Dynamic Regulation of the NMDA Receptor Coagonist D-serine in the Mammalian Retina

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Robert F. Miller

September, 2011

Acknowledgements

I thank my advisor Robert Miller for creating a lab environment that is always open to new ideas and emphasizes the importance of understanding your experiment from the ground up.

I thank my current lab members: Manny Esguerra, "the cerebellum whisperer", for his skill and patients in teaching hands on science; Eric Gustafson, for his philosophy and keen intuition; Gabriel Romero, for always questioning things.

I thank Anusha Mishra, for reading my manuscripts and stressing the importance of fine detail.

I thank my committee members, Eric Newman, Paulo Kofuji, and Michael Bowser, for keeping their doors open and for sharing their equipment and techniques.

I dedicate this work to my Mother and Father for teaching me that achievement is meaningless without love

Abstract

The N-methyl D-aspartate (NMDA) receptor coagonist D-serine is important in a number of different processes in the central nervous system, ranging from synaptic plasticity to disease states, including schizophrenia. In the retina, light-evoked responses of retinal ganglion cells are shaped in part by NMDA receptors which require a coagonist for activation. There is debate over whether glycine or D-serine is the endogenous coagonist of retinal ganglion NMDA receptors. I used a mutant mouse lacking functional serine racemase (SRKO), the only known D-serine synthesizing enzyme in mammals, to show that retinal ganglion cells depend on D-serine for NMDAR activation (chapter 1).

Most changes in NMDA receptor currents during synaptic activity have been attributed to glutamate fluctuations against a steady background of coagonist, excluding the possibility of dynamic coagonist release. The retina is a particularly useful system to determine if coagonist release occurs in the nervous system, because it can be naturally stimulated with light. By saturating the glutamate binding site of NMDA receptors, I was able to measure coagonist release during light-evoked responses. Coagonist release was detected in retinal ganglion cell light responses and depended on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic AMPA receptors. Coagonist release was significantly lower in SRKO mice (chapter 2). By directly measuring extracellular D-serine using capillary electrophoresis, I demonstrated that D-serine can be released from the intact mouse retina through an AMPA receptor dependent mechanism (chapter 3). The collective works put forth in this thesis imply that activity-dependent modulation of D-serine availability may add an extra dimension to NMDA receptor coincidence detection in the central nervous system.

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Introduction

NMDA receptors

Iontropic glutamate receptors mediate fast excitatory neurotransmission throughout the mammalian nervous system. The ligand binding domain of all known glutamate receptors shares homology to a family of bacterial periplasmic binding proteins (1). Glutamate gated ion channels are found in plants (2) and some species of prokaryotes (3), implying that glutamate receptors mediated cellular signaling events in eukaryotes long before the evolutionary emergence of nervous systems approximately 600 million years ago (4). Three major classes of ionotropic glutamate receptors are found in the nervous system: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl D-aspartate (NMDA) receptors.

The biophysical properties of NMDA receptors (NMDARs), compared to AMPA and kainate receptors, allow for relatively large charge transfer following activation. This in part is due to high peak channel conductance and slow deactivation (5). The majority of NMDARs in the CNS are tetramers, comprised of two GluN1 subunits and two GluN2 subunits (GluN2A or GluN2B) which contain the glutamate binding site. NMDARs comprised of GluN2A or GluN2B subunits exhibit similar peak channel conductance, but GluN2B containing receptors are more sensitive to glutamate and display slower deactivation (6). Early in postnatal cortical development GluN2B containing receptors are more abundant, but as synapses mature GluN2A receptors become more prevalent (7).

Gated NMDARs are highly conductive to Ca^{2+}, which upon entry interacts with Ca^{2+} binding proteins tethered to the receptor, subsequently activating a plethora of intracellular signaling cascades. These signaling events can lead to changes in synaptic efficacy via phosporylation of AMPARs or can lead to long-term changes in synaptic function by altering gene expression. The amount of Ca^{2+} entering through NMDARs is critical in determining which signaling pathways become activated. Small Ca^{2+} influx through NMDARs results in synaptic depression, while larger Ca^{2+} influxes lead to the downstream potentiation of synapses (8). On the other hand, excessive levels of Ca^{2+} entering through NMDARs may cause excitotoxic cell death by activating apoptotic pathways (9).

Given the wide range of neuronal modifications brought on by NMDAR activation, it is no surprise that that their gating is regulated by a number of factors. For this reason, NMDARs can be thought of as molecular coincidence detectors, requiring the simultaneous occurrence of cellular signaling events to become active. One such requirement is sufficient neural depolarization to remove Mg^{2+} blocking the NMDAR pore. Furthermore, NMDARs are unique among glutamate receptors in that they require the simultaneous binding of four agonists to become activated, which, in part, is responsible for their slow activation kinetics (10). Channel gating requires glutamate bound to each GluN2 subunit and, more recently discovered, a coagonist must be bound to each GluN1 subunit (6). Over the years, two candidates have emerged as endogenous activators of the coagonist site, glycine and D-serine. In 1961 it was discovered that the application of D-serine potentiated excitatory field potentials in frog spinal cord (11). Despite these findings D-serine, being a mirror image enantiomer of the biologically

more common amino acid L-serine, was overlooked as an endogenous agonist candidate for over two decades, given the scarcity of D-amino acids found in animals.

Molecular asymmetry in biology

The concept of molecular handedness began in 1811 when the French scientist Francois Argago discovered that polarized light passing through particular substances was rotated by a specific angle (12). Using this method of molecular finger printing, in 1848 Louis Pastuer discovered that certain compounds isolated from life would rotate light in one direction, but when synthesized in the laboratory these compounds were not optically active. Pastuer realized when observing the macroscopic crystals of tartaric acid that they existed as mirror images of each other. No matter how one crystal was oriented, it could not be overlayed with its mirror image crystal. Pastuer segregated the mirror-image crystals out from each other, and suspended each type in solution. The compounds were now optically active, rotating light by the same angle, but one to the left (levrorotatory, L-) and the other to the right (dextrototory, D-) (13).

It has since been discovered that molecular asymmetry is found in biological systems, whereas the majority of reactions in the lab do not favor L-isomers over D-isomers and thus form racemic mixtures containing equal quantities of each enantiomer. In life, L-amino acids are more common than D-amino acids, forming the building blocks of proteins. Also, sugars and nucleic acids are predominantly D-isomers. It remains to be determined if biological asymmetry emerged 3.5 billion years ago as an arbitrary assembly of one enantiomer over the other from a racemic primordial soup, preserved by

the earliest self-replicators, or if molecular asymmetry existed on earth prior to life. Indeed, L-amino acids are more abundant in uncontaminated meteorite samples (14).

NMDA Receptor Coagonist

In 1987, Johnson and Ascher discovered that NMDAR currents in cultured neurons were potentiated by glycine (15). Shortly after, Klechner and Dingledine found that it was essential for the glycine binding site to be liganded for NMDAR activation and that D-serine could also serve this role (10). At the time it was known that D-serine was present in the blood, but it was thought to originate from the ingestion of bacteria, which use D-amino acids as a component of their cell walls (16). Accordingly, it was tacitly concluded that glycine, given its abundance in the nervous system and role as an inhibitory neurotransmitter, was the endogenous ligand.

The potency of glycine and D-serine may depend on both the subtype of NMDAR (GluN2A or GluN2B) and the species it belongs to. Rat NMDARs expressed in *Xenopus* oocytes, consisting of a mixed population of GluN2A and GluN2B receptors, display an ED_{50} of 0.5 μM and 1 μM for glycine and D-serine, respectively, with similar efficacies (17). Another study specifically compared the potency of each coagonist in oocytes separately expressing mouse GluN2 (A-D) receptors and found that D-serine was about three times more potent than glycine for each receptor (18). In cloned human NMDARs D-serine is slightly more potent at GluN2A receptors, while glycine is a more potent coagonist for GluN2B receptors (19). Although there appears to be modest differences in coagonist potencies for the NMDARs of different species, as an approximation, D-serine and glycine are comparably effective coagonists for GluN2A or GluN2B receptors. More

noteworthy is that the affinity of glycine and D-serine for GluN2A receptors is several fold greater than GluN2B receptors (20).

The prospects of D-serine as an endogenous coagonist began when Hashimoto and colleagues devised a technique to better resolve brain L-amino acids from D-amino acids (21). They discovered the brain contains high levels of free D-serine (22), comparable to that of glycine (7 μM) (18). Furthermore, histochemical staining revealed that D-serine overlapped with the expression of NMDARs (23). The high levels of D-serine found in cortex could not be entirely attributed to bacterial origin and pointed towards a mechanism of D-serine synthesis.

D-serine synthesis

Work done in 1965 showed that certain eukaryotic organisms, including silkworms, were capable of synthesizing large quantities of D-serine from added L-serine consisting of carbon isotopes (24). The enzyme responsible for this conversion remained elusive until 1998 when the protein was partially purified (25). Wolosker and colleagues showed that a similar racemization process was conserved in an isolated protein from rat brain (26), and went on to clone and sequence human serine racemase (27). Three different mouse lines have since been generated that lack functional serine racemase, all showing an approximately 90% reduction in cortical levels of D-serine (28). Serine racemase is expressed throughout the brain, and on a cellular level was initially shown to be expressed in astrocytes which also express high levels of D-serine (29). This picture has been complicated by the more recent discovery of serine racemase and D-serine in neurons (30).

D-serine uptake and degradation

The abundance of free D-serine found in the brain raised questions about factors that might also limit D-serine availability. Studies have shown that intracerebroventricularly injected D-serine is taken up preferentially by glial cells (31). The sodium dependent slow uptake transporter arganine-serine-cysteine-threonine (ASCT2) is expressed in astrocytes (32) and is responsible for D-serine uptake in astrocyte cultures (33). However, the sodium independent fast amino acid transporter alanine-serine-cysteine (ASC-1) is also present in neurons (34) and using ASC-1 knockout mice, it was shown that 30% of D-serine uptake from isolated synaptosomes was through ASC-1 (35). Collectively, these findings suggest that global D-serine levels may be regulated by glial ASCT2, while the rapid uptake of D-serine occurs near synaptic sites via ASC-1. It is unclear which of these transport mechanisms are functionally relevant in regulating NMDAR coagonist availability.

D-serine levels are also regulated by the flavin adenine dinucleotide (FAD) dependent enzyme D-amino acid oxidase, which to date is the only known D-serine degrading enzyme found in mammals, D-serine dehydratase being found in birds (36). Extensive studies of DAO began in 1935 when Sir Hans Kreb noted that an enzyme present in kidney showed preference for D-amino acid substrate over L (37). It was assumed that the purpose of D-amino acid oxidase was to degrade D-amino acids originating from the diet or from bacteria in the gut. Later Hashimoto discovered that DAO is present in the brain (22). Histochemical staining reveals a negative correlation between DAO expression and D-serine. Rostral brain regions, including neocortex and hippocampus, show robust labeling for D-serine and no staining for DAO, whereas caudal regions,

including cerebellum and brainstem, show minimal D-serine and high DAO expression (38). On a cellular level, DAO is localized to the peroxisomes of cerebellar astrocytes and Bergman glia (39). Interestingly, in lower vertibrates DAO expression is relatively high in rostral brain, suggesting an evolutionary divergence in the utilization of D-serine (38). Mice with a naturally occurring point mutation in DAO lose enzymatic function and consequently have dramatically elevated D-serine levels in these caudal but not rostral brain structures, stressing the importance of DAO in regulating D-serine (40). On the other hand, SR expression is relatively uniform in the brain, including expression in cerebellum, and therefore cannot account for the difference observed in D-serine levels (41). Although DAO is not expressed rostrally, it has been proposed that D-serine levels might be regulated by the B-eliminase activity of SR which converts D-serine into pyruvate, thus preventing an overaccumulation of D-serine in cells (42). In addition, D-serine levels can be indirectly depleted by NMDAR activity which reduces the activity of constitutively active SR by translocating it to the membrane (43) where it is inhibited by PIP2 (44).

Ontogeny of D-serine regulation

In mice, D-serine levels increase several fold from postnatal day 1 (P1) to P7 in cortex, striatum, and cerebellum. These changes correlate with a dramatic increase in SR expression (45). During rat P1, D-serine is predominantly present in hindbrain, midbrain, and spinal cord (38). By P14 D-serine is absent in hindbrain and spinal cord, showing the highest levels in forebrain (38). D-serine reduction during the course of cerebellar development has also been observed in mice, correlating with an increase in DAO, while SR expression remained constant (45). Early in the development of cerebellum, granule

cells migrate along Bergman glia, a class of radial glia, to the appropriate layer of cerebellar cortex. SR expressed in Bergmann glia provides D-serine allowing for activation of granule cell NMDARs, which are essential for migration (46). However, in adulthood, Bergmann glia heavily express DAO (47), thus reducing D-serine levels, while SR remains relatively stable. Collectively, these studies suggest that the D-serine increases throughout the brain due to increased SR expression, but later in development DAO is expressed in hindbrain, reducing D-serine in this area.

Physiological role for D-serine as NMDA receptor coagonist

The extensive mechanisms regulating D-serine synthesis, uptake, and degradation imply that it plays an important role in CNS function, but levels of glycine are comparable to D-serine when measured in CSF (38). However, it is possible that the concentrations of glycine and D-serine could vary drastically at the synapse, where NMDARs are present. A number of electrophysiology studies have looked at the role of D-serine in regulating NMDAR activity during synaptic responses. The application of exogenous DAO dramatically reduced NMDAR currents in slice recordings from hippocampus, which could be rescued by applying D-serine (48). In isolated retinal recordings, light-evoked retinal ganglion cell NMDAR currents are completely abolished by bath applied DAO (49) or the highly specific D-serine degrading enzyme D-serine deaminase (50), implying that D-serine can serve as coagonist during physiological stimuli.

Astrocytes are responsible for providing the D-serine acting on NMDARs in multiple brain regions. In the supraoptic nucleus of the hypothalamus, D-serine is the primary coagonist. During lactation the astrocytic processes enwrapping synapses retract and

NMDAR currents plummet. This effect can be rescued by applying coagonist, suggesting the drop in excitatory input was caused by D-serine depletion, as the source became more distant from synapses (51). Similarly, in hippocampus, patch-loading astrocytes with an inhibitor of SR reduced NMDAR currents in CA1 pyramidal neurons, consequently preventing the induction of LTP (52). In prefrontal cortex, the glial toxin fluoroacetate reduced NMDAR currents in pyramidal neurons and this effect is rescued by adding D-serine to the bathing medium (53).

Coagonist release

Multiple studies have shown that the coagonist site of NMDARs is unsaturated during synaptic responses in the same regions where NMDARs appear to depend on D-serine. A picture where some but not all NMDARs are occupied by coagonist lends the possibility that a decrease or increase of D-serine could limit or recruit, respectively, NMDARs in synaptic responses. When D-serine expression was first shown in astrocytes, studies revealed that the application of AMPAR agonist evoked D-serine release from astrocyte cultures (29). Indeed, astrocytes display vesicular glutamate release, sharing many features with vesicular release from neurons (54). Upon investigation, AMPAR mediated D-serine release from cultured astrocytes also shared many of the mechanisms of neural-like vesicular release. This process depended on extracellular Ca^{2+} levels, with the novelty of also depending on release of Ca^{2+} from intracellular stores. Furthermore, D-serine release depended on SNARE proteins and the proton pumps necessary for loading vesicles with transmitter (55). Immunolabeling in astrocytes shows D-serine colocalization with vesicular SNARE proteins, which are recruited to the membrane following glutamate receptor stimulation (56). It is unknown

if vesicular release of D-serine from astrocytes occurs in intact tissue, but in hippocampal slices D-serine release depends on the elevation of intracellular Ca^{2+} in astrocytes (52). AMPAR stimulation may also elevate extracellular D-serine by activating serine racemase. Under basal conditions, Glutmate Receptor Interacting Protein 1 (GRIP-1) is bound to AMPARs. Upon AMPAR activation, GRIP dissociates from AMPARs and associates with SR, allowing it to become active (46).

Mechanisms for D-serine release independent of AMPAR activation have also been proposed. Activation of mGluR5 receptors has been shown to phosphorylate PIP2, relieving its inhibition of SR and increasing levels of D-serine production (44). D-serine release through the sodium dependent neutral amino acid transporter ASCT2 has also been proposed (33). The addition of L-serine or cysteine, a substrate for the ASCT2, favors the sodium dependent release of D-serine via an antiport mechanism (57). Therefore, changes in the extracellular environment, including elevations of amino acids known to act on ASCT2, or relative increases in the intracellular sodium concentration in glia, decreasing the driving force for sodium, may consequently elevate D-serine levels.

When D-serine and serine racemase were found in neurons, studies revealed that neuronal cultures also release D-serine in response to AMPAR stimulation (30). However, additional experiments argued that the underlying factor was more likely neural depolarization. Direct depolarization by applying potassium or activators of voltage gated sodium channels evokes D-serine release from cultured neurons but not from cultured astrocytes. Similarly, D-serine release in response to depolarization was observed in cortical slices by making real-time measurements of D-serine using online capillary electrophoresis. Removal of external Ca^{2+} or chelation of intracellular Ca^{2+} had

no effect on depolarization-evoked release, suggesting the depolarization dependent D-serine release was non-vesicular (58).

