Type VI), 0.005% 3-3' diaminobenzidine, 0.065% sodium azide, 0.6% nickel ammonium sulfate, and 22mM D-proline. Reactions were stopped with 2 rinses of 0.1M PBS. Sections were mounted on poly-lysine coated slides, dehydrated through an ethanol-xylene series and coverslipped. Bright- and dark-field images were acquired on an Olympus VANOX microscope equipped with a SPOT camera. Dark-field images with contrast reversed were used so that HRP reaction product is black. Images were taken with identical settings and edited simultaneously in Adobe Photoshop.

Capillary Electrophoresis

We used capillary electrophoresis (CE) to separate and measure the D-serine levels in tissue homogenates of isolated ddY/DAO^- mutant and wild-type mouse retinas. Retinas were homogenized in 0.6M perchloric acid (PCA) using a sonicator probe. KOH was added to neutralize the PCA. The mixture was spun down in a tabletop centrifuge at 14 000 rpm for 5 minutes and the supernatant was removed. The remaining pellet was re-suspended in 2M NaOH for protein determination using a Pierce (Rockford, Illinois) bicinchoninic acid assay. Amino acids in the supernatant were fluorescently derivatized

at 60ºC for 15 min with 4-fluro-7-nitrobenz-2oxa-1,3-diazole (NBD-F; Molecular Probes, Eugene, OR). CE separations were performed on a commercial CE instrument (Bechman-Coulter MDQ, Fullerton, CA) with laser-induced fluorescence (LIF) detection. Separations were performed in a (2-Hydroxypropyl)-b-cyclodextrin buffer at 15 kV (70 μA). 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 PMT and digitally plotted as the magnitude of the fluorescence signal versus time (electropherogram). Quantification of the data was achieved by integrating the D-serine peak, divided by the integrated peak of the amino adipic acid internal standard. The conversion to concentration was done by comparing the results with a series of D-serine standards. Data was displayed and peak integration was performed using 32Karat software (Beckman-Coulter).

Chemicals and agents

DsdA was provided by Dr. Herman Wolosker (Technion-Israel Institute of Technology, Haifa, Israel). All other chemicals and agents used in these experiments were purchased from Sigma Chemical (St. Louis, MO), unless noted: hyaluronidase and collagenase (Worthington Chemical, Lakewood, NJ); KCH3SO4 (Pfaltz & Bauer); tetrodotoxin, D,L-2-amino-phosphonoheptanoate (AP7), 5, 7-dichlorokynurenic acid (5, 7-DCK) from Tocris (Ellisville, MO).

Statistics

All results are reported as mean ± SEM. One directional t-tests were used for comparisons. A p-value < 0.05 was considered significant.

Results

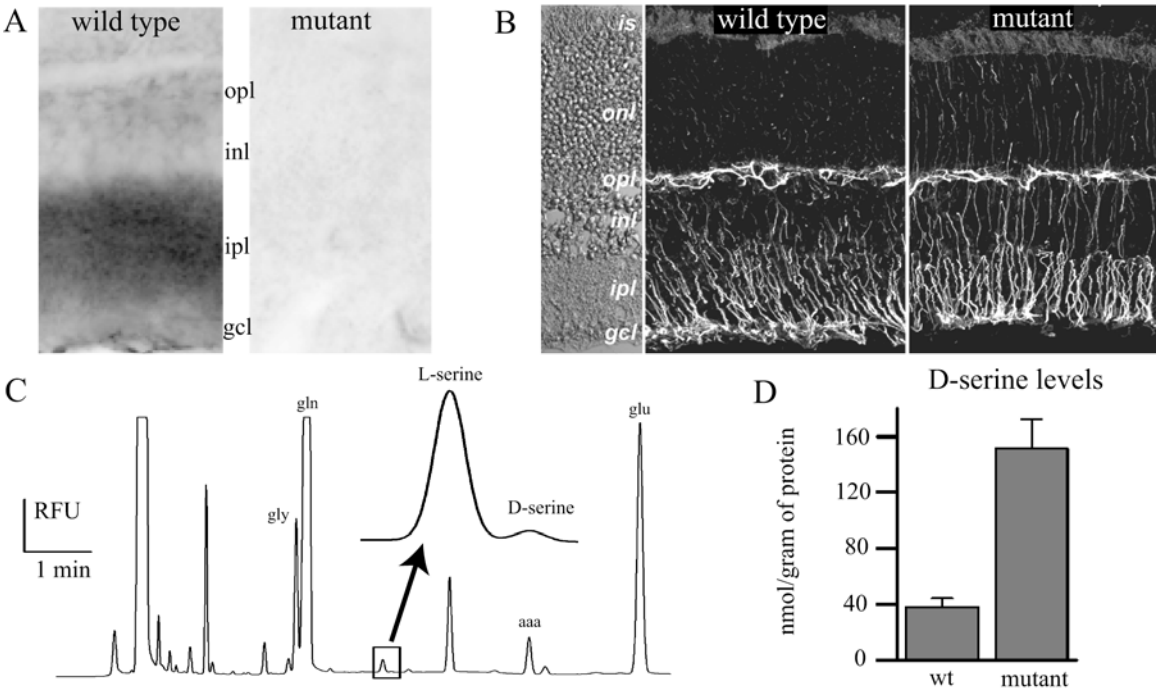
Retinas from ddY/DAO$^-$ mutant mice have increased levels of D-serine