The direct biochemical measurement of D-serine following pharmacological manipulations has provided a number of potential mechanisms of coagonist release, but it is unclear which, if any, of these mechanisms is utilized during synaptic transmission. The measurement of coagonist release using electrophysiological recordings has been complicated by the fact that glutamate release is also simultaneously occurring. Furthermore, for coagonist release to be detected through its activation of NMDARs, glutamate must also be present, but glutamate is only transiently available during synaptic transmission. Therefore, sophisticated techniques have been developed to measure coagonist release during synaptic transmission. By pharmacologically preventing glycine release in spinal cord slices, where D-serine is absent due to high levels of DAO expression, there was a reduction in NMDAR currents but not AMPAR currents. This effect was absent when the experiment was repeated in the continuous presence of saturating coagonist (59). The reduction in NMDAR current caused by inhibiting glycine release was proportional to the stimulus intensity, suggesting that the glycine effecting NMDAR currents was being released during stimulation (59). Slice recordings of hippocampal pyramidal neurons have shown that the potentiation of NMDAR currents by the addition of coagonist were reduced as afferent stimulation was increased. The authors concluded that larger stimulus intensities were causing more endogenous coagonist release and therefore less potentiation of NMDAR currents by exogenous coagonist (60). However, it is possible that stronger stimuli cause more glutamate release, which then activates NMDARs that are basally occupied by coagonist.

Another study, in cerebellum, utilized a competitive antagonist for the coagonist binding site to estimate the levels of coagonist acting on granule cell NMDARs following mossy fiber stimulation. The estimated coagonist concentration during stimulation exceeded the values of free glycine measured in cerebellum, suggesting that coagonist was released (61). However, this experiment does not rule out the possibility that coagonist is constantly released, independent of stimulation, near synaptic NMDARs.

Coagonist regulation in Retina

Retinal phototransduction is initiated when the chromophore 11-cis retinal in photoreceptors is converted to all-trans retinal by the absorption of a single photon, resulting in the downstream closure of constitutively active cyclic nucleotide gated cation channels. The photoreceptor membrane potential becomes more negative, preventing their tonic release of glutamate. ON bipolar cells, carrying information about the onset of light, are typically inhibited by glutamate released in the dark via activation of $mGluR_6$ receptors. Thus, the cessation of glutamate release following the onset of light depolarizes ON bipolar cells. Off bipolar cells, on the other hand, are activated by glutamate through AMPA and kainate receptor activity. Therefore, OFF bipolar cells are activated by the offset of light and suppressed by the onset of light. The glutamate released from bipolar cell terminals activates the AMPA and NMDARs of retinal ganglion cells (RGCs), whose axons form the optic nerve, relaying information to the brain in the form of spikes.

NMDARs contribute to RGC light-evoked currents for both ON and OFF responses (62). GluN2A and GluN2B are both expressed by RGC dendrites in the ON and OFF

sublamina of the inner plexiform layer, although GluN2B expression is slightly more prevalent at ON synapses and GluN2A at OFF synapses (63). Spontaneous excitatory post synaptic currents in OFF cells are insensitive to GluN2B antagonist, while ON cells are only sensitive to an GluN2B antagonist when glutamate transporters are blocked, suggesting that GluN2B receptors are localized perisynaptically and activated by glutamate spillover during synaptic transmission (64).

The retina contains relatively high steady state levels of glycine (65), which alone would be high enough to saturate the coagonist binding site of NMDARs (18). Also, during light responses, the receptive field properties of RGCs are fine-tuned via direct glycinergic inhibition from amacrine cells (66). Early studies measuring the effects of exogenously applied coagonist on RGC NMDAR currents in retinal slice preparations showed no evidence for augmentation and concluded that endogenous glycine must saturate the coagonist binding sites (67). However, later studies in intact retina showed that the addition of glycine or D-serine to the bathing medium potentiated NMDAR currents. In intact retina, RGCs were more sensitive to D-serine than glycine by several-fold (49), whereas these coagonists show comparable potency and efficacy in NMDAR expression systems (17). The apparent lower coagonist potency of glycine has been attributed to the glycine transporter 1 (GlyT1), which is expressed by Müller and amacrine cells in the inner plexiform layer (68). Indeed, the coagonist site becomes saturated during light responses if GlyT1 is blocked (69), suggesting GlyT1 prevents glycine from spilling into the synaptic cleft.

Initial studies found that D-serine and SR were present in astrocytes and Müller cells of the retina (49), but a more recent report found that SR mRNA may also be expressed by

amacrine, bipolar, and retinal ganglion cells (70). Irrespective of the cellular origins of D-serine, it appears to play a critical role in regulating RGC activity during light responses. Degradation of D-serine with bath applied DAO (49) or the highly specific bacterial D-serine degrading enzyme D-serine deaminase (50) eliminates NMDAR currents in RGCs, suggesting that D-serine is the major coagonist acting on RGC NMDARs.

The uptake of D-serine in isolated retinas is sodium dependent and blocked by cystine and threonine, suggesting that it is regulated by the ASCT type transporter and not the sodium independent transporter ASC-1 (71), which plays a role in cortical uptake of D-serine (35). Islolated Müller cells express both ASCT1 and ASCT2 transporters, but D-serine uptake is precluded by glutamine, suggesting ASCT2 is responsible for uptake in Müller cells (72). Adding ASCT substrate causes D-serine release from isolated Müller cells through heteroexchange (73). It is possible that the D-serine acting on RGC NMDAR coagonist sites is released through ASCT transporters.

There is evidence for coagonist release during synaptic activity in the retina. Diamond and colleagues used an $mGluR_6$ antagonist to pharmacologically activate ON bipolar cells and measured NMDAR currents in RGCs. To measure coagonist release, they saturated the glutamate binding site of NMDARs, removing the need for coincident glutamate release and preventing changes in NMDAR currents due to glutamate release. A substantial bipolar cell-evoked current persisted in the presence of saturating NMDA, while the addition of saturating D-serine prevented this current, suggesting it was due to coagonist release. It remains unclear if the coagonist released was glycine or D-serine and whether actual light stimulation evokes D-serine release (74).

Chapter 1

Serine racemase deletion abolishes light-evoked NMDA receptor currents in retinal ganglion cells

Introduction

NMDA receptor activation requires coincident binding of glutamate and a coagonist, either glycine or D-serine (15). It was known for some time that D-serine was capable of exciting neural tissue (11), but given the paucity of D-amino acids in eukaryotes, it was ruled out as an endogenous neurotransmitter. Later, it was discovered that D-serine is present in the brain (22), near postsynaptic sites abundant in NMDA receptors (29), and that reducing extracellular D-serine by perfusion with exogenously applied D-amino acid oxidase reduced NMDA receptor activity (51).

The discovery of the D-serine synthesizing enzyme, serine racemase (SR), proved seminal to understanding coagonist regulation in the nervous system (26). SR is a vitamin B_6-dependent enzyme which catalyzes the synthesis of D-serine from L-serine (75). Cortical regions high in D-serine also show marked SR expression (76). Mice lacking functional SR have diminished cortical D-serine, resulting in failed induction of long-term potentiation (77). Behaviorally, SR mutants have shown impairments in spatial memory (77) and recollection of event order (78).

SR was thought to be predominately expressed by astrocytes (76) possessing high levels of D-serine (79). Congruently, inhibiting SR activity in only a few astrocytes locally

impairs cortical LTP induction (52). However, there is evidence that SR is also expressed in neurons (30). A number of groups have shown that glutamatergic signaling can lead to downstream activation (46) or inhibition (43) of SR, consequently regulating the levels of extracellular D-serine. These findings gain functional relevance in light of the fact that the NMDAR coagonist site is not saturated at many CNS sites, including retina (80).

D-serine is present in the inner retina, where it serves as an endogenous coagonist of NMDA receptors (49). Although retinal ganglion cells (RGCs) receive substantial inhibitory input from glycinergic amacrine cells, degradation of D-serine with bath applied enzyme virtually eliminates retinal ganglion cell NMDAR currents (50). Also, application of a serine racemase inhibitor has been shown to reduce RGC NMDAR activity in salamander retina (81). SR is predominantly expressed in astrocytes and Müller cells of the adult retina (49) and AMPAR dependent D-serine release in whole-mount retinas requires glial cell function (82).

Little is known about the involvement of SR in inner retinal function or what consequences diminished D-serine throughout development might have on vision. In the present study, we use a transgenic mouse line with the SR gene deleted (SRKO) (77) and show that serine racemase synthesizes most of the retinal D-serine. Whole-cell recordings from SRKO RGCs revealed that NMDARs contribute very little to light-evoked responses, and SRKO RGCs display reduced NMDA/AMPA receptor ratio even after rescuing NMDAR currents with exogenous D-serine. Yet, SRKO mice displayed no apparent signs of visual impairment in behavioral testing.

Results

SRKO retinas have reduced D-serine

To measure the levels of retinal D-serine, we homogenized isolated retinas and used capillary electrophoresis to quantify the amino acid content, which were then normalized to total retinal protein. SRKO retinas displayed an 85% percent reduction in D-serine compared to wt (Fig 1B,C), whereas no difference in L-serine was detected (Fig 1C).

Figure 1

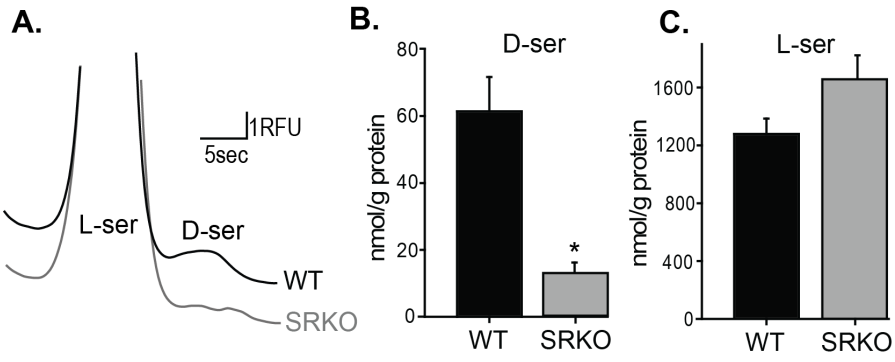

Figure 1. SRKO mice have reduced retinal D-serine. (A) Capillary electrophoresis electropherogram showing the separation of L-serine from D-serine in wt and SRKO retinal homogenates. **(B)** Total retinal D-serine normalized to protein content. SRKOs (n=5) have significantly less D-serine than wt (n=5). **(C)** No significant difference in L-serine was detected between SRKOs and wt retinas (n=5). *indicates $p<0.01$.

NMDARs contribute less to light-evoked impulses in SRKO RGCs

To determine the effects of diminished D-serine levels on retinal ganglion cell activity, we measured light-evoked synaptic currents and impulse activity using whole cell

recordings. Patch pipettes were loaded with fluorescent dye (Alexa 594) and RGCs were morphologically identified using fluorescence microscopy (Fig 2A). When injected with a series of hyperpolarizing currents, SRKO RGCs displayed no significant difference in input resistance compared to wt, suggesting there was no difference in the size or passive properties of RGCs between the genotypes (Fig 2B). We next tested whether NMDAR contribution to RGC light-evoked action potentials was altered in SRKO retinas. Current clamp recordings were made in a physiological concentration of Mg^{2+} (1 mM) and the number of light-evoked spikes for ON responses was averaged over repeated stimuli. The addition of the NMDAR antagonist AP7 significantly reduced light-evoked spiking in wt (39.1±8.5 % of control spiking, $p<0.0001$) but not in SRKOs (91.2±9.1% control, $p=0.19$), with only 2 out of 6 showing a reduction greater than 5% (Fig 2C,D). The effects of AP7 were markedly greater in wt than SRKO cells ($p<0.01$). Although D-serine increased RGC light-evoked activity significantly in both SRKO and wt (wt=117.1±6.6%, $p<0.01$; SRKO=156.9±8.0%, $p<0.01$; Fig 2C,D), this effect was greater in SRKOs than in wt RGCs ($p<0.01$; Fig 2D).

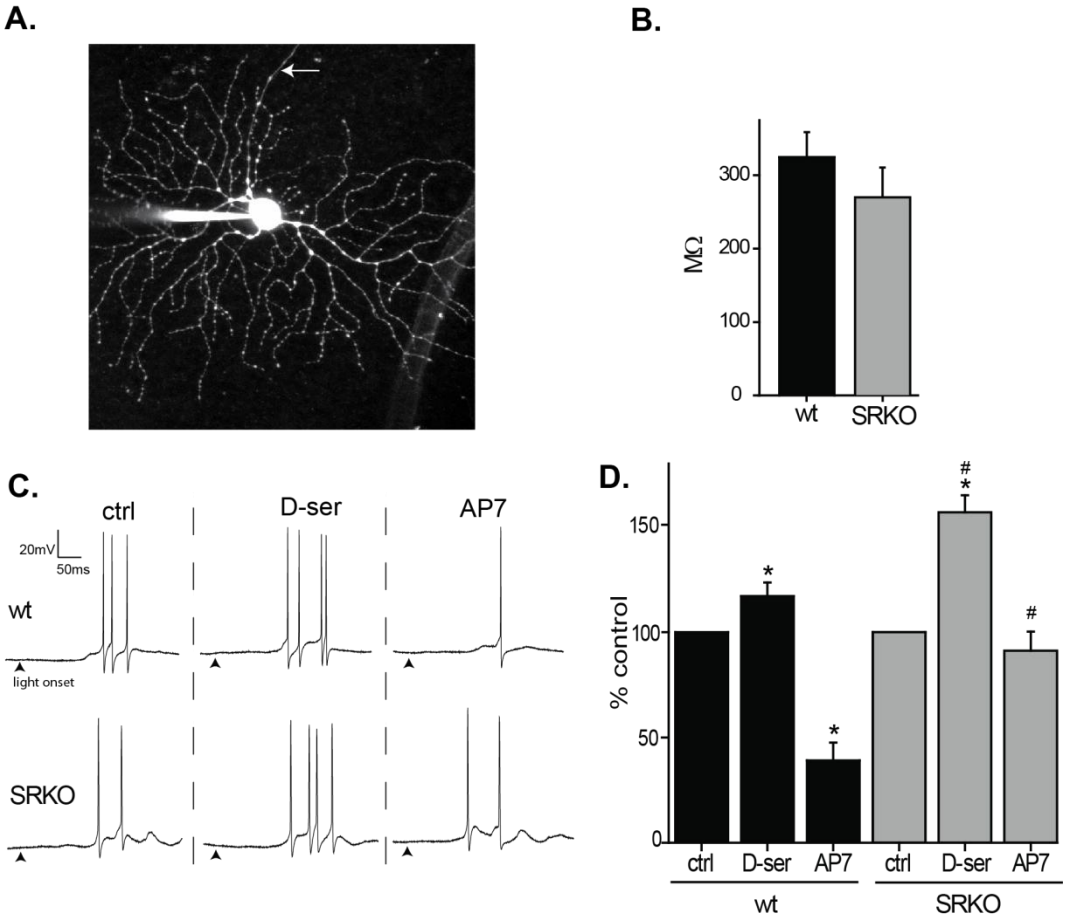

Figure 2. SRKO RGC NMDARs have less coagonist occupancy and contribute less to light-evoked spiking. (A) A flattened multi-photon Z-stack image of a RGC patch-filled with Alexa 594. Following whole-cell recordings, the identity of RGCs was confirmed by the presence of an axon (arrow). **(B)** The input resistance, calculated by a series of hyperpolarizing current injections in the passive range of conductance, was similar between wt (n=27) and SRKO RGCs (n=27). **(C)** ON responses, recorded in current clamp (0 pA holding), from wt and SRKO RGCs showing the effects of bath applied D-ser (100 µM) and AP7 (50 µM) on light-evoked (600ms, 600 lux) action potentials. Traces shown are from the same cell in each animal. Recordings were made under physiological Mg^{2+} (1 mM). (Arrowheads, light stimulus onset). **(D)** D-serine potentiated ON response spiking in both wt (n=6) and SRKO (n=6) RGCs, but this effect was greater in SRKOs. AP7 attenuated RGC spiking in wt but not SRKOs. For a given cell, spikes were averaged over a series of 8 repeated exposures to light. The responses under pharmacological conditions were normalized to those under control conditions (% control). *denotes $p<0.01$ compared to control within genotype; # denotes $p<0.01$ between genotypes for the same condition).

SRKO RGCs lack light-evoked NMDAR currents and have lower NMDA/AMPA receptor ratios

To confirm that NMDAR currents underlie the spiking differences observed in SRKOs, voltage clamp recordings of RGCs were performed at the calculated chloride reversal potential (-65mV). No Mg^{2+} was added to the superfusate to maximize NMDAR current. In addition, TTX was added to prevent cell spiking and strychnine was added to minimize inhibitory currents. Under these conditions, AP7 significantly reduced the peak current amplitude of light-evoked ON responses in wt RGCs (33.3±5.9% control, $p<0.001$; Fig 3A). SRKO ON responses were much less sensitive to NMDAR antagonist (88.8±12.5% control, $p=0.20$) compared to wt ($p<0.001$). Bath applied D-serine significantly potentiated ON responses in both wt and SRKOs (wt=122.6± 4.4% control, $p<0.01$; SRKO=174.4± 18.6% control, $p<0.01$) but to a greater extent in SRKOs ($p<0.01$, compared to wt increase; Fig 3A). A similar result was observed for OFF responses (Fig 3B), where AP7 attenuated wt but not SRKO inward currents (wt=36.3±6.1% control, $p<0.001$; SRKO=84.7±11.6% control, $p=0.12$; $p<0.01$ between genotypes), while the potentiation by D-serine was significantly greater in SRKOs (wt=126.7±15.2% control, $p<0.05$; SRKO=171.0±8.0% control, $p<0.001$; $p<0.05$ between genotypes). The pooled ON and OFF light-evoked charge transfer (Fig 3C) was comparable to the peak amplitude results (D-serine: wt=143.6± 14.0%, $p<0.001$; SRKO=190.6± 17.7%, $p<0.001$; $p<0.05$ between genotypes) (AP7: wt=44.3± 9.4%, $p<0.001$; SRKO=93.7±17.7%, $p=0.3$, $p<0.001$ between genotypes). Collectively, these findings

suggest that SRKOs have less NMDAR activity, at least in part because they are deficient in coagonist.

Blocking AMPARs and NMDARs with NBQX and AP7, respectively, nearly abolished all inward currents (Fig 3D). Because RGC excitatory inputs are predominantly AMPA and NMDAR mediated, we were able to derive NMDA/AMPA response ratios (see methods). SRKO retinas had a significantly lower NMDA/AMPA ratio in the presence of saturating coagonist (wt=3.7± 0.8; SRKO=1.3± 0.3; $p<0.05$) (Fig 3E), implying that a reduction in D-serine availability might alter the expression of synaptic glutamate receptors.

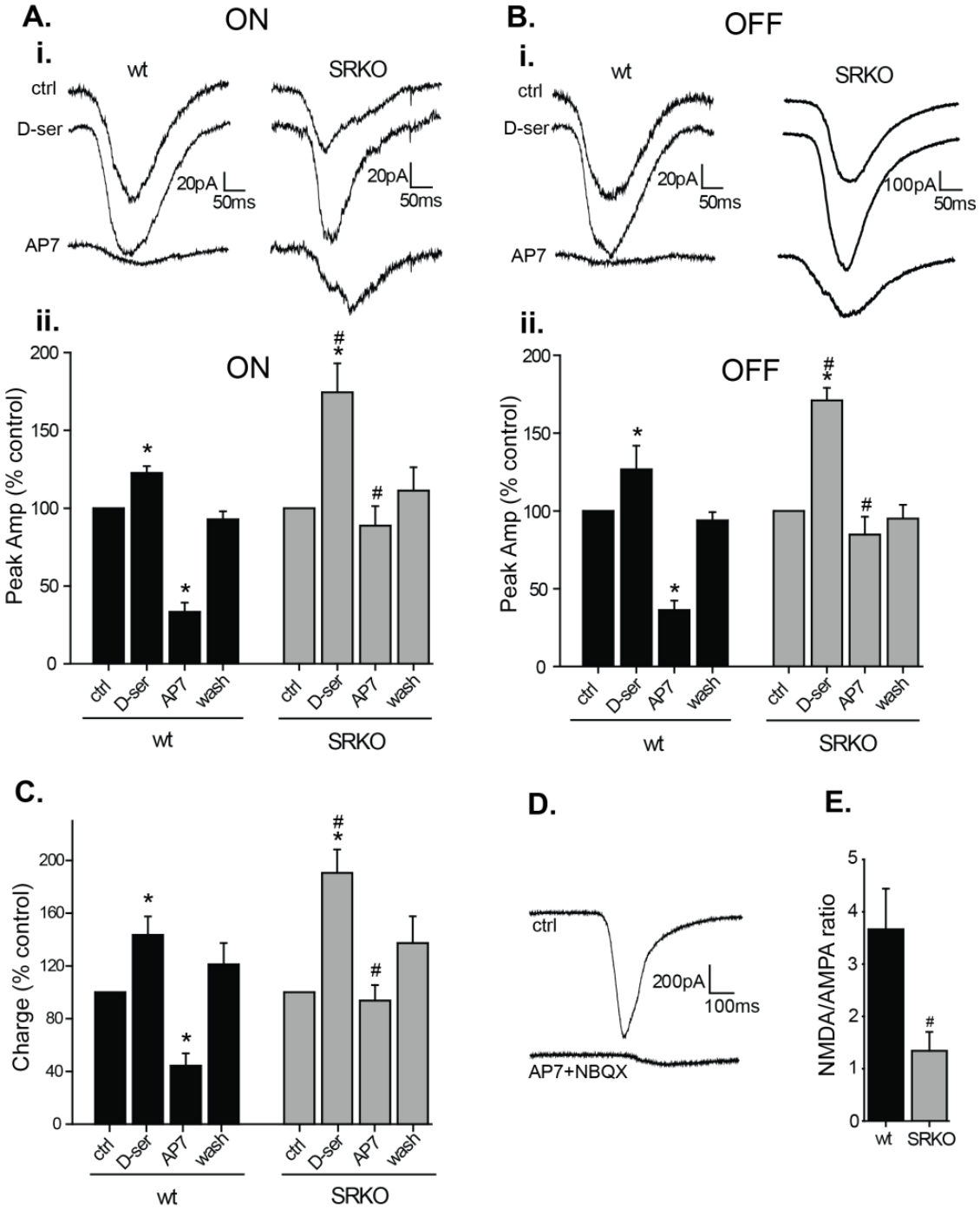

Figure 3. SRKO RGCs have less light-evoked NMDAR currents and lower NMDA/AMPA receptor ratios. Whole-cell voltage clamp recordings from retinal ganglion cells held at -65 mV, in 1 µM TTX, 10 µM strychnine and 0 Mg^{2+}. **(A) i.** Light-evoked inward currents from the ON

responses of wt and SRKO mice (averaged over 8 sweeps) and the effects of bath applied D-ser and AP7. **ii.** ON response peak inward currents were increased by D-serine in both wt (n=9) and SRKO mice (n=9), but to a significantly greater extent in SRKOs. Blocking NMDAR with AP7 reduced currents more in wt than in SRKO RGCs. AP7 washout (wash) **(B) i.** OFF response inward currents compared between wt and SRKOs. **ii.** Potentiation by D-serine of peak inward currents in OFF responses was significantly greater in SRKOs (n=6) than in wt (n=5). AP7 reduced inward currents in wt but not SRKO RGCs. **(C)** The ON and OFF pooled light-evoked charge was similar to peak amplitude. **(D)** Raw trace showing that a combination of AMPAR antagonist (10 μM NBQX) and AP7 blocks nearly all light-evoked inward currents. **(E)** The light-evoked NMDA/AMPA current ratio (see methods) was significantly lower in SRKOs (n=15) than in wt (n=14); data from ON and OFF responses pooled. All data are normalized to control value within genotype (% control). *denotes $p<0.05$ compared to control within genotype; #$p<0.01$ between genotypes for the same condition).

SRKO mice have normal low level vision

The optokinetic reflex (OKR) stabilizes moving images on the retina by moving the eyes relative to the head or the head relative to the body. The OKR has been used as a reliable behavioral test of low level visual function (83). We utilized OKR head movements to probe for potential differences in visual function in the SRKO mice. A drum patterned with alternating white and black bars was rotated around a fixed platform where the mouse was stationed. The number of head tracking movements elicited over a given time interval was used to gauge visual sensitivity (Fig 4A). Visual acuity in wt and SRKO

Figure 4

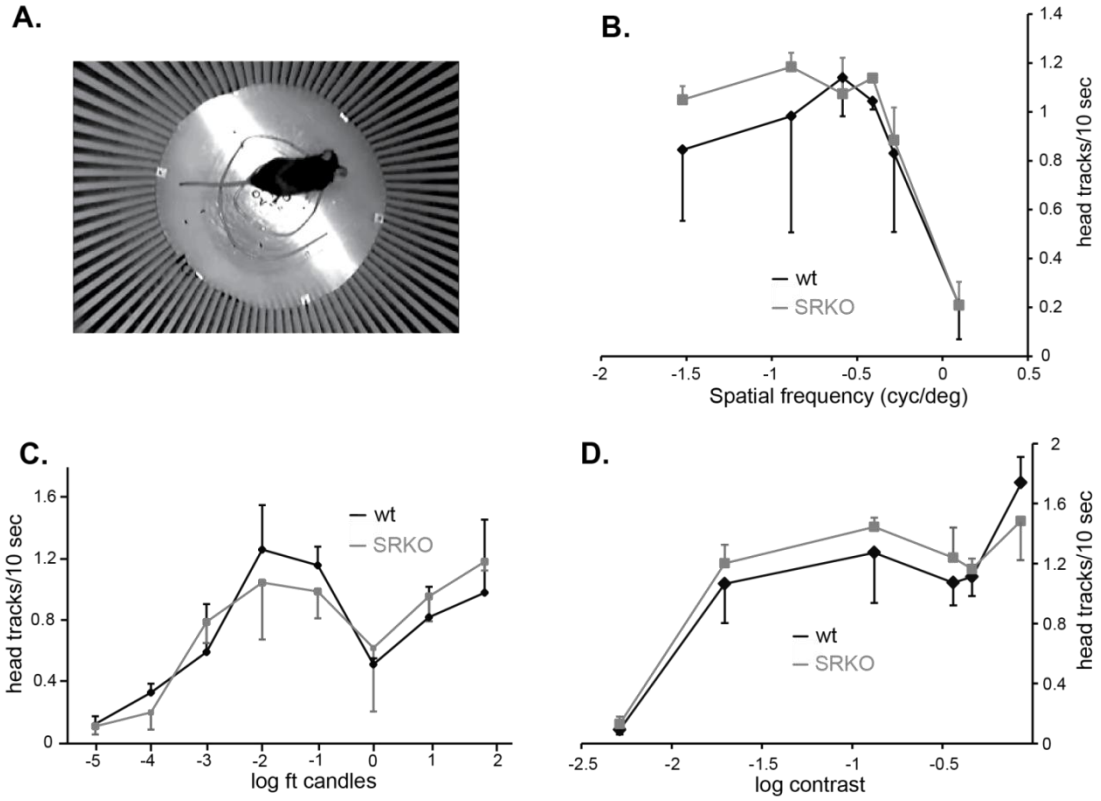

Figure 4. SRKO mice have normal vision. (A) Overhead view of a mouse in the optokinetic reflex (OKR) testing chamber. Mice stood on a stationary elevated platform while a pattern of alternating white and black stripes rotated around them and the OKR was measured as the number of tracking head turns over a given time interval. **(B)** Under photopic lighting (40 ft candles), the number of black-white alterations per degree of visual field (cycles/degree) was altered to measure visual acuity. The OKR elicited was similar between wt and SRKO mice over a wide range of spatial frequencies. **(C)** Using a 0.13 cycles/degree visual stimulus, varying overhead light intensities (foot candles) produced a similar OKR in wt and SRKOs. **(D)** The OKR in SRKOs was similar to controls when tested over a range of Michelson contrast values for a 0.13 cycles/degree pattern with 40 ft candles of overhead light.

mice was compared by varying the spatial frequency of the grating on the rotating drum and measuring the OKR under photopic lighting conditions (40 ft candles). There appeared to be no difference in visual acuity between wt and SRKO mice within the range of stimuli tested (Fig 4B). Using the optimal spatial grating measured from the visual acuity experiments (0.13 cycles per degree), we tested to see if there was any difference in the OKR under varying light intensities. SRKO mice appeared to have normal OKRs with brightness values ranging from photopic to scotopic vision (Fig 4C). Additionally, we tested contrast sensitivity under the photopic lighting condition over a 100 fold range of Michelson contrast values, but no apparent differences were detected between the genotypes. (Fig 4D).

Discussion

These studies demonstrate that serine racemase is essential for providing D-serine for proper retinal ganglion cell NMDAR activity. SRKO mice displayed a marked reduction in retinal D-serine levels and an absence of NMDAR drive in light-evoked currents and impulse activity, indicating that D-serine, and not glycine, is the predominant endogenous co-agonist of RGC NMDARs. A substantial portion of the NMDA receptor response could be rescued in the SRKO mice by adding exogenous coagonist, but a difference remained in the NMDA/AMPA receptor ratios in the SRKO mice. Surprisingly, SRKO mice had no apparent deficit in basic visual function when tested behaviorally.

SRKO mice displayed an 85% reduction in retinal tissue D-serine compared to wt controls, similar to the 90% reduction in D-serine found in cortex (77). However, the total level of D-serine that we measured in the wt retina was substantially lower than that found in the cortex. This, in part, might be a consequence of elevated expression of the D-serine degrading enzyme D-amino acid oxidase (DAO) in retina compared to cortex. Mutant mice lacking functional DAO have increased retinal D-serine (82), while having an even greater increase of D-serine in cerebellum but very little change in the cortex (84). However, DAO cannot solely account for this difference because D-serine levels in the retinas of DAO mutant mice are still much lower than in cortex. Perhaps serine racemase expression levels are lower in the retina than in cortex but the D-serine it synthesizes is precisely distributed at RGC synapses or less stringently regulated by transporters (71;72).

Both ON and OFF light-evoked currents were less sensitive to NMDAR antagonist in SRKO RGCs, suggesting NMDARs contribute less to SRKO RGC currents. This apparent difference could not have been due to a lack of synaptic NMDARs in the SRKOs, because an NMDAR component could be rescued in these animals by adding D-serine. Although wt RGC NMDAR currents were still significantly increased by coagonist, the magnitude of potentiation in SRKOs was greater. A major contributor to the smaller SRKO NMDAR currents must therefore be reduced occupancy of coagonist sites.

Glycine, another NMDAR coagonist, is found in high concentrations in the retina where it serves as an inhibitory neurotransmitter. Measurements of extracellular amino acids in

the retina have shown that glycine levels are much higher than those of D-serine (82), yet the findings here demonstrate that D-serine is the major coagonist of synaptic RGC NMDARs. Previously, we demonstrated that mice heterozygous for a null mutation of the glycine transporter GlyT1 have saturated RGC NMDARs (85). Thus, GlyT1 appears to be responsible for maintaining low concentrations of glycine in the synaptic cleft in the retina and permitting D-serine to serve as the predominant coagonist.

The light-evoked synaptic responses of SRKO RGCs displayed reduced NMDA/AMPA receptor ratios, raising the possibility that D-serine is involved in determining the balance of ionotropic glutamate receptors at excitatory synapses during development. At the initial phase of synapse formation in hippocampus, neurons only express NMDARs, which must be activated for AMPARs to be delivered to synapses (86), and adult SRKO mice have shown elevated expression of NMDAR subunits in the postsynaptic density of hippocampal neurons (87). In the retina, D-serine is present in Müller cells prior to the expression of synaptic proteins (88) where it might serve a role in synaptogenesis. If D-serine is important in driving AMPAR insertion in retinal synapses, then one might expect to find an increase in the NMDA/AMPA ratio, the opposite of what we observed. However, the retina differs from cortex in that neurons initially possess AMPARs during early development and expresses NMDARs later on (89). Perhaps D-serine is required to activate newly inserted NMDARs, which in turn regulate the number of AMPARs at retinal synapses.

SRKO mice had a significantly reduced NMDAR component in light-evoked RGC impulse activity, yet behavioral tests of visual function demonstrated that SRKOs had

normal photopic visual acuity, scotopic detection, and contrast sensitivity. SRKO mice may undergo some form of developmental compensation to adequately relay visual input, such as increased expression of AMPARs, which would also account for the lower NMDA/AMPA ratios observed in these studies. An alternative explanation is that RGC NMDARs and their interrelation with D-serine might serve a more specialized role in retinal processing, features which were not tested in this study.

Methods

Isolation of retinas

Experiments were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Adult C57 B1 mice were killed by an overdose of Nembutal (0.1 mL of 50 mg/mL, i.p.) followed by pneumo-thorax. Eyes were enucleated and placed in bicarbonate Ringer's solution (111 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 32 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 15 mM dextrose; bubbled with 95% O_2, 5% CO_2) for the surgical isolation of the retina.

Capillary Electrophoresis

We used capillary electrophoresis (CE) to measure D-serine levels in retinal homogenates. Both retinas from an animal were pooled and homogenized in 0.6 M perchloric acid (PCA) using a sonicator probe. 35 µl of 2M KOH was added to neutralize the acid. The mixture was spun down in a tabletop centrifuge and the supernatant was

removed. The remaining pellet was re-suspended in 2 M NaOH for protein determination using a Pierce (Rockford, Illinois) bicinchoninic acid assay. Amino acids in the supernatant were fluorescently derivatized at 60^{o}C for 15 min with 0.7 mg/mL 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 in a (2-Hydroxypropyl)-β-cyclodextrin buffer at 15 kV (70 μA). Fluorescent signals were detected by a photomultiplier tube and digitally plotted as fluorescence versus time (electropherogram). Mass of D-serine was determined by comparing samples to known standards. Peak integration was performed using 32 Karat software (Beckman-Coulter).