Retinal slices from fixed eyecups were used to measure D-amino acid oxidase (DAO) activity in wild-type and mutant retinas. Figure 5.1A shows contrast-reversed, dark-field images of sections of mouse retina that had been assayed for oxidase activity. Following exposure to D-proline, an HRP-DAB reaction product was visible in the IPL of wild-type retina but not in the retinal slice from the ddY/DAO$^-$ mutant animal. The lack of product in the mutant retinas likely results from an absence of hydrogen peroxide synthesis that results from the oxidation of the D-proline by endogenous DAO activity in the wild-type retinas.

We used immunohistochemistry to examine the expression of the D-serine synthesizing enzyme, serine racemase (SR) in the two mouse types. Retinal slices from adult wild-type and mutant animals showed a similar retinal structure. SR labeling was found to be expressed strongly in processes which extend radially through the retina with particularly high expression in the ganglion cell and inner plexiform layers (figure 5.1B). This staining pattern, which resembles Müller cells, was found in both the wild-type and mutant retinas, suggesting that SR expression was not drastically effected by the lack of a functioning D-serine degradation pathway.

To directly determine the effect of the DAO mutation on retinal D-serine levels in the retina, we used capillary electrophoresis to measure the D-serine levels of retinal tissue homogenates from the ddY/DAO$^-$ mutant and wild-type retinas. An example trace

Figure 5.1



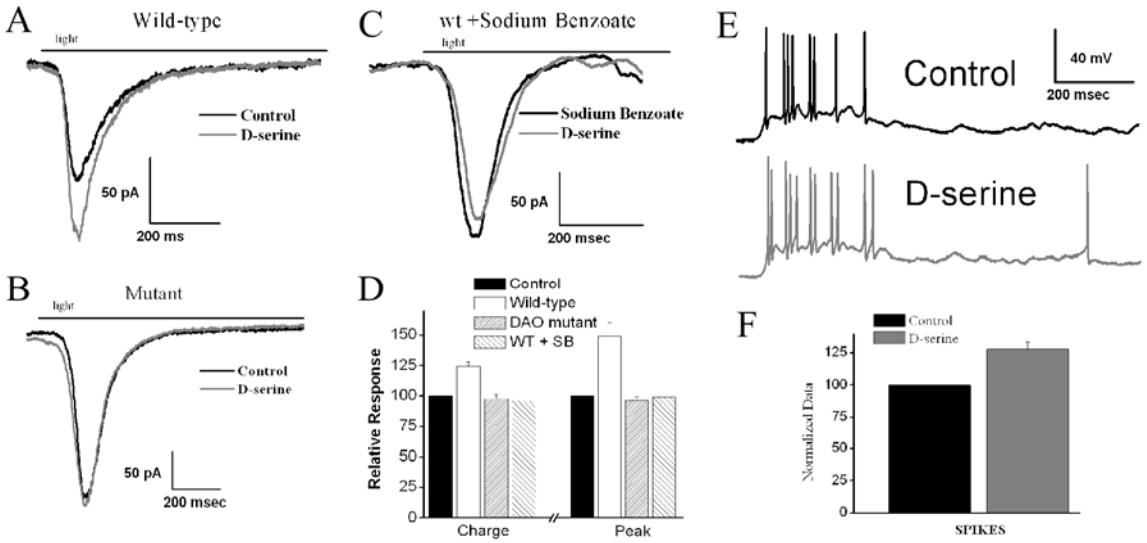
A lack of DAO activity results in an increase in D-serine. A, An HRP-DAB reaction product, the end product in a DAO activity assay, was visible in the ipl (inner plexiform layer) of wild-type retina but not in the retinal slice from the ddY/DAO$^-$ mutant animal. **B**, Immunoreactivity for serine racemase was seen along radial processes that extend through the retina, especially in the opl (outer plexiform layer), ipl (inner plexiform layer), and gcl (ganglion cell layer). **C**, A CE electropherogram, time versus RFU (relative fluorescent units) showing the separation of L-serine and D-serine from homogenized mouse retina. Also noted: gly-glycine, gln-glutamine, aaa-amino adipic acid, glu-glutamate. **D**, Cumulative results from CE analysis shows a four-fold increase in D-serine in the DAO mutant retinas.