Whole-cell recordings

Following vitreous removal, isolated retinas were treated with an enzyme solution containing collagenase (120 units/ml) and hyaluronidase (465 units/ml). The edges of the retina were maltese-cross cut and flattened over nitrocellulose paper containing a hole in it for imaging. The retina preparation was placed over a glass cover slip of a perfusion chamber and secured using a platinum ring crossed with nylon threading. The chamber was perfused continuously with bicarbonate Ringer's solution bubbled with 95% O_2, 5% CO_2 at a flow rate of ~2 ml/minute. Patch pipettes (3-8MΩ) contained, in mM, 128 KCH_3SO_4, 5 $NaCH_3SO_4$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, 1 Glutathione, 2 ATP-Mg^{2+}, 0.2 GTP (3Na) and Alexa 594. Ganglion cell bodies were identified using IR-DIC prior to patching (Fig 1A) and, following recordings, the presence of an axon was confirmed with multi-photon or epifluorescence imaging (800 nm) (Fig 1B).

Current-clamp recordings were made in bicarbonate Ringer's containing 1 mM Mg^{2+}, while voltage-clamp recordings (-65 mV holding) were made in Mg^{2+} free Ringer's solution which also contained 1 µM TTX and 10 µM strychnine. Data were acquired using an Axoclamp 700A amplifier with a 10-kHz low-pass Bessel filter at a sampling frequency of 10 kHz, digitized with a Digidata 1320, and recorded in pClamp 9.0 (all Molecular Devices). All experiments were performed at room temperature.

Voltage clamp sweeps were averaged (4-8) for a given pharmacological condition and the resulting trace was used to determine peak amplitude and area. NMDA/AMPA ratios were calculated by subtracting the residual current after adding AP7 from the control current (NMDA), divided by the AP7 residual current (AMPA). In current clamp, spike counts were made by averaging the number of spikes generated over 8 sweeps for the On response. These values were normalized to the response evoked in the control condition prior to statistical analysis.

For each sweep retinas were exposed to a single flash of light (~100 µm diameter spot, 600 lux, 600 ms duration, 10 sec inter-stimulus interval) generated using a digital projector controlled by custom software.

Optomotor response

Methods were adopted from (90). Briefly, paper drums (30 cm diameter x 40 cm high) were constructed with printed alternating black and white bars. Mice were centered in the drum on a stationary platform while the drum rotated at a constant rate of two rotations per minute. To control for directional bias, the drum was rotated 2 minutes

clockwise, followed by a 30 sec period of no rotation, and then 2 minutes of counter-clockwise rotation. The light intensities were adjusted to the desired intensity using a neutral density filters before each experiment. For testing in scotopic conditions, mice were first dark adapted for 10 minutes and night vision camera was used. Michelson contrast measurements were made by measuring the luminance (candelas/m^2) of the white and black bars with a Minolta CS-100 meter. For all data, two independent observers, blind to the conditions, analyzed each video by counting the number of head tracks (defined as head motions matching the angular velocity of the drum) over the testing period. Time when the mice were grooming was subtracted in analysis.

Statistics

All comparisons between groups were made using a Student's one-tailed t-test. Z-tests were used when the null hypothesis stated no change from a fixed value. All data are expressed as mean ± standard error, significance defined as $p<0.05$.

Chapter 2

AMPA receptor dependent light-evoked D-serine release acts on retinal ganglion cell NMDA receptors

Introduction

NMDARs serve as molecular coincidence detectors, requiring sufficient depolarization for the removal of Mg^{2+}, which blocks the ion channel, and glutamate binding to the GluN2 subunit. Early studies showed that NMDARs are also modulated by coagonist (15) and later it was discovered that coagonist binding to the GluN1 subunit was necessary for ion channel gating (10;38). Numerous studies have since demonstrated that the coagonist site is unsaturated, including work in hippocampal slices (91), prefrontal cortex *in vivo* (92), and the intact retina (49), lending the possibility that changes in coagonist levels determine the number of NMDARs available during glutamatergic transmission.

It was originally assumed that glycine was the endogenous coagonist acting on NMDARs, because of its abundance in nervous tissue. However, D-serine is present in the brain (22) and bath application of D-serine degrading enzymes reduces NMDAR currents (48;49). D-serine and its synthesizing enzyme serine racemase are found in both glia (29) and neurons (30). Biochemical measurements of extracellular D-serine from glial (29;55) and neuronal (30) cultures have shown that AMPA receptor (AMPAR) stimulation evokes D-serine release. D-serine levels may increase following AMPAR stimulation by activating serine racemase via the Glutmate Receptor Interacting Protein

(GRIP) (46). Though AMPAR stimulation is sufficient for D-serine release, it is unknown if this mechanism is utilized during synaptic transmission. There is electrophysiological evidence for coagonist release acting on postsynaptic NMDARs during evoked potentials in hippocampus (60), cerebellar slices (61), spinal cord (59), and retina (74), but it is unclear if glycine or D-serine is being released.

In the retina, D-serine is present in Muller cells and astrocytes and is essential for the activation of RGC NMDARs (49;50). AMPAR stimulation causes release of D-serine from intact retinas (82), but it is unknown if this mode of release occurs during light responses. Pharmacological activation of ON bipolar cells evokes coagonist release capable of acting on RGC NMDARs (74), but the mechanism of this release and whether or not it happens during light stimulation is unestablished. Furthermore, it is unclear if the coagonist released in these studies was glycine or D-serine.

In this study we detected light-evoked coagonist release in the isolated retina which acts on RGC NMDARs. To determine if the released coagonist was D-serine, we utilized a serine racemase knockout (SRKO) with reduced retinal D-serine (93), and found that these mice display markedly diminished coagonist release. We also found that the coagonist reaching RGC NMDARs during light responses or following the direct application of NMDA was reduced by AMPAR antagonist, although this effect was substantially greater for light-evoked responses. NMDA-evoked currents were surprisingly similar in SRKO mice, which have little to no coagonist contribution to light responses (93). Based on these findings, we present a model for coagonist regulation in the retina where AMPAR dependent light-evoked D-serine release serves as coagonist at

synaptic NMDARs and static or saturating glycine levels occupy extrasynaptic NMDARs.

Methods

Retina preparation: Experiments were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Adult C57 B1 mice were killed by an overdose of Nembutal (0.1 mL of 50 mg/mL, i.p.) followed by pneumo-thorax. Eyes were enucleated and placed in bicarbonate Ringer's solution (111 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 32 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 15 mM dextrose; bubbled with 95% O_2, 5% CO_2) for the surgical isolation of the retina. Following vitreous removal, retinas were treated with an enzyme solution containing collagenase (120 units/ml) and hyaluronidase (465 units/ml). Retinas were flattened over a glass cover slip of a perfusion chamber and secured using a platinum ring crossed with nylon threading. The chamber was perfused continuously with bicarbonate Ringer's solution bubbled with 95% O_2, 5% CO_2 at a flow rate of ~2 ml/minute. RGCs were identified using IR-DIC prior to patching and, following recordings, by fluorescently imaging cells patch loaded with alexa 594 (0.1 mg/ml).

Light-evoked inward currents: Patch pipettes (3-8MΩ) contained, in mM, 128 KCH_3SO_4, 5 $NaCH_3SO_4$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, 1 Glutathione, 2 ATP-Mg^{2+}, and 0.2 GTP (3Na). RGCs were voltage clamped at the estimated chloride reversal potential (-65 mV). The bathing medium contained 0 Mg^{2+}, 10 uM strychnine, and 1 uM TTX.

For each recorded sweep, retinas were exposed to a single flash of light (~100 μm diameter spot, 600 lux, 600 ms duration, 10 sec inter-stimulus interval) generated using a digital projector controlled by custom software (Vision Egg). A trace averaged over eight repeated responses was used for data analysis.

NMDA picospritz: Whole cell-recording were made at -65 mV. Conditions were identical to those for measuring light-evoked inward currents, except the bathing solution also contained TPMPA (50 uM) and picrotoxinin (50 uM). 3-5 MΩ pipettes were loaded with oxygenated (95% O_2, 5% CO_2) bicarbonate Ringer's solution containing 10mM NMDA. RGCs were identified as ON, OFF, or ON-OFF depending on their response to flashes of light. The puff-pipette was positioned in the inner-plexiform layer above the patched cell, and ejections were made at 10 psi every 45 seconds. Ejection times were adjusted (40-80 ms) to obtain the peak maximum inward current. All other drugs were applied by switching the perfusion. The response to two repeated puffs was averaged for data analysis.

Measuring light-evoked coagonist release: Outward currents were measured by clamping RGCs at +40 mV. The light stimulation protocol was the same as for measuring inward currents. The extracellular solution was identical to that for recording inward currents with the addition of Mg^{2+} (1mM), TPMPA (50 uM), and picrotoxinin (50 uM). Recordings were made using 3-7 MΩ pipettes containing the standard intracellular solution, except KCH_3SO_4 was substituted with 108 mM $CsCH_3SO_4$ and 20 mM TEA-Cl. The light-evoked outward current was measured after which 100 uM NMDA was adding to the bathing media, evoking a baseline increase in outward DC. Once the baseline

stabilized, light responses were recorded. This was followed by adding 100 uM D-serine plus 100 uM NMDA to the bathing medium. Once the DC response stabilized, the light-evoked response was recorded again. The average response in NMDA and D-serine was subtracted from the average response in NMDA alone, to derive a resultant trace that represented the time course and magnitude of coagonist release. For data analysis, the integrated peak area (charge) of the resultant trace was normalized to the initial light-evoked current. Un-normalized resultant traces were averaged to generate Fig 3B. All traces used for subtracting were averages of eight light responses in the specified condition. To calculate the percent potentiation of bath applied D-serine, the baseline shift when switching from NMDA to NMDA plus D-serine was normalized to the baseline shift going from control to NMDA.

Statistics: All comparisons between groups were made using Student's one-tailed t-test. Z-tests were used when the null hypothesis stated no change from a fixed value. All data are expressed as mean ± standard error, significance defined as $p<0.05$.

Results

Blocking AMPARs reduces coagonist availability during light-evoked RGC responses

To determine if AMPARs influence coagonist levels during light responses, we first measured excitatory ON responses from RGCs of isolated retinas and determined their sensitivity to NBQX. OFF responses were excluded from analysis because OFF bipolar cell excitation is driven in part by AMPARs, whereas ON bipolar cell activity is mediated

by $mGluR_6$. RGCs were clamped at the calculated chloride reversal potential and light-evoked inward currents were measured in the presence of TTX and strychnine, with Mg^{2+} absent to optimize NMDAR currents. ON responses were substantially reduced by 10 uM NBQX (25.5±5.9% of control light response, n=14; $p<0.01$) (Fig 1A, B). Reports have shown that AMPARs contribute no more than 30-50% to RGC light-evoked currents (93). To test if the exaggerated block of excitatory RGC currents by NBQX was due to reduced coagonist, we applied D-serine (100 uM) to the bathing medium, which rescued light-evoked ON responses (125.7±19.67% ctrl, n=6; $p<0.005$ for NBQX vs. NBQX+D-serine) (Fig 1B, C). The rescued currents were blocked by the NMDAR antagonist AP7 (11.3±3.0% ctrl; $p<0.005$) (Fig 1B, C), showing that D-serine was acting through NMDARs. In control conditions bath applied D-serine increased light responses by 22.8±12.8% (n=11; $p<0.05$) (Fig 1A, D), but in the continuous presence of NBQX the potentiation was substantially greater (678.0±204.0%, $p<0.05$ comparing control and NBQX) (Fig 1B, D), indicating that NBQX reduced coagonist occupancy of synaptic NMDARs during light responses. For simplicity, we refer to the RGC NMDARs active during light responses as "synaptic", although there is evidence that perisynaptic receptors are also activated (63).

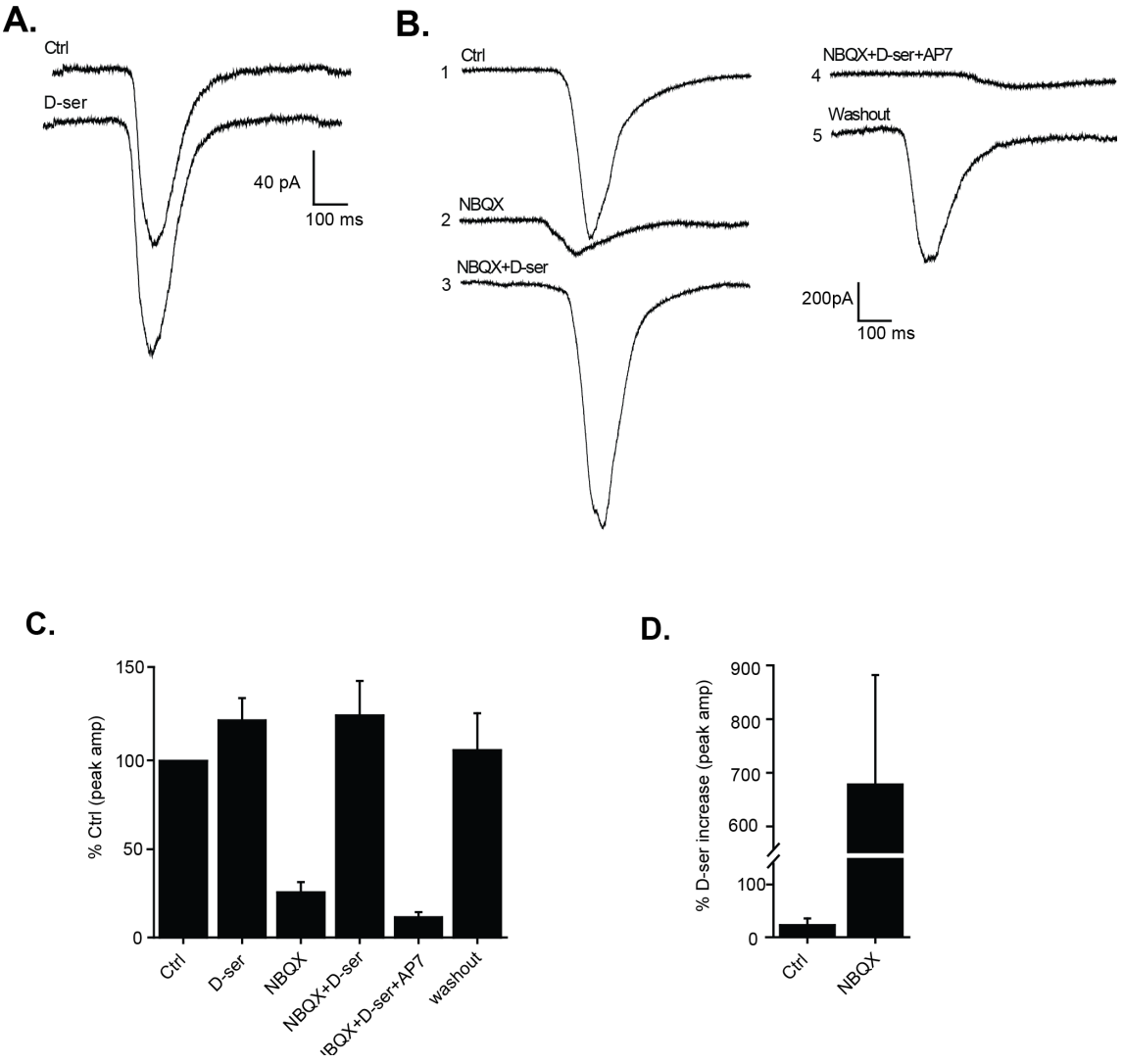

Figure 1: Voltage clamp recordings of RGC responses to the onset of light. Cells were held at -65 mV and recordings were made in 0 Mg^{2+}, 1uM TTX, and 10 uM strychnine. Light stimulation began at the start of traces and lasted the entire duration. **A.)** The addition of 100 uM D-serine potentiated ON responses. **B.)** 10 uM NBQX diminished ON responses and this current was rescued by D-serine. Rescued currents were abolished by 50 uM AP7. Inward currents returned to control values following washout of NBQX, D-serine, and AP7. Numbers to left of trace indicate the order of drug application. **C.)** Summary of the pharmacological series shown in B, expressed as the peak light-evoked current amplitude normalized to control values (%Ctrl). **D.)** When NBQX was in the bathing medium, the percent increase in the ON response by D-serine was significantly larger than in control conditions.

RGC extrasynaptic NMDARs are less dependent on AMPARs than synaptic NMDARs

It was unclear if AMPARs regulate the tonic release of coagonist, resulting in a steady background of coagonist, or if their phasic activation during light stimulation caused coagonist release. We first tested whether AMPARs regulated tonic coagonist availability in the absence of light by measuring NMDAR currents from RGCs in response to picospritzed NMDA. Puff-evoked currents were abolished by bath applying the NMDAR antagonist AP7 (Fig 2A), confirming that the currents were generated by NMDARs. No significant decrease in puff-evoked NMDAR currents was observed when NBQX was bath applied to the retina (ctrl=-262.6±41.8 pA, n=11; NBQX=-271.6±44.7 pA, n=11; p=0.89) (Fig 2B, C). These findings indicate that the effects of NBQX on puff-evoked NMDAR currents were not as pronounced as that observed for light-evoked currents. This leaves two possible explanations involving coagonist regulation; either the AMPARs involved in providing synaptic NMDARs with coagonist during light responses are not active in basal conditions, or NBQX reduces synaptic coagonist levels but this effect is masked in NMDA puff-evoked responses by the activation of extrasynaptic NMDARs that do not depend on AMPARs for coagonist.