from one CE experiment is shown in figure 5.1C, with a close-up of the region of interest showing the separation of D- and L-serine. Cumulative results from six sets of experiments showed that D-serine levels were roughly four times as high in the mutant retinas when compared to those in the wild-type (figure 5.1D; mutant = 151.3 ± 20.9, wild-type 38.1 ± 6.5 nmol/g of protein, n = 6, p = 0.0002).

NMDAR coagonists sites are saturated in retinal ganglion cells of ddY/DAO$^-$ mutant mice

Given the role that D-serine plays as an endogenous coagonist in the retina (Stevens et al. 2003) (Gustafson et al. 2007), we examined what effect the lack of DAO activity and the subsequent increase in D-serine levels had on retinal ganglion cell activity. Figure 5.2A shows the whole-cell, voltage-clamp response of a retinal ganglion cell from a wild-type retina to the onset of a light stimulus. The addition of exogenous D-serine (100 μM, gray trace) to the bathing solution led to an increase in the light response of this cell when compared to that in control Ringer (black trace). The story was not the same when exogenous D-serine was added to the bathing media of ddY/DAO$^-$ mutant mice. Figure 5.2B displays traces that were recorded from a RGC in a mutant mouse in which the addition of D-serine (gray trace) failed to affect the light response. In an attempt to mimic the effects of the DAO mutation, we incubated some wild-type retinas in the DAO inhibitor sodium benzoate (SB, 200 μM) (Moses et al. 1996) for two hours before recording RGC responses. Figure 5.2C illustrates how SB incubation eliminated the ability of D-serine to enhance the light-evoked responses in a

Figure 5.2



DAO activity keeps NMDAR coagonist sites unsaturated. **A**, D-serine (100 μM) potentiated the light response in a wild-type retina, indicating that the NMDAR site is not saturated. **B**, D-serine did not affect the light response in a mutant retina. **C**, Following incubation in the DAO inhibitor sodium benzoate, D-serine did not increase the light response in a wild-type (wt) retina. **D**, Cumulative results of voltage-clamp responses from retinas of wild-type, DAO mutant, and wild-type incubated in sodium benzoate (WT + SB), shown as total charge and peak amplitude. **E**, Current-clamp recordings of light responses from RGCs of wild-type retinas in both normal magnesium Ringer and with the addition of 100 μM D-serine. **F**, Average results showing the increase in relative spiking rate following the addition of D-serine containing Ringer.

wild-type retina. Cumulative results from these experiments show that in RGCs from wild-type retinas, D-serine significantly enhanced the light responses with the total charge increased by 24.5 ± 3.8 % ($p = 0.0001$) and the peak of the response increased by 49.2 ± 11.3 % ($p = 0.001$, $n = 9$) when compared to control. In the ddY/DAO$^-$ mutant mouse, D-serine failed to potentiate the responses. Ganglion cells from mutant retinas showed insignificant decreases of 2.4 ± 3.7 % of the total charge ($p = 0.7$) and 3.6 ± 2.3 % of the peak amplitude ($p = 0.9$, $n = 7$) in the presence of exogenous D-serine. Likewise, in the wild-type animals incubated with SB, the addition of D-serine did not affect the responses (decreases of 4.2 ± 3.3 %, $p = 0.9$ for the total charge and of 1.3 ± 1.9 %, $p = 0.7$ for the peak amplitude, $n = 5$). Together, these findings suggest that in the mutant and SB treated wild-type retinas, the NMDAR coagonist sites have a higher state of occupancy compared to the wild-type retinas.

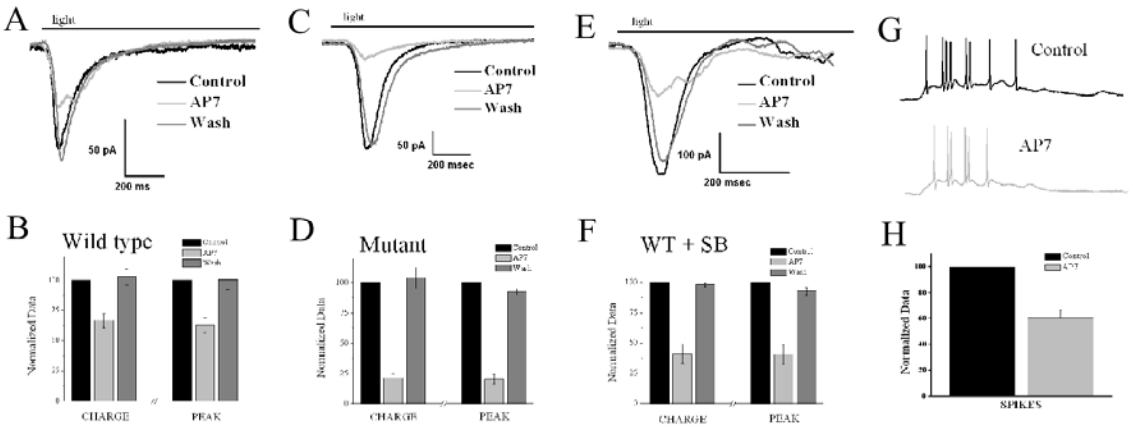
In our voltage clamp experiments we use a magnesium-free ringer solution to maximize the NMDAR currents as well as TTX and strychnine. To investigate whether the unsaturated state of the NMDARs we found under these conditions were exhibited under more physiological conditions, we studied light-evoked ganglion cell impulse activity in current-clamp configuration from wild-type retinas in the absence of TTX and strychnine with an external Mg^{2+} concentration of 1.0 mM. Figure 5.2E shows example traces from a RGC in normal Ringer solution. In response to the light stimulus, this RGC fired eight action potentials. Following the addition of D-serine (100 μM), the number of spikes resulting from an identical stimulus increased to 12. On average, the number of action potentials recorded from wild-type RGCs in the presence of D-serine was 28.0 ±

5.8 % greater when compared to responses in normal Ringer (Figure 5.2F, $p = 0.004$, $n = 6$). Thus, the enhanced excitability of retinal ganglion cells in the presence of D-serine was not created by the presence of drugs used to isolate and enhance NMDAR mediated synaptic events.