It was previously shown that the synaptic RGC NMDARs involved in light-evoked responses depend on the coagonist D-serine (50). However, there are also NMDARs present on RGC dendrites distant from synaptic sites (63) and on cell bodies (94). It is likely that our puff-applied NMDA also recruits these extrasynaptic NMDARs, which may be activated by different source of coagonist, such as ambient glycine. To determine

the contribution of D-serine to ambient coagonist levels, we measured puff-evoked NMDAR current in serine racemase knockout mice (SRKO). These mice display a ~85% reduction in retinal D-serine and subsequently have no NMDAR contribution to light-evoked responses (93). No difference ($p=0.47$) was detected in puff-evoked inward currents between wt (-326.7±20.7 pA, n=28) and SRKOs (-316.5±32.8 pA, n=25) when sampling a mix of ON, OFF, and ON/OFF cells (Fig 2D). When we compared different RGC types separately, a marginal, but significant, decrease was only detected in the ON RGCs of SRKOs (wt=-322.8±34.5 pA, n=11; SRKO= -212.7±32.5 pA, n=7, $p<0.05$). This finding implies that, unlike synaptic NMDARs, extrasynaptic RGC NMDARs are primarily occupied by a coagonist other than D-serine, most likely glycine.

To more closely examine the effects of NBQX on coagonist availability during puff-evoked NMDAR currents, we measured the potentiating effects of bath applied D-serine on these responses. In control conditions, D-serine significantly potentiated NMDA currents by 11.5±3.4% (n=13, $p<0.005$)(fig 2E, F). In the presence of NBQX, D-serine potentiated NMDA-evoked currents significantly more than in control conditions (NBQX=24.9±4.0% increase, n=11; $p<0.01$ between ctrl and NBQX), although this effect was still much lower than the potentiation of light-evoked currents in NBQX. SRKOs were more potentiated by D-serine than wts in control conditions (SRKO=28.27±7.4% increase; $p<0.05$ between genotypes). However, unlike wt, coagonist potentiation in SRKOs was not increased in the presence of NBQX (22.14±5.5% increase; $p=0.26$ compared to control) (fig 2E, F), suggesting that NBQX reduces ambient D-serine.

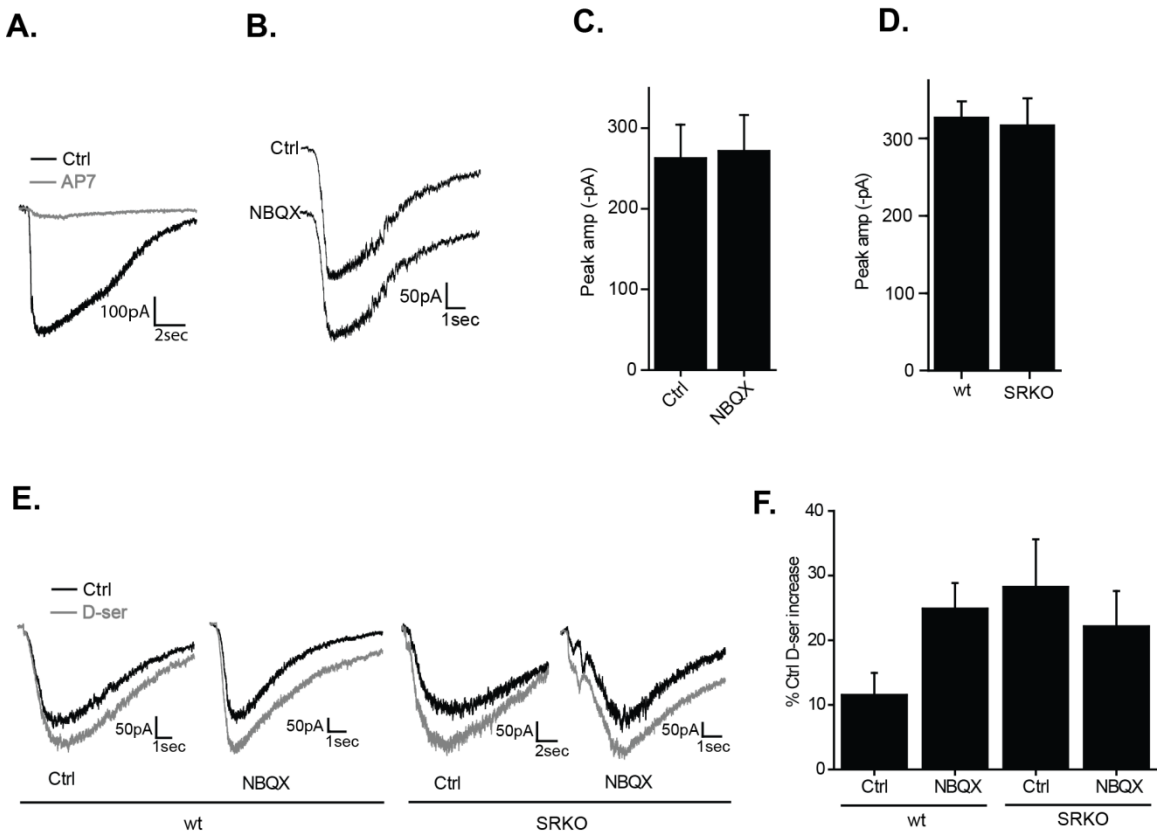

Figure 2: Inward currents evoked by picrospritzing 10 mM NMDA (40-80 ms duration) onto RGCs. Cells were held at -65 mV and recordings were made in 0 Mg^{2+}, 1 uM TTX, 10 uM strychnine, 50 uM TPMPA, and 50 uM picrotoxinin. Puffs were initiated 300 ms into traces. **A.)** Raw trace showing that puff-evoked inward currents are blocked by bath applied AP7. **B.)** Bath applied NBQX did not alter puff-evoked NMDAR currents. **C.)** There was no significant decrease in the average puff-evoked peak inward current by NBQX. D.) No significant difference in the average puff-evoked peak inward current was detected between wt and SRKOs. **E.)** Raw traces showing the potentiation of NMDAR currents by D-serine in wt and SRKO RGCs, with or without NBQX in the bathing medium. **F.)** Summary of E. In wt RGCs, bath applied D-serine significantly potentiated NMDA currents in ctrl conditions, but in the presence NBQX this potentiation was larger. In SRKO RGCs, D-serine potentiated NMDA currents greater than in wt RGCs. Unlike wt, there was no further potentiation by D-serine in the presence of NBQX.

AMPAR dependent light-evoked D-serine release

It was unclear if the effect of NBQX on light-evoked responses was reducing steady-state ambient levels of coagonist, or preventing light-evoked coagonist release. Previous studies have shown that AMPAR activation can evoke D-serine release from the isolated retina (82), although it is unclear if this mechanism of coagonist release occurs during light stimulation. To test this possibility, we utilized a method previously established for measuring coagonist release (74). Retinas were bathed in a cocktail of inhibitory antagonists (strychnine, picrotoxinin, and TPMPA) and light-evoked outward currents were measured from RGCs voltage clamped at +40 mV (Fig 3A). To mask the changes in NMDAR currents caused by glutamate release and to assure that any released coagonist would activate NMDARs, we saturated the glutamate binding site of NMDARs by bath applying 100 uM NMDA. The bathing solution also contained 1mM Mg^{2+} to reduce the global effects of NMDA perfusion. The addition of NMDA caused an increase in the baseline outward current recorded from RGCs (Fig 3A). With the glutamate binding site saturated, a residual outward light-evoked current persisted. To determine if any residual current was due to coagonist release, we saturated the NMDAR coagonist site in addition to the glutamate binding site (NMDA plus D-serine) so any released coagonist would not cause a change in current. Bath applied D-serine 100uM, resulted in a further baseline increase (Fig 3A) and a reduction in the light-evoked outward current recorded. By subtracting the light-evoked current obtained in the presence of only NMDA from that in the presence of both NMDA and D-serine, we were able to observe coagonist release in the resultant trace (Fig 3A). Averaging the resultant

traces from multiple cells showed a clear trend of coagonist release (Fig 3B). The charge induced by coagonist release in wt retinas was 52.1±4.7% (n=13) of the charge measured in control light responses, without NMDA added. To test if the released coagonist was D-serine, we repeated these experiments in SRKO mice. We discovered that the charge transfer due to coagonist release in SRKO ON responses was substantially lower than wt (14.9±3.9% ctrl, n=8; $p<0.005$ compared to wt) (Fig 3B, C). Coagonist release during wt ON responses was blocked when these experiments were repeated in the continuous presence of NBQX (8.5±3.6% ctrl, n=8; $p<0.005$ compared to wt) (Fig 3B, C). Coagonist release was minimal in OFF responses and showed no significant difference between SRKO (6.7±4.6% ctrl, n=4) and wt animals (13.6±2.5% ctrl, n=6; $p=0.56$ between genotypes) (Fig 3C).

The relative potentiation of NMDAR currents by the coapplication of NMDA and D-serine to the bath was comparable to our observations using puff-applied NMDA (Fig 3D). Specifically, D-serine significantly potentiated NMDAR currents in wt (28.0±3.4% increase) and SRKOs (52.8±10.7% increase), but to a greater extent in SRKOs ($p<0.05$ between genotypes). In wt RGCs, NBQX enhanced the potentiating effects of D-serine (50.6±5.6% increase; $p<0.01$ between ctrl and NBQX), suggesting that NBQX reduces the baseline level of coagonist that is present in the absence of light stimulation, in addition to preventing evoked coagonist release.

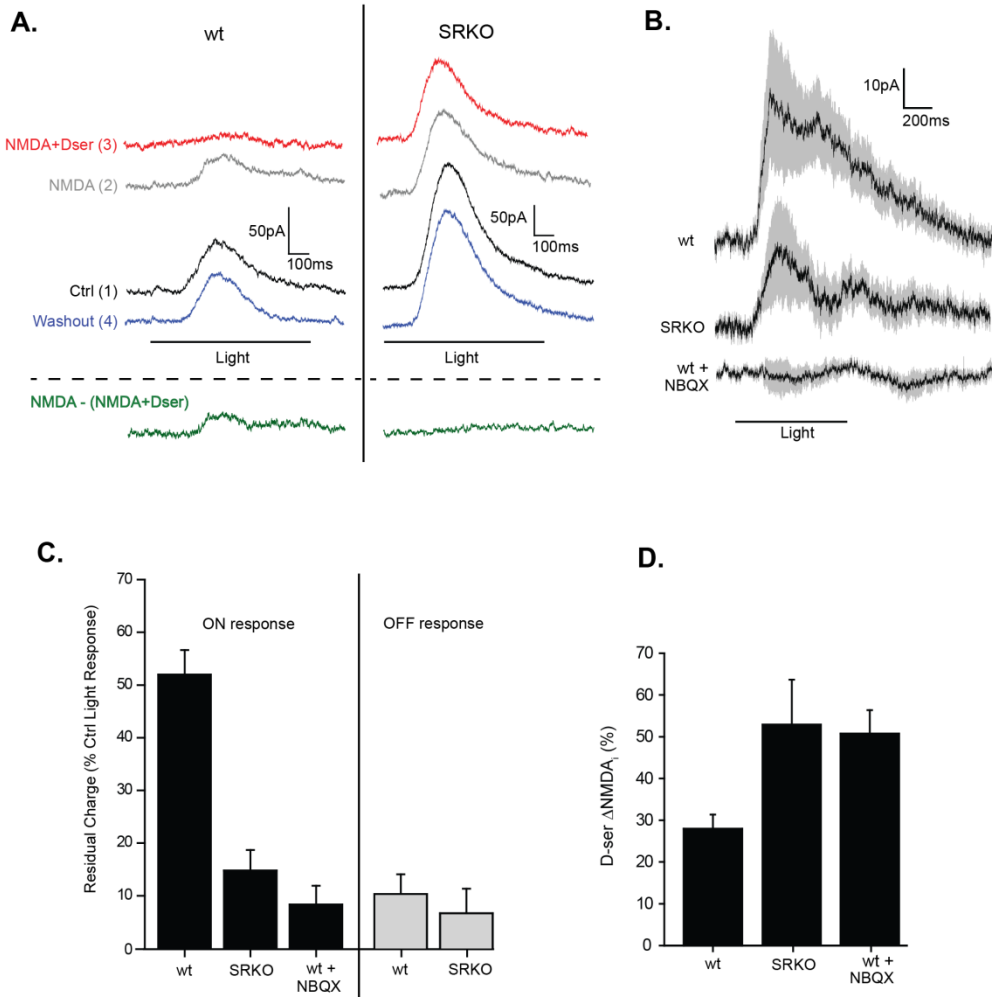

Figure 3: Light-evoked RGC responses recorded at +40 mV. Recordings were made in 1 mM Mg^{2+}, 1 uM TTX, 10 uM strychnine, 50 uM TPMPA, and 50 uM picrotoxinin. **A.)** Raw trace showing the effects of bath applied NMDA (100 uM) or NMDA plus D-serine (100 uM) on light-evoked ON responses. Numbers indicate the order of drug application. Traces are offset to show the baseline shift in current caused by the different pharmacological conditions (arrows). The green trace, representing coagonist release, was obtained by subtracting the NMDA plus D-serine trace from the NMDA trace. **B.)** Average coagonist release traces (SE shown in gray) for ON cells compared between wt and SRKO RGCs in control conditions and wt RGCs in the continuous presence of NBQX. **C.)** The response area (charge) of coagonist release traces normalized to the charge in control conditions. SRKOs displayed significantly less coagonist release than wt for ON responses but not OFF responses. Coagonist release was significantly

lower for both genotypes during OFF responses than in ON responses. NBQX significantly reduced coagonist release during ON responses in wt. **D.)** The percent increase in the baseline shift (see A) caused by NMDA after adding NMDA plus D-serine. The D-serine induced potentiation of NMDAR currents was larger in SRKOs than wt. In the continuous presence of NBQX, D-serine potentiation was enhanced it wt.

Discussion

Our study is the first to demonstrate AMPAR-dependent coagonist release during retinal light responses. Using a SRKO mouse, we provide evidence that the released coagonist acting on RGC NMDARs during ON responses was D-serine. Light-evoked NMDAR currents were abolished by NBQX but could be rescued by adding D-serine, demonstrating that AMPARs are critical in providing synaptic NMDARs with coagonist. RGC NMDAR coagonist site occupancy was also reduced by blocking AMPARs during puff-evoked NMDAR currents but to a far lesser extent than that observed in light responses, suggesting a difference between coagonist regulation at synaptic and extrasynaptic sites. NMDA-puff responses in SRKOs were fairly robust, but still showed lower coagonist site occupancy, suggesting ambient coagonist levels are at least in part regulated by D-serine. Overall these findings illustrate a complex system of coagonist regulation, where synaptic NMDAR receive D-serine from AMPAR-dependent light-evoked coagonist release, while extrasynaptic NMDARs are regulated primarily by relatively stable levels of glycine.

NBQX dramatically reduces the NMDAR currents in RGCs evoked by the pharmacological activation of ON bipolar cells, which can be rescued by exogenous D-

serine (74). Our findings show that NBQX also reduces the coagonist contribution to light-evoked ON responses in RGCs. The potentiation of NMDA puff-responses by D-serine was also increased in NBQX, suggesting a reduction in coagonist occupancy, but to a far lesser extent than that observed in response to light. In fact, NBQX did not noticeably alter puff-evoked NMDAR currents (Fig 2C). These findings imply that synaptic NMDARs depend on AMPARs for coagonist supply, while extrasynaptic coagonist is primarily regulated via an unknown mechanism.

Enzymatic degradation of extracellular D-serine abolishes RGC NMDAR currents during ON and OFF light responses (50). However, we found that SRKO RGC responses to direct NMDA application were relatively normal, only showing a slightly augmented potentiation by coagonist application compared to wt. Congruently, enzymatic removal of extracellular D-serine only reduces NMDA puff-evoked currents (49) and NMDA-induced Ca^{2+} responses to bath applied NMDA by ~40% (95). These findings provide evidence for heterogeneity in coagonist distribution in the retina. The synaptic NMDARs involved in light responses require D-serine for activation. However, when extrasynaptic NMDAR are also activated by puff-applying NMDA to RGCs, a population of NMDARs that do not depend on D-serine for activation, potentially occupied by glycine, is revealed.

Direct measurement of global glycine concentrations in the retina (82;85) show that glycine reaches levels high enough to saturate NMDARs (17). The glycine transporter GlyT1 is critical in limiting these high concentrations of glycine from saturating the RGC NMDARs involved in light responses (69;85). However, it is possible that glycine is

regulated less stringently away from synaptic sites, where it could serve as a coagonist to extrasynaptic NMDARs. Consistent with this model, GlyT1 expression is relatively high in the inner plexiform layer, where bipolar cells synapse onto RGCs, but is absent in ganglion cell bodies (68), where NMDARs are also expressed (96). Perhaps exploiting the differences in the coagonist utilized by different NMDAR populations in the CNS might serve to target extrasynaptic NMDA receptors, which are suspect in excitoxic cell death (97) and Alzheimer's disease (98).