NMDARs have a significant role in retinal ganglion cell light responses

The NMDAR contribution to the light response was determined by measuring the response of RGCs in the presence of the NMDAR antagonist D,L-AP7 (100μM). In figure 5.3A, three traces show the light responses of a RGC from a wild-type retina. The addition of AP7 to the bathing media (light gray) resulted in a measurable decrease in the light-evoked current when compared to the control response (black). The light-response returned to control levels after a short wash in control Ringer (dark gray). Reversible decreases were also seen in recordings of RGCs from ddY/DAO$^-$ mutant retinas (3C) and in those from wild-type retinas that had been incubated in SB (5.3E). Blocking NMDAR currents with AP7 had a significant effect on the current responses in all RGCs recorded (cumulative results 5.3B,D,F). Light responses decreased by about one-third in wild-type RGCs following NMDAR blockade (33.4 ± 6.1 % charge, 37.0 ± 6.2 % peak, $n = 8$). In mutant RGCs the decreased response in AP7 was far greater (decreases of 78.8 ± 3.3 % charge and 79.7 ± 3.8 % peak, $n = 7$), while the responses in wild-type retinas exposed to the DAO inhibitor SB showed an intermediate level of reduction in AP7 (58.7 ± 8.3 % and 59.3 ± 7.9 %, $n = 5$, all $p < 0.001$). We again used current-clamp recordings of RGCs from wild-type retinas to verify that NMDAR blockade had a similar effect in normal Ringer. Spike traces in figure 5.3E show a RGC that fired eight spikes in control

Figure 5.3



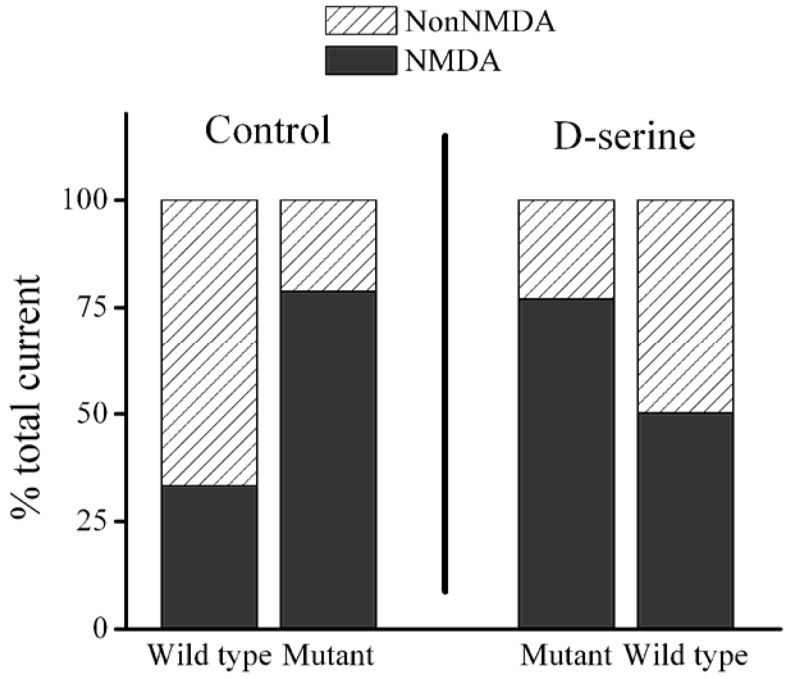
NMDARs have a significant role in retinal ganglion cell light responses. A,C,E, The application of 100 µM D-L AP7 reversibly reduced the light responses recorded in voltage-clamp from RGCs: **A,** wild-type, **C**, mutant, and **E**, wild-type incubated in sodium benzoate (WT + SB). **B, D, F**, Cumulative results of AP7 and its wash out from each mouse type: **B**, wild-type, **D**, mutant, and **F,** WT + SB. **G**, AP7 reduced spiking in a RGC from a wild-type retina. **H,** Effect of AP7 on light induced spiking in wild-type retinas.

Ringer, while responding with only six when AP7 was added. On average, AP7 application decreased RGC spike count by 39.4 ± 5.8 % (Figure 5.3F, p = 0.001, n = 6).

NMDAR component of the light response is greater in ddY/DAO$^-$ mutant mice

When we examined the data showing the reduction in light responses following AP7 application, it was clear that the mutant animals had a much more pronounced decrease following NMDAR blockade. But it was also evident from the D-serine application experiments (figure 5.2) that the control level we used as a starting point represented a saturated coagonist response in the mutants while in the wild-types a significant number of NMDAR coagonist sites were not fully occupied. To determine if the difference in the effect of AP7 on light responses seen between wild-type and mutant mice was due to the availability of D-serine, we reanalyzed the data and compared the responses in AP7 to the maximum responses that were recorded in a saturating dose of exogenous D-serine. These results are presented in figure 5.4. The columns represent the total current measured in each condition with the black portion representing the percentage of that current mediated by NMDARs. On the left side of the graph, the NMDAR and non-NMDAR currents measured using the magnesium free Ringer as the control are displayed, showing the same two-fold increase in the percentage of current mediated by NMDARs in the mutant versus the wild-type shown above in figure 5.3. The two columns on the right use, as a starting point, the saturated responses seen in the presence of 100 μM D-serine. The currents measured in AP7 are then compared to those in the saturating dose of D-serine. While the percent of the RGC current response

Figure 5.4



NMDAR component of the light response is greater in ddY/DAO$^-$ mutant mice. The graph displays the percent of the current mediated by NMDA (black) and non-NMDA (hatched) receptors under different conditions. The left side represents the percentage of NMDAR and non-NMDAR currents as recorded in control Ringer for both the wild-type and mutant mice. The right side compares the percentages in relation to a Ringer solution containing 100 μM D-serine.

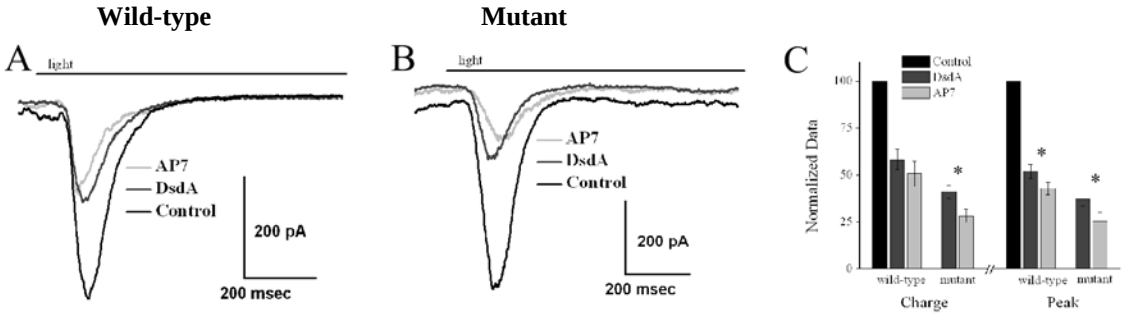
mediated by NMDARs in the wild-type retina increased to 50.3 ± 5.2 % under these conditions, it was still far less than that seen in the ddY/DAO$^-$ mutant retina.

D-serine is an essential ligand for NMDA receptors

The d-serine degrading enzyme D-serine deaminase (DsdA) was used to determine the actions of endogenous D-serine on light evoked responses from RGCs. Figure 5.5A shows the results of an experiment in which whole-cell, voltage-clamp recordings were made from a ganglion cell from a wild-type retina. DsdA (10 μM) was added to the control Ringer followed by the NMDAR antagonist AP7. The application of DsdA (dark gray trace) resulted in a large decrease in the current response when compared to the control (black). The subsequent addition of AP7 (light gray) had a modest depressing effect on top of the reduction seen in DsdA. A very similar result from a recording from a mutant retina is displayed in figure 5.5B. The cumulative results of RGC responses from 6 wild-type and 7 mutant retinas are presented in figure 5.5C. Wild-type responses decreased significantly following DsdA (dark gray) application with the total charge dropping by 41.7 ± 5.4 % and the peak by 49.3 ± 6.7 % ($p < 0.001$). When AP7 was added there was a further drop in both the total charge to 50.7 ± 3.6 % and in peak amplitude to 42.9 ± 3.3 % of the control response. The difference between the DsdA and AP7 responses was significant for the peak response ($p = 0.04$) but not for the charge ($p = 0.2$). A similar picture was painted with respect to the results from the DAO mutant retinas. Like in figure 5.3, the NMDAR component in the mutants was increased when compared to that in the wild-type. When compared to the control response, the total charge was reduced by 58.8 ± 3.4 % in DsdA and 72.0 ± 3.4 % in AP7

and the peak responses were reduced by 62.6 ± 3.8 % in DsdA and 74.7 ± 4.6 % in AP7. All reductions are significant when compared to control ($p < 0.001$) and when compared across treatments, DsdA to AP7 ($p = 0.01$ charge, 0.04 peak).