We found evidence for released coagonist acting on RGC NMDARs during ON responses. Coagonist release was abolished by blocking AMPARs, implying that NBQX reduces coagonist availability during light responses (Fig 1) by blocking coagonist release, as opposed to merely reducing ambient coagonist levels set by tonic AMPAR activity in the absence of light. Work in hippocampus (60), cerebellum (61), and retina (74) have provided evidence for activity dependent coagonist release acting on NMDARs but it was unclear whether this coagonist was D-serine or glycine. Indeed, RGCs receive inhibitory glycinergic input from amacrine cells which depend on AMPARs for activation. On the other hand, AMPAR activation evokes D-serine release from isolated retinas (82). We used SRKO mice to test if the coagonist released during light responses was D-serine and found the amount of coagonist released during ON responses was reduced dramatically, suggesting a role for D-serine release in RGC responses.

The coagonist released during ON responses in our study was relatively rapid, overlapping with excitatory glutamatergic input. Although the retinal cell-type releasing D-serine cannot be determined from our findings alone, D-serine is present in retinal glia

(49) and possibly neurons (70). Rapid vesicular release of neurotransmitter has been demonstrated in glia (54), but it is unclear how effectors downstream of AMPAR activation could elevate Ca^{2+} levels fast enough in glia, unless the Ca^{2+} originated from the AMPARs themselves. Indeed, AMPA induced D-serine release in retina depends on Ca^{2+} permeable AMPARs (82). Alternatively, D-serine could be released from neurons during light responses. Irrespective of the cell type(s) responsible for releasing D-serine in the retina, our work emphasizes that coagonist release should be considered as one of the coinciding factors required for NMDAR activation during excitatory transmission in the CNS.

Chapter 3

AMPA receptor mediated D-serine release from retinal glial cells

Introduction

Activation of N-methyl-D-aspartate receptors (NMDARs) requires glutamate bound to the NR2 subunit as well as a separate coagonist bound to the NR1 subunit (10;15). Glycine was originally thought to be the endogenous coagonist, but in recent years D-serine has emerged as the more likely coagonist at several CNS sites, including the retina. D-serine is now known to be abundant in the brain (22), especially in regions rich in NMDARs (29). The prospects of D-serine as an endogenous coagonist were further strengthened when Wolosker discovered serine racemase, the enzyme that synthesizes D-serine from L-serine (26). D-serine has since been implicated in numerous mechanisms ascribed to NMDARs, including neuroplasticity (51;77) and learning and memory (99). Furthermore, abnormalities in D-serine regulation have been implicated in the NMDAR hypofunction theory of schizophrenia (100;101).

Early immunohistochemical and ultrastructural studies localized D-serine to astrocytes adjacent to neurons expressing NMDARs (79). A similar result was later confirmed in the retina, where D-serine was found in astrocytes and Müller glia (49). However, the recent discovery of D-serine in neurons throughout the brain (30) and serine racemase mRNA in retinal neurons (70) brings into question the idea that D-serine originates solely from glia in the CNS.

While the cellular origins of D-serine are presently unclear, it serves as an endogenous coagonist of retinal ganglion cell (RGC) NMDARs. In the intact retina, applying D-serine deaminase (DsDa), a highly selective D-serine degrading enzyme, has the same effect on light-evoked RGC NMDAR currents as completely blocking NMDARs with conventional antagonist. Adding exogenous coagonist augments RGC NMDAR currents (49;50) demonstrating that the coagonist site is not saturated and additional NMDARs would be recruited by D-serine release.

Studies on cultured cortical astrocytes suggest that α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) activation stimulates the synthesis (46) and release of D-serine (55). However, cultured cortical neurons (30) and, more recently, neurons from cortical slice have displayed similar AMPA-evoked D-serine release (102). It remains unclear which cell type contributes to the endogenous pool of D-serine in intact CNS tissue and whether the same glutamatergic release mechanisms are employed.

Previous attempts at measuring extracullar D-serine in the retina have proven difficult because of the relatively low levels of D-serine (103), despite the fact that D-serine modulates retinal ganglion cell NMDARs. To aid us in our study of D-serine release, we utilized a mutant mouse with a point mutation in the only known mammalian D-serine degrading enzyme, D-amino acid oxidase (DAO-), rendering the enzyme inactive (40). Direct measurements of extracellular D-serine in the intact retina via capillary electrophoresis demonstrate glutamate-evoked D-serine release through an AMPAR-

dependent pathway. This D-serine release persisted in the presence of neural inhibitors but was abolished by a glial toxin.

Materials and Methods

Materials

AMPA, cyclothiazide, TFB-TBOA, GYKI 52466 hydrochloride, TTX, and suramin were purchased from Tocris Bioscience (Ellisville, MO). NBD-F and Fluo-4 AM were purchased from Invitrogen (Eugene, OR). All other chemicals were purchased from Sigma (St Louis, MO).

Isolation and pharmacological treatment of retinas

Experiments were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Adult ddY mice were euthanized by an overdose of Nembutal (0.1ml of 50mg/ml, i.p.) and pneumo-thoraxed. Eyes were enucleated and placed in bicarbonate Ringer's solution (111 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 32 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 15 mM Dextrose; bubbled with 95% O_2, 5% CO_2) for the surgical isolation of the retina. For each data point, two isolated retinas were incubated in 100 μL of Ringer's solution and oxygenated in a humidified chamber containing 95% O_2 and 5% CO_2 gas with shaking for 50 minutes, unless indicated otherwise. Following incubation in the drug solution or control, the bathing media from the retinas was

extracted and partitioned into three equal volumes. These samples were used for the identification of D-serine via capillary electrophoresis.

Capillary electrophoresis

Capillary electrophoresis (CE) with a commercial LIF detection system (MDQ, Beckman-Coulter, Fullerton, CA, USA), was used to separate and measure D-serine as described previously (71). 25 µL of the sample was treated with 2.5 µL of the internal standard α-aminoadipic acid (5 µM final concentration). D-glutamate was used as an internal standard in experiments using α-aminoadipic acid as a glial toxin. The amino acids from extracellular retinal samples were fluorescently derivitized by adding 2.5 µL of 4-fluro-7-nitrobenz-2oxa-1,3-diazole (NBD-F) dissolved in acetonitrile (3.6 mM final concentration; 30 µL final volume) and reacting at 60°C for 15 minutes.

Samples were pressure injected for 5 s at 0.5 psi into a fused cilica capillary and run at -15 kV (70 µA) for 30 minutes. Before each run, the capillary was rinsed with 1M NaOH and loaded with 34 mM hydroxypropyl-β-cyclodextrin in 165 mM borate pH 10.2 to separate D-serine from its enantiomeric partner L-serine. A 4 mW argon laser (488 nm excitation) was used to detect fluorescence. A beam splitter combined with two separate photo multiplier tubes allowed simultaneous recording of samples at different gain settings. Amino acids were quantified by integrating the generated electropherogram using 32 karat analysis software.

Retinal protein determination

Total protein measurement was used to normalize all CE amino measurements (expressed as nmol/g protein). Retinas were homogenized by sonication and centrifuged at 11,000 g for 5 minutes. The pellets from each sample were re-suspended in 120 µL of 2M NaOH and diluted (1:20) in water. Protein concentration in the diluted samples was quantified using a Pierce (Rockford, IL) bicinchoninic acid assay.

Identification of D-serine

The D-serine peak was identified by spiking a fraction of the extracellular sample with an additional 5 µM D-serine. To be sure that another molecule with a similar migration time was not interfering with our D-serine measurements, D-serine was also measured after treating the sample with DsDa (50 µg/ml, 20 minutes). DsDa specifically eliminated D-serine from a series of amino acid standards (Figure 1A) and also completely removed the peak matching the D-serine migration time in samples (Figure 1B). The mass of D-serine in each sample was computed from a standardized curve.

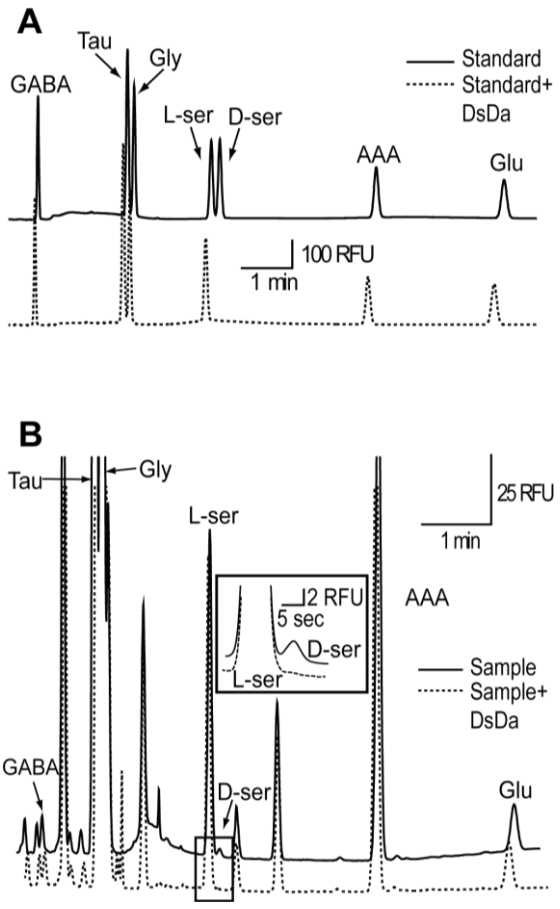

Figure 1. Identification of D-serine via capillary electrophoresis. **(A)** Electropherogram showing the separation of D-serine (D-ser), from a series of amino acid standards; Gamma-aminobutyric acid (GABA), Taurine (Tau), Glycine (Gly), L-serine (L-ser), α-aminoadipic acid (AAA), and Glutamate (Glu). Treatment with D-serine deaminase (DsDa) (50 ug/ml, 20 min) specifically eliminated D-serine (dotted trace). **(B)** Separation of amino acids from mouse retina extracellular media, with L-serine and D-serine expanded. D-serine was completely eliminated following DsDa incubation.

Glial toxin α-aminoadipic acid

Retinas were preincubated in Ringer's solution containing 10 mM α-aminoadipic acid (AAA) for 3 hours, while control retinas were incubated for the same period without the toxin. The treated retinas were then incubated for 50 minutes in presence of 1mM AAA (10 mM AAA added too much noise to the CE trace) and the extracellular media was sampled.

Ca^{2+} imaging

Retinas were incubated in Ringer's solution containing 230 μM Fluo-4 AM for 45 minutes to label glial cells (104). Whole-mount retinas were flattened on nitrocellulose paper, with the ganglion cell side up, and perfused in bicarbonate Ringer's solution. Whole field fluorescence in the ganglion cell layer revealed Müller cell end feet surrounding dark unlabeled ganglion cell bodies. Images were acquired every 2 seconds with a multiphoton laser (λ=820 nm) (Prairie Technologies). Data were expressed as ΔF/F, where F is the average baseline fluorescence measured over the first 30 seconds. Drugs blocking neural signaling were applied 1 minute prior to imaging. Figure 4A (bottom) was reconstructed from a Z-series of images taken at 1um intervals.

Statistical analysis

Each data point collected (n) for CE data was derived from an incubation of two retinas, while those for Ca^{2+} imaging denotes the response of a single retina. Student's one-tailed t-test was used to calculate significance. All data are expressed as mean ± SE. Significance defined as $p<0.05$.

Results

AMPA induced D-serine release

DAO- mice have significantly elevated levels of D-serine in brain regions rich in DAO expression, including the brainstem and cerebellum (84). In this study, we found that DAO- retinas incubated for 50 min in oxygenated Ringer's solution had significantly

greater extracellular D-serine levels compared to wild-type (wt) controls (Table 1, Figure 2B). In contrast, no significant difference was observed in any of the other amino acids measured, including L-serine, glycine, taurine, GABA, or glutamate (Table 1).

	D-serine	L-serine	Glycine	Glutamate	GABA	Taurine
wt	0.69 ± 0.12	492.68 ± 57.61	336.36 ± 70.37	86.02 ± 56.58	31.17 ± 16.59	4579.72 ± 883.25
DAO-	4.66* ± 1.13	715.40 ± 148.08	395.11 ± 127.38	118.29 ± 48.61	42.14 ± 18.23	5667.64 ± 1252.42

Table 1. Extracellular amino acid comparison between wt and DAO- mice. Data expressed as the mean extracellular amino acid measurement in nmol/g protein ± S.E. *, statistically different from wt at $p<0.05$. n=5.

We examined the possibility that D-serine is released through glutamatergic mechanisms. To carry out this objective, we exposed retinas to AMPA+cyclothiazide (which prevents AMPAR desensitization (105)) and sampled the extracellular media. AMPA+cylcothiazide treatment resulted in an approximately twofold increase in extracullar D-serine in both wt and DAO- retinas (figure 2B), implying that there is no difference in the D-serine release mechanism between the genotypes. DAO- mice were used in all subsequent pharmacological experiments to more readily detect changes in D-serine.

We tested whether the D-serine release induced by AMPA+cyclothiazide was detectable over shorter exposures. Retinas were moved to a new well containing fresh Ringer's

solution every 10 minutes for five consecutive incubations, and the incubation sample measured for D-serine at each point (50 minutes total). Over this time period, the AMPA+cyclothiazide-induced D-serine release was steady and inexhaustible (Figure 2C). This finding suggests that the source of releasable D-serine was continuously replenished, presumably by serine racemase. Coapplication of 1-naphthyl acetyl spermine (NAS), a Ca^{2+} permeable AMPAR antagonist (106), significantly reduced the AMPA+cyclothiazide-induced D-serine release observed over time (Figure 2C, 2D). AMPA alone, in the absence of cyclothiazide, was incapable of increasing extracellular D-serine, suggesting that this mode of D-serine release is prone to AMPAR desensitization (Figure 2C, 2D). Cyclothiazide alone had no effect on D-serine levels (data not shown).

Blocking Glutamate uptake elevates extracellular D-serine by acting through AMPA receptors

Our findings raised the possibility that endogenous glutamate is capable of evoking D-serine release. In healthy retinas, extracellular glutamate concentrations are tightly regulated by excitatory amino acid transporters (EAAT1-EAAT5) and removal of EAAT1 or EAAT2 significantly elevates extracellular glutamate (107). We treated the retinas with TFB-TBOA, a high affinity EAAT1 and EAAT2 blocker (108), which dramatically elevated extracellular glutamate over the course of 50 minutes (Figure 3A, 3B).

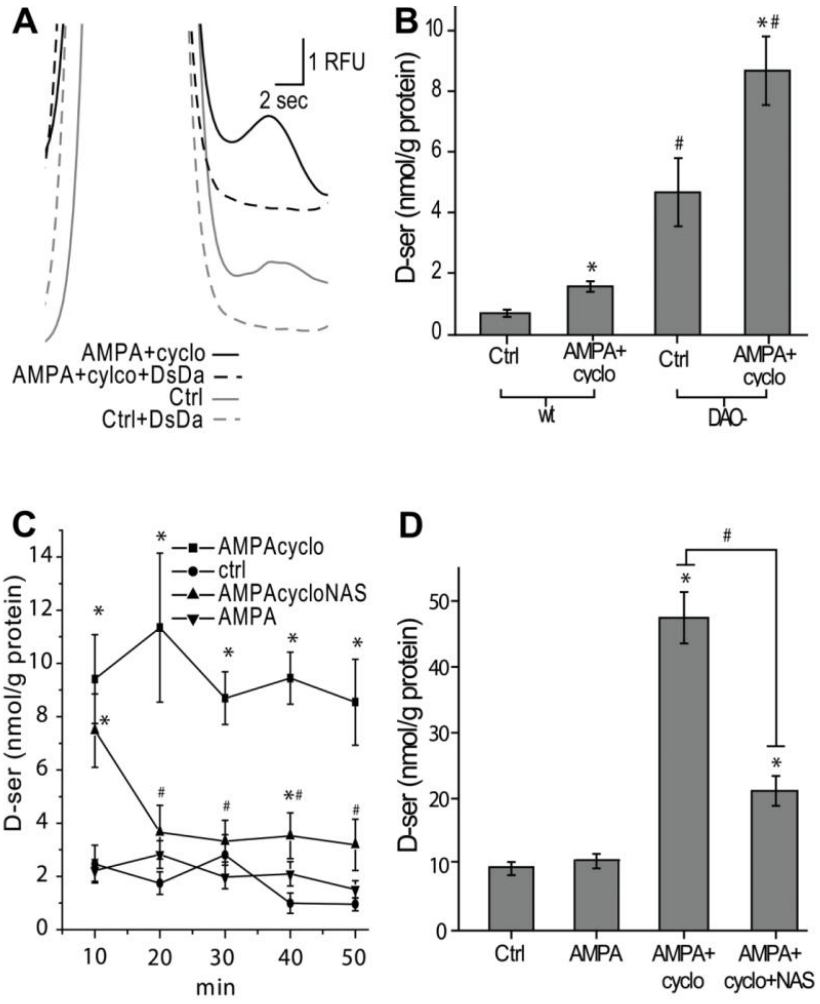

Figure 2. AMPA+cyclothiazide evokes D-serine release in wt and DAO- retinas. **(A)** Extracellular D-serine in DAO- retinas incubated for 50 minutes (Ctrl) or with 50 µM AMPA plus 50 µM cyclothiazide (AMPA+cylco). Both peaks were eliminated by DsDa. **(B)** Comparison of extracellular D-serine between wild-type (wt) and DAO- mice, ctrl or with AMPA+cyclo. *, statistically different from control at $p<0.05$. #, statistically different from wt under same drug condition at $p<0.05$. **(C)** D-serine release measured in DAO- retinas every 10 minutes, for 5 times total. **(D)** sum of D-serine release at each 10 minute time point in C. C-D, AMPA alone failed to elevate D-serine (*, significantly different from control, $p<0.05$) and AMPA+cyclo release was significantly attenuated by 1-naphthyl acetyl spermine (NAS) (#, comparison of two groups, $p<0.05$). Data reported as the mean ± S.E. n=3-6.