Figure 5.5



D-serine is a major activator of NMDA receptors. **A**, Degradation of D-serine by DsdA (10 µg/ml) sharply reduced the light response in a RGC from a wild type retina. D-L AP7(100 µM) had a small additional effect. **B,** A similar pattern was seen from a RGC in a mutant retina. **C**, Cumulative results showing the effect of DsdA and a subsequent addition of AP7 on the charge and peak responses of retinal ganglion cells, * denotes significant difference between DsdA and AP7 treatments.

Discussion

Previous studies have investigated the role of D-serine in amphibian and rat retinas and have shown that D-serine is present in Müller cells (Stevens et al. 2003) and acts as the major NMDAR coagonist in light-evoked responses of RGCs (Gustafson et al. 2007). In this study, we present the first results of D-serine action on light-induced RGC activity in the intact mouse retina. Additionally, we have utilized a mutant mouse (ddy/DAO$^-$) which lacks the ability to degrade D-serine and show the importance of the enzyme DAO in maintaining D-serine levels in the retina and the functional consequences of increased D-serine levels.

A lack of D-amino acid oxidase function increases D-serine levels in the retina

Using an assay of DAO activity we have shown that the ddY/DAO$^-$ mouse failed to oxidize exogenously applied D-proline. While in the wild-type counterpart, DAO activity was found to be concentrated in the IPL—which is a region where NMDARs are abundant. This is an important observation for two reasons. One, it helps to illustrate the functional difference between the mutant and wild-type retinas, but more fundamentally, it provides the first evidence of functional DAO activity in the mammalian retina. Up until now, DAO activity has only been reported in the frog and amphibian retina (Beard et al. 1988a) (St Jules et al. 1992) without any demonstration of its function. The finding that the DAO activity is strong in a region of NMDAR expression also suggests that retinal DAO is well positioned to affect D-serine action. CE measurements of amino acid levels in retinal homogenates showed D-serine levels approximately four times that observed in wild-type retinas. Together these results are similar to those found across

several brain regions. In regions of the brain where DAO activity was present, like the cerebellum and midbrain, the ddy/DAO$^-$ mice had considerably higher D-serine levels. In addition, no obvious compensatory changes have been found in studies of D-serine and NMDAR associated proteinsin these mice, including serine racemase (SR), (Almond et al. 2006). Likewise, using an antibody against D-serine's synthesizing enzyme, SR, we report that no obvious changes have occurred in its expression. While we cannot be certain that compensatory changes have not been made in the synthesis of D-serine in the ddY/DAO$^-$ mice, it is clear that the lack of DAO activity results in a substantial increase in D-serine levels in the retina.

D-amino acid oxidase maintains D-serine below saturation levels for the NMDAR coagonist site

With the discovery that NMDARs required a second coagonist for ion channel gating, it was assumed that relatively high levels of glycine in tissue would be sufficient to saturate the responses, making modulation at the coagonist site unlikely. While early results from retinal slice preparations suggested that retinal NMDARs were saturated (Gottesman and Miller 1992) (Lukasiewicz and Roeder 1995), more recent studies from intact retinas have reported increases in RGC responses after the application of exogenous D-serine in both rat and amphibian (Stevens et al. 2003), leaving open the potential for modulation at this site. The results presented here extend this finding to mice. In whole-cell recordings from the wild-type ddY/DAO$^+$ retinas, RGCs showed increases in both their firing rate and in currents measured in voltage-clamp in response to light stimuli (figure 5.2). This increase in response suggests that under control