We found that elevating glutamate alone was insufficient to measurably increase D-serine, consistent with our findings that AMPA alone did not elevate extracellular D-serine (Figure 2D). However, using cyclothiazide, in addition to TFB-TBOA, resulted in a significant elevation of D-serine. This increase was blocked by the selective non-competitive AMPAR antagonist GYKI 52466, confirming that glutamate was acting through an AMPAR-dependent pathway to elevate D-serine. GYKI did not bring D-serine levels significantly below baseline (Figure 3C), which implies that the baseline levels of D-serine measured in these experiments were set by a non-AMPAR mechanism, possibly by the alanine-serine-cysteine transporter (ASCT2) as described previously (71;72).

AMPA induced D-serine release is independent of neural activity

We next wanted to evaluate the cell type responsible for AMPAR-dependent D-serine release. Given that D-serine could be present in both neurons and glia, there are three basic possibilities: (1) AMPA could act directly on neurons stimulating them to release D-serine; (2) AMPA could act on neurons which then signal to glial cells to release D-serine; or (3) AMPA could act directly on glial cells to release D-serine. We addressed possibilities (1) and (2) by adding pharmacological inhibitors of neuronal activity and neurotransmitter release, combined with an inhibitor of neuron to glia signaling.

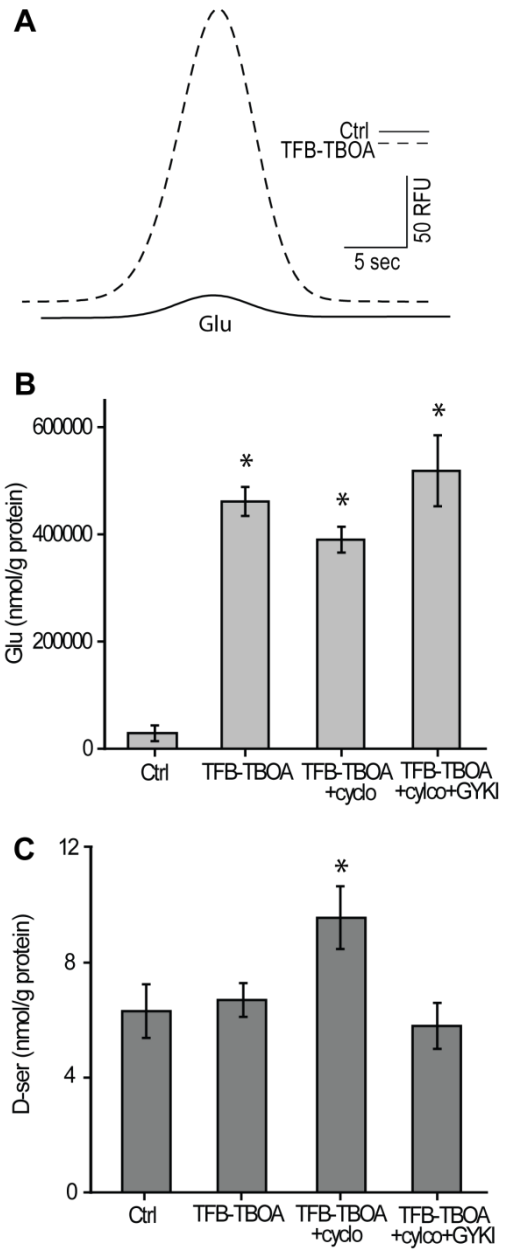

Figure 3. Blocking Glutamate uptake elevates extracellular D-serine by acting through AMPA receptors. **(A)** Raw trace showing glutamate (Glu) increase following a 50 minute incubation in 10 µM TFB-TBOA. **(B)** TFB-TBOA significantly elevated Glu in all conditions tested. There was no difference in Glu between experiments where TFB-TBOA was added. **(C)** TFB-TBOA elevated D-serine in the presence of cyclothiazide which was blocked by the application of 50 µM GYKI. A, B, C DAO- retinas (*, statistically different from control, $p<0.05$, n=6-7).

For possibility (3) to hold true, AMPA must have some direct effect on glia. Müller cells appear to express AMPA receptors (109) and are responsive to AMPA in isolation (110). To test this in the intact retina, we looked at intracellular Ca^{2+} in retinal glia in

response to AMPA+cyclothiazide treatment in the presence of a cocktail of neural signaling inhibitors. TTX was used to prevent neural impulses, and cadmium chloride (Cd^{2+}) was also added to prevent synaptic transmission in retinal neurons with graded potentials by blocking voltage gated Ca^{2+} channels. To inhibit neuron-to-glia and glia-to-glia communication, which is primarily carried out by ATP in the retina (111), the purenurgic (P2) antagonist suramin was added. Whole-mount retinas were incubated in Fluo-4 AM over time periods favoring glial loading (104). We observed that Fluo-4 AM loaded astrocytes and Müller cells but not neurons (Figure 4A-B). Imaging with a multiphoton laser we found that retinal Müller cell endfeet in the ganglion cell layer of the retina show significant increases in intracellular Ca^{2+} in response to AMPA+cylothiazide in the absence of neural signaling which was blocked by the AMPAR antagonists GYKI and NAS (Figure 4C-E), suggesting that AMPA is capable of directly acting on glia, perhaps through Ca^{2+} permeable AMPARs. There was no difference in the Ca^{2+} response between wt and DAO- retinas (Figure 4E), implying that the DAO mutation did not indirectly influence D-serine levels by altering glial sensitivity to agonist. AMPA+cyclothiazide application still evoked D-serine release in the presence of suramin and the neural signaling inhibitors (Figure 4F), which implies that neurons are not contributing to the AMPAR-dependent D-serine release in these studies.

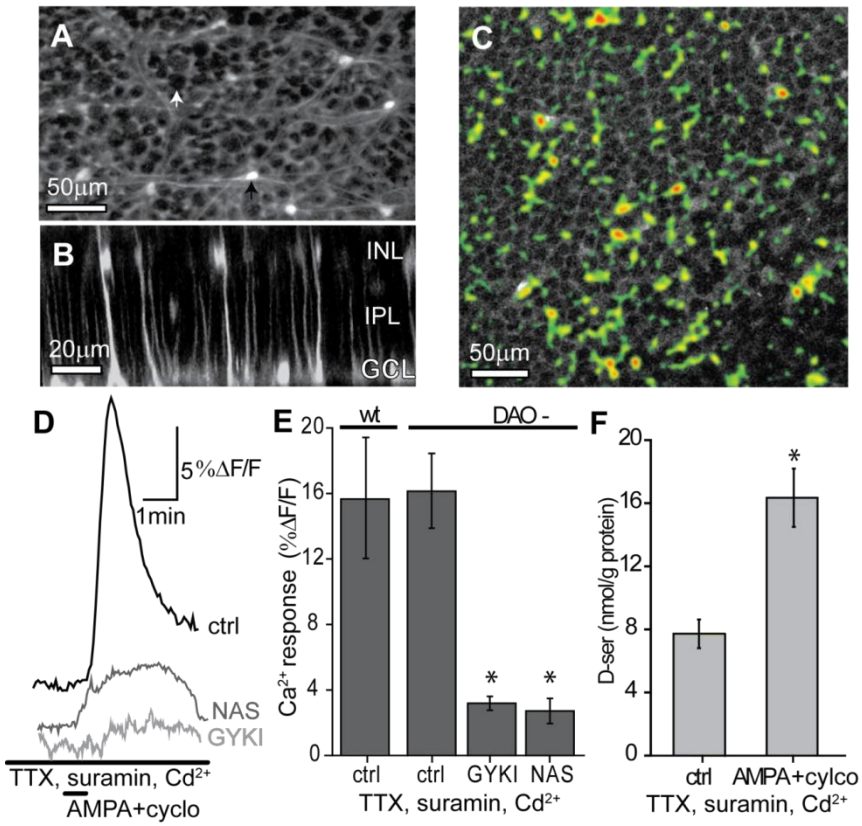

Figure 4. AMPA+cyclothiazide D-serine release is not blocked by inhibition of neural activity. **(A)** Fluo-4 AM loaded retina showing labeling of Astrocytes (black arrow) and Müller cell endfeet surrounding unlabeled ganglion cell bodies (white arrow). **(B)** Cross-section of retina in A reconstructed from a Z-series showing Müller cell labeling of endfeet near the ganglion cell layer (GCL), with stalks spanning the inner plexiform layer (IPL), and cell bodies in the inner nuclear layer (INL). **(C)** Ca^{2+} response of Müller cell endfeet to AMPA+cylco. Retinas were bulk loaded with Fluo-4 AM then treated with 1µM TTX, 100 µM suramin, and 200 µM Cd^{2+} 2 minutes prior to a 30 second bath application of AMPA+cylco. Image shows the change in Ca^{2+} in response to AMPA+cylco as a thresholded maximal projection (pseudocolor) overlaid on the average baseline image. Ca^{2+} elevations excluded ganglion cell bodies. **(D)** Change in Ca^{2+} over time from the region in C. **(E)** No significant difference was observed in the Ca^{2+} increase between wt (n=4) and DAO- (n=3) retinas following AMPA+cylco application. GYKI (n=5) and NAS (n=6) significantly reduced the AMPA+cylco induced Ca^{2+} response in DAO- retinas (*, $p<0.05$, compared to ctrl). **(F)** DAO- retinas exposed to AMPA+cyclo still released D-serine in the combined presence of TTX, suramin, and Cd^{2+}. (*, statistically different from control, $p<0.05$, n=4-5).

Glial cells regulate baseline as well as evoked D-serine release

To address whether or not AMPA+cyclothiazide can act directly on glia to evoke D-serine release, we utilized the glial toxin α-aminoadipic acid (AAA). AAA toxicity is thought to originate by causing free radical buildup in glial cells (112), and eliminates retinal Müller cell function, while retaining the retina's responsiveness to light (113;114). Preincubating retinas in 10 mM AAA significantly reduced extracellular glutamine (Gln) concentrations compared to control retinas (Figure 5A), indicating that the glial cells, the major source of Gln (115), were impaired. In the same preparations, D-serine release evoked by AMPA+cyclothiaizide was dramatically reduced (Figure 5B). Glial impairment also significantly lowered baseline D-serine (Figure 5B), implying that glia also contribute to basal concentration of D-serine in the extracellular environment.

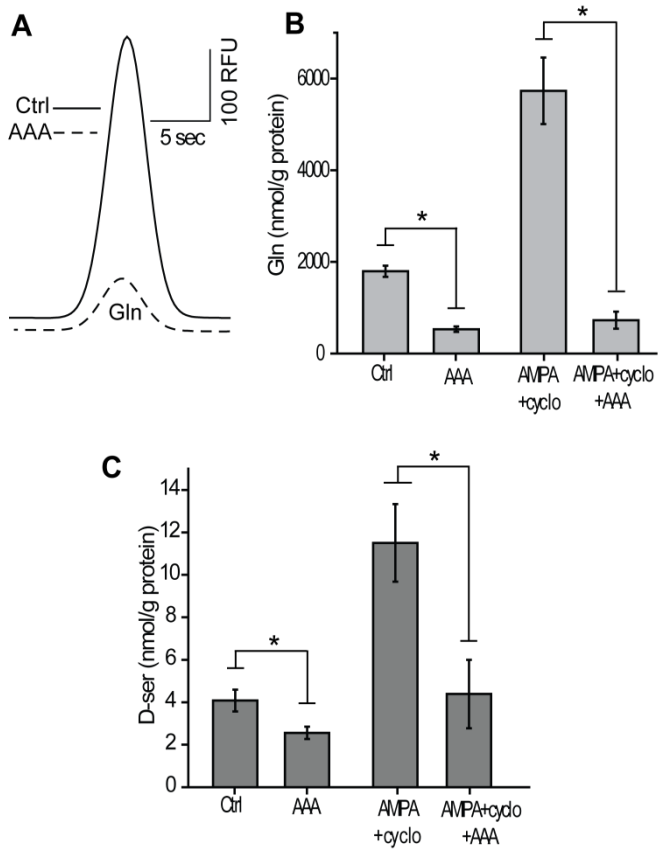

Figure 5. Glial toxin prevents AMPA receptor dependent D-serine release. **(A)** Electropherogram showing the reduction of Glutamine (Gln) following a 3 hour incubation in 10 mM AAA. **(B)** Gln was significantly reduced by AAA irrespective of AMPA+cyclo application. **(C)** Baseline D-serine and AMPA+cyclo induced D-ser release was reduced by AAA. A, B, C DAO- retinas (*, $p<0.05$, $n=5$).

Discussion

We have provided the first direct measurements of dynamic D-serine release from the isolated retina. This pathway for D-serine release involved the activation of AMPARs, a model consistent with previous findings in cortical astrocytes (55). Because the coagonist site is not saturated on RGC NMDARs (49;50;74;95), stimulated D-serine release would influence the output of the retina to the brain.

Our results reveal that DAO- retinas have significantly greater D-serine than wt, while a number of other amino acids are unaffected. The similar increase in D-serine evoked by AMPA+cylcothiaizide in DAO- and wt suggests that the mechanism of D-serine release is unaltered in the mutants, making them ideal for studying D-serine release in the retina. In other areas of the nervous system, DAO expression appears to correlate negatively with D-serine levels. Brain regions that typically show the lowest levels of D-serine, such as the cerebellum, display the greatest relative increase in DAO- mice, whereas brain regions with high levels of D-serine appear to be unaffected by the mutation (84). Based on our results, we would predict the utilization of DAO in the regulation of retinal D-serine to most likely fall somewhere between the extremes of cortex and cerebellum.

We showed that raising extracellular glutamate by blocking glutamate transport can induce D-serine release via an AMPAR-dependent pathway. Many retinal stressors are known to increase extracellular glutamate, including: hypoglycemia, anoxia (116), optic nerve crush (117;118), and models of glaucoma (119). A leading theory as to how these stressors pathologically develop is that the excess glutamate becomes excitotoxic through its action on NMDARs. Our studies suggest that glutamate elevations could also cause retinal glia to release D-serine, which would recruit more NMDARs for the same level of glutamate to act on, thus exacerbating excitotoxicity. Congruently, oxygen-glucose deprivation in the cerebrum leads to efflux of glutamate and D-serine (120) and serine racemase knockout mice are protected against ischemia (121), while the addition of coagonist exaggerates NMDA excitotoxicity in the retina and D-serine removal attenuates it (122).

The fact that the glial toxin AAA reduced but did not completely eliminate baseline extracellular D-serine might mean that AAA only partially impaired the glial metabolic machinery required for D-serine synthesis. Alternatively, neurons might also contribute to baseline extracellular D-serine levels. However, retinal D-serine uptake is thought to occur solely through ASCT2 (71), expressed primarily by glial cells (72). If there was a neural source of D-serine, disrupting the main mechanism of D-serine uptake by functionally eliminating glia would be expected to elevate the levels of extracellular D-serine, but the opposite was observed. Although a potential role of neurons in the regulation of D-serine cannot be ruled out, the fact that there was no apparent D-serine release observed when glia were functionally eliminated, and that inhibitors of neurons and neuron-to-glia signaling had no effect on D-serine release, suggests that the AMPAR-dependent D-serine release observed in our studies was exclusively from glia.

Electrophysiological observations in our lab have shown that increasing the contrast of light stimuli leads to a greater occupancy of the NMDAR coagonist site on RGCs (unpublished findings). Another group has shown, in rat retinal slice, that a pharmacologically mimicked light stimulus is still capable of recruiting additional RGC NMDA current in the presence of saturating NMDA concentrations, again implying dynamic coagonist release (74). In this report, stimulated coagonist release was unaffected by the addition of DAO, leading the authors to conclude that glycine was enhancing NMDAR currents. However, previous studies in the salamander retina showed that using an enzyme with greater specific activity against D-serine, DsDa,

abolished light-evoked RGC NMDAR (50), implying that released glycine was not reaching the coagonist site.