conditions, there is insufficient D-serine to match the amount of glutamate being released, thereby leaving the coagonist site a potential site for modulation. Interestingly, the increase was greater for the peak amplitude than for the total charge, suggesting that there was less coagonist available at the immediate onset of the response. In the mutant ddY/DAO$^-$ mice, on the other hand, application of exogenous D-serine had no effect on the light-evoked responses measured in RGCs. Additionally, when wild-type retinas were incubated with the DAO inhibitor sodium benzoate, NMDAR responses were not significantly changed by application of exogenous D-serine. Further study will be needed to determine what affect this saturation has on retinal function. Recently, a study has reported that synaptic stimulation can result in the direct release of coagonist, which in turn can activate NMDARs (Kalbaugh et al. 2009). There is much interest as well in pharmacologically targeting DAO for treatment of psychiatric disorders including schizophrenia (Almond et al. 2006). Since it may be assumed that any drug passing the blood-brain barrier to treat these disorders may also pass the blood-retinal border, it will be important to understand the implications increased coagonist availability might have on the retina.

NMDAR contributions to light-evoked responses are altered in mice lacking DAO activity

NMDARs are expressed in amacrine and ganglion cells of the inner retina and contribute to light-evoked activity described in both cell types (Massey and Miller 1990; Dixon and Copenhagen 1992; Mittman et al. 1990; Slaughter and Miller 1983). This was found to be true in the mice studied here as well. In wild-type mice, the NMDAR

antagonist AP7 decreased both the light-evoked spiking activity of ganglion cells as well as had a substantial effect on the currents measured in voltage-clamp experiments. Unexpectedly, the decreases observed in the mutant ddY/DAO$^-$ mice to AP7 application were much greater (>75 % decrease) than those in the wild-type mice (~35 % decrease). This meant that while in the wild-type RGCs only one-third of the light-evoked current response was carried by NMDARs, more than three-fourths of the current was NMDAR-mediated in the ddY/DAO$^-$ mutant mice. This difference was diminished, but not eliminated, when we compared the AP7 responses to those of the wild-type retinas during a period of coagonist site saturation with D-serine. From this we can conclude that the differences in the NMDAR contribution to the responses seen in the ddY/DAO$^-$ versus the ddY/DAO$^+$ mice were partially the result of an increase in coagonist availability, but were also the result of a greater percentage of the response being derived from NMDARs versus non-NMDARs. This may not be so surprising since the activity and the expression of NMDARs has been tied to development. In addition, the expression of D-serine and DAO play a major role in shaping the synaptic connections of the cerebellum, where early expression of D-serine in Bergmann glia correlates with transient NMDAR expression in Purkinje cells and their subsequent synaptic connections with parallel and climbing fibers (Rabacchi et al. 1992) (Schell et al. 1997). The difference in NMDAR activity may not simply be a reflection in the number of NMDARs; it may also be a function of the type of NMDAR that is expressed. The size of currents generated by NMDARs depends on their subunit composition, with the NR1/NR2B combination (Kutsuwada et al. 1992) having larger currents than the NR1/NR2A subunits (Monyer et

al. 1994). The expression of these subunits is developmentally regulated, early stages favoring the large-current NR2B with later expression dominated by the NR2A (Watanabe et al. 1992). It is possible that the high levels of D-serine have affected the expression of NMDARs in the retinas of the mutant mice. Recently, the differential expression of the glutamate-binding NR2 subunits, including a possible distinct role for NR2B subunits in the ON versus OFF pathway has been reported in the retina (Kalbaugh et al. 2009). Further investigation of NMDAR contributions to the RGC light response and the impact it may have under different stimulus conditions and at different developmental stages may prove fruitful for developing a better understanding of the roles of NMDAR function and the expression of its subunits on the ganglion cells of the retina.

D-serine is essential for light-evoked NMDAR currents in retinal ganglion cells

While the lack of DAO activity had a large impact on the percentage of the light response mediated by NMDARs, it did not seem to have much of an effect on the percentage of the NMDAR response that was dependent on endogenous D-serine. Similar to previous studies in the salamander retina (Gustafson et al. 2007), the majority of the NMDAR light response is dependent on D-serine, as illustrated by the small differences seen between the current responses in DsdA and those in AP7. The further reductions that were seen may have been the result of the shorter exposure time for the DsdA and the faster perfusion rate used in the mouse versus the salamander. Another possibility, however, is that there is another endogenous coagonist which is functioning at

the coagonist site in these mice, perhaps glycine as was recently suggested (Kalbaugh et al. 2009).

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