Glycine serves as a major inhibitory neurotransmitter in the retina (66). It is noteworthy that our CE measurement of the extracellular retinal environment showed a significantly higher baseline concentration for glycine than D-serine. It is plausible that the high affinity glycine transporter GlyT1 expressed in amacrine (123) and Müller (124) cells keeps glycine levels low at the local micro-environment of RGC NMDARs. Coexpression of both GlyT1 and NMDARs in Xenopus oocytes dramatically reduces the level of glycine capable of reaching the coagonist site (125), despite the fact that glycine and D-serine have comparable potencies in expression systems void of amino acid transporters (17). Similarly, mutant mice with reduced expression of the GlyT1 transporter display greater coagonist site occupancy in the retina (85). On the other hand, transport of D-serine in the retina is thought to occur almost exclusively through the relatively slow neutral amino acid transporter ASCT2 (71;72), unlike the cortex where the high affinity transport ASC-1 is also expressed (35). It is possible that the absence of a high affinity transport system for D-serine, combined with spatially precise release, allows such small extracellular levels of D-serine to serve as the major coagonist in the retina.

The mechanism of D-serine release in response to light stimulation remains to be elucidated. Our findings provide a model for D-serine release whereby glutamate released from bipolar cells during light stimulation could act on Müller cell processes in the IPL, which in turn release D-serine onto RGC NMDARs. The added NMDAR

currents recruited by releasable D-serine could serve to increase the dynamic range of light-evoked RGC responses, or may lead to more long-term changes in ganglion cell activity by the activation of intracellular signaling pathways.

Bibliography

1. Madden, D. R. (2002) The structure and function of glutamate receptor ion channels. *Nat.Rev.Neurosci.* 3, 91-101

2. Michard, E., Lima, P. T., Borges, F., Silva, A. C., Portes, M. T., Carvalho, J. E., Gilliham, M., Liu, L. H., Obermeyer, G., Feijo, J. A. (2011) Glutamate receptor-like genes form Ca2+ channels in pollen tubes and are regulated by pistil D-serine. *Science* 332, 434-437

3. Chen, G. Q., Cui, C., Mayer, M. L., Gouaux, E. (1999) Functional characterization of a potassium-selective prokaryotic glutamate receptor. *Nature* 402, 817-821

4. Denes, A. S., Jekely, G., Steinmetz, P. R., Raible, F., Snyman, H., Prud'homme, B., Ferrier, D. E., Balavoine, G., Arendt, D. (2007) Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. *Cell* 129, 277-288

5. Howe, J. R., Colquhoun, D., Cull-Candy, S. G. (1988) On the kinetics of large-conductance glutamate-receptor ion channels in rat cerebellar granule neurons. *Proc.R.Soc.Lond B Biol.Sci.* 233, 407-422

6. Paoletti, P. (2011) Molecular basis of NMDA receptor functional diversity. *Eur.J.Neurosci.* 33, 1351-1365

7. Liu, X. B., Murray, K. D., Jones, E. G. (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *J.Neurosci.* 24, 8885-8895

8. Malenka, R. C., Bear, M. F. (2004) LTP and LTD: an embarrassment of riches. *Neuron* 44, 5-21

9. Manev, H., Favaron, M., Guidotti, A., Costa, E. (1989) Delayed increase of Ca2+ influx elicited by glutamate: role in neuronal death. *Mol.Pharmacol.* 36, 106-112

10. Kleckner, N. W., Dingledine, R. (1988) Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science* 241, 835-837

11. Curtis, D. R., Phillis, J. W., Watkins, J. C. (1961) Actions of aminoacids on the isolated hemisected spinal cord of the toad. *Br.J.Pharmacol.Chemother.* 16, 262-283

12. Mathieu, J. P. Activit Optique Naturelle, Handbuch der Physik. Spectroscopie 28. 1957. Spectroscopie.

13. Pasteur, L. Researches on the molecular asymmetry of natural organic products. Alembic Club Reprints 14, 1-46. 1848.

14. Pizzarello, S., Huang, Y., Alexandre, M. R. (2008) Molecular asymmetry in extraterrestrial chemistry: Insights from a pristine meteorite. *Proc.Natl.Acad.Sci.U.S.A* 105, 3700-3704

15. Johnson, J. W., Ascher, P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325, 529-531

16. Mehta, P. K., Christen, P. (2000) The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. *Adv.Enzymol.Relat Areas Mol.Biol.* 74, 129-184

17. McBain, C. J., Kleckner, N. W., Wyrick, S., Dingledine, R. (1989) Structural requirements for activation of the glycine coagonist site of N-methyl-D-aspartate receptors expressed in Xenopus oocytes. *Mol.Pharmacol.* 36, 556-565

18. Matsui, T., Sekiguchi, M., Hashimoto, A., Tomita, U., Nishikawa, T., Wada, K. (1995) Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. *J.Neurochem.* 65, 454-458

19. Priestley, T., Laughton, P., Myers, J., Le Bourdelles, B., Kerby, J., Whiting, P. J. (1995) Pharmacological properties of recombinant human N-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. *Mol.Pharmacol.* 48, 841-848

20. Mori, H., Mishina, M. (1995) Structure and function of the NMDA receptor channel. *Neuropharmacology* 34, 1219-1237

21. Hashimoto, A., Nishikawa, T., Oka, T., Takahashi, K., Hayashi, T. (1992) Determination of free amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with N-tert.-butyloxycarbonyl-L-cysteine and o-phthaldialdehyde. *J.Chromatogr.* 582, 41-48

22. Hashimoto, A., Nishikawa, T., Hayashi, T., Fujii, N., Harada, K., Oka, T., Takahashi, K. (1992) The presence of free D-serine in rat brain. *FEBS Lett.* 296, 33-36

23. Hashimoto, A., Nishikawa, T., Oka, T., Takahashi, K. (1993) Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. *J.Neurochem.* 60, 783-786

24. SRINIVASAN, N. G., CORRIGAN, J. J., Meister, A. (1965) BIOSYNTHESIS OF D-SERINE IN THE SILKWORM, BOMBYX MORI. *J.Biol.Chem.* 240, 796-800

25. Uo, T., Yoshimura, T., Shimizu, S., Esaki, N. (1998) Occurrence of pyridoxal 5'-phosphate-dependent serine racemase in silkworm, Bombyx mori. *Biochem.Biophys.Res.Commun.* 246, 31-34

26. Wolosker, H., Sheth, K. N., Takahashi, M., Mothet, J. P., Brady, R. O., Jr., Ferris, C. D., Snyder, S. H. (1999) Purification of serine racemase: biosynthesis of the neuromodulator D-serine. *Proc.Natl.Acad.Sci.U.S.A* 96, 721-725

27. De Miranda, J., Santoro, A., Engelender, S., Wolosker, H. (2000) Human serine racemase: moleular cloning, genomic organization and functional analysis. *Gene* 256, 183-188

28. Mori, H., Inoue, R. (2010) Serine racemase knockout mice. *Chem.Biodivers.* 7, 1573-1578

29. Schell, M. J., Molliver, M. E., Snyder, S. H. (1995) D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. *Proc.Natl.Acad.Sci.U.S.A* 92, 3948-3952

30. Kartvelishvily, E., Shleper, M., Balan, L., Dumin, E., Wolosker, H. (2006) Neuron-derived D-serine release provides a novel means to activate N-methyl-D-aspartate receptors. *J.Biol.Chem.* 281, 14151-14162

31. Wako, K., Ma, N., Shiroyama, T., Semba, R. (1995) Glial uptake of intracerebroventricularly injected D-serine in the rat brain: an immunocytochemical study. *Neurosci.Lett.* 185, 171-174

32. Broer, A., Brookes, N., Ganapathy, V., Dimmer, K. S., Wagner, C. A., Lang, F., Broer, S. (1999) The astroglial ASCT2 amino acid transporter as a mediator of glutamine efflux. *J.Neurochem.* 73, 2184-2194

33. Ribeiro, C. S., Reis, M., Panizzutti, R., De Miranda, J., Wolosker, H. (2002) Glial transport of the neuromodulator D-serine. *Brain Res.* 929, 202-209

34. Matsuo, H., Kanai, Y., Tokunaga, M., Nakata, T., Chairoungdua, A., Ishimine, H., Tsukada, S., Ooigawa, H., Nawashiro, H., Kobayashi, Y., Fukuda, J., Endou, H. (2004) High affinity D- and L-serine transporter Asc-1: cloning and dendritic localization in the rat cerebral and cerebellar cortices. *Neurosci.Lett.* 358, 123-126

35. Rutter, A. R., Fradley, R. L., Garrett, E. M., Chapman, K. L., Lawrence, J. M., Rosahl, T. W., Patel, S. (2007) Evidence from gene knockout studies implicates Asc-1 as the primary transporter mediating d-serine reuptake in the mouse CNS. *Eur.J Neurosci.* 25, 1757-1766

36. Grillo, M. A., Fossa, T., Coghe, M. (1965) D-serine dehydratase of chicken kidney. *Enzymologia*. 28, 377-388

37. Krebs, H. A. (1935) Metabolism of amino-acids: deamination of amino acids. *Biochem.J.* 29, 1620-1644

38. Schell, M. J. (2004) The N-methyl D-aspartate receptor glycine site and D-serine metabolism: an evolutionary perspective. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 359, 943-964

39. Horiike, K., Tojo, H., Arai, R., Nozaki, M., Maeda, T. (1994) D-amino-acid oxidase is confined to the lower brain stem and cerebellum in rat brain: regional differentiation of astrocytes. *Brain Res.* 652, 297-303

40. Konno, R., Yasumura, Y. (1983) Mouse mutant deficient in D-amino acid oxidase activity. *Genetics* 103, 277-285

41. Verrall, L., Walker, M., Rawlings, N., Benzel, I., Kew, J. N., Harrison, P. J., Burnet, P. W. (2007) d-Amino acid oxidase and serine racemase in human brain: normal distribution and altered expression in schizophrenia. *Eur.J.Neurosci.* 26, 1657-1669

42. Wolosker, H. (2007) NMDA receptor regulation by D-serine: new findings and perspectives. *Mol.Neurobiol.* 36, 152-164

43. Balan, L., Foltyn, V. N., Zehl, M., Dumin, E., Dikopoltsev, E., Knoh, D., Ohno, Y., Kihara, A., Jensen, O. N., Radzishevsky, I. S., Wolosker, H. (2009) Feedback inactivation of D-serine synthesis by NMDA receptor-elicited translocation of serine racemase to the membrane. *Proc.Natl.Acad.Sci.U.S.A* 106, 7589-7594

44. Mustafa, A. K., van Rossum, D. B., Patterson, R. L., Maag, D., Ehmsen, J. T., Gazi, S. K., Chakraborty, A., Barrow, R. K., Amzel, L. M., Snyder, S. H. (2009) Glutamatergic regulation of serine racemase via reversal of PIP2 inhibition. *Proc.Natl.Acad.Sci.U.S.A* 106, 2921-2926

45. Wang, L. Z., Zhu, X. Z. (2003) Spatiotemporal relationships among D-serine, serine racemase, and D-amino acid oxidase during mouse postnatal development. *Acta Pharmacol.Sin.* 24, 965-974

46. Kim, P. M., Aizawa, H., Kim, P. S., Huang, A. S., Wickramasinghe, S. R., Kashani, A. H., Barrow, R. K., Huganir, R. L., Ghosh, A., Snyder, S. H. (2005) Serine racemase: activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration. *Proc.Natl.Acad.Sci.U.S.A* 102, 2105-2110

47. Horiike, K., Tojo, H., Arai, R., Yamano, T., Nozaki, M., Maeda, T. (1987) Localization of D-amino acid oxidase in Bergmann glial cells and astrocytes of rat cerebellum. *Brain Res.Bull.* 19, 587-596

48. Mothet, J. P., Parent, A. T., Wolosker, H., Brady, R. O., Jr., Linden, D. J., Ferris, C. D., Rogawski, M. A., Snyder, S. H. (2000) D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc.Natl.Acad.Sci.U.S.A* 97, 4926-4931

49. Stevens, E. R., Esguerra, M., Kim, P. M., Newman, E. A., Snyder, S. H., Zahs, K. R., Miller, R. F. (2003) D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. *Proc.Natl.Acad.Sci.U.S.A* 100, 6789-6794

50. Gustafson, E. C., Stevens, E. R., Wolosker, H., Miller, R. F. (2007) Endogenous D-serine contributes to NMDA-receptor-mediated light-evoked responses in the vertebrate retina. *J.Neurophysiol.* 98, 122-130

51. Panatier, A., Theodosis, D. T., Mothet, J. P., Touquet, B., Pollegioni, L., Poulain, D. A., Oliet, S. H. (2006) Glia-derived D-serine controls NMDA receptor activity and synaptic memory. *Cell* 125, 775-784

52. Henneberger, C., Papouin, T., Oliet, S. H., Rusakov, D. A. (2010) Long-term potentiation depends on release of D-serine from astrocytes. *Nature* 463, 232-236

53. Fossat, P., Turpin, F. R., Sacchi, S., Dulong, J., Shi, T., Rivet, J. M., Sweedler, J. V., Pollegioni, L., Millan, M. J., Oliet, S. H., Mothet, J. P. (2011) Glial D-Serine Gates NMDA Receptors at Excitatory Synapses in Prefrontal Cortex. *Cereb.Cortex*

54. Araque, A., Li, N., Doyle, R. T., Haydon, P. G. (2000) SNARE protein-dependent glutamate release from astrocytes. *J.Neurosci.* 20, 666-673

55. Mothet, J. P., Pollegioni, L., Ouanounou, G., Martineau, M., Fossier, P., Baux, G. (2005) Glutamate receptor activation triggers a calcium-dependent and SNARE

protein-dependent release of the gliotransmitter D-serine. *Proc.Natl.Acad.Sci.U.S.A* 102, 5606-5611

56. Martineau, M., Galli, T., Baux, G., Mothet, J. P. (2008) Confocal imaging and tracking of the exocytotic routes for D-serine-mediated gliotransmission. *Glia* 56, 1271-1284

57. O'Brien, K. B., Bowser, M. T. (2006) Measuring D-serine efflux from mouse cortical brain slices using online microdialysis-capillary electrophoresis. *Electrophoresis* 27, 1949-1956

58. Rosenberg, D., Kartvelishvily, E., Shleper, M., Klinker, C. M., Bowser, M. T., Wolosker, H. (2010) Neuronal release of D-serine: a physiological pathway controlling extracellular D-serine concentration. *FASEB J.* 24, 2951-2961

59. Ahmadi, S., Muth-Selbach, U., Lauterbach, A., Lipfert, P., Neuhuber, W. L., Zeilhofer, H. U. (2003) Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. *Science* 300, 2094-2097

60. Li, Y., Krupa, B., Kang, J. S., Bolshakov, V. Y., Liu, G. (2009) Glycine site of NMDA receptor serves as a spatiotemporal detector of synaptic activity patterns. *J.Neurophysiol.* 102, 578-589

61. Billups, D., Attwell, D. (2003) Active release of glycine or d-serine saturates the glycine site of NMDA receptors at the cerebellar mossy fibre to granule cell synapse. *Eur.J.Neurosci.* 18, 2975-2980

62. Coleman, P. A., Miller, R. F. (1988) Do N-methyl-D-aspartate receptors mediate synaptic responses in the mudpuppy retina? *J.Neurosci.* 8, 4728-4733

63. Zhang, J., Diamond, J. S. (2006) Distinct perisynaptic and synaptic localization of NMDA and AMPA receptors on ganglion cells in rat retina. *J.Comp Neurol.* 498, 810-820

64. Zhang, J., Diamond, J. S. (2009) Subunit- and pathway-specific localization of NMDA receptors and scaffolding proteins at ganglion cell synapses in rat retina. *J.Neurosci.* 29, 4274-4286

65. Coull, B. M., Cutler, R. W. (1978) Light-evoked release of endogenous glycine into the perfused vitreous of the intact rat eye. *Invest Ophthalmol.Vis.Sci.* 17, 682-684

66. Miller, R. F., Dacheux, R. F., Frumkes, T. E. (1977) Amacrine cells in Necturus retina: evidence for independent gamma-aminobutyric acid- and glycine-releasing neurons. *Science* 198, 748-750

67. Gottesman, J., Miller, R. F. (1992) Pharmacological properties of *N*-methyl-D-aspartate receptors on ganglion cells of an amphibian retina. *J.Neurophysiol.* 68, 596-604

68. Pow, D. V., Hendrickson, A. E. (1999) Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. *Vis.Neurosci.* 16, 231-239

69. Stevens, E. R., Gustafson, E. C., Miller, R. F. (2010) Glycine transport accounts for the differential role of glycine vs. D-serine at NMDA receptor coagonist sites in the salamander retina. *Eur.J.Neurosci.* 31, 808-816

70. Takayasu, N., Yoshikawa, M., Watanabe, M., Tsukamoto, H., Suzuki, T., Kobayashi, H., Noda, S. (2008) The serine racemase mRNA is expressed in both neurons and glial cells of the rat retina. *Arch.Histol.Cytol.* 71, 123-129

71. O'Brien, K. B., Miller, R. F., Bowser, M. T. (2005) D-Serine uptake by isolated retinas is consistent with ASCT-mediated transport. *Neurosci.Lett.* 385, 58-63

72. Dun, Y., Mysona, B., Itagaki, S., Martin-Studdard, A., Ganapathy, V., Smith, S. B. (2007) Functional and molecular analysis of D-serine transport in retinal Muller cells. *Exp.Eye Res.* 84, 191-199

73. Dun, Y., Duplantier, J., Roon, P., Martin, P. M., Ganapathy, V., Smith, S. B. (2008) Serine racemase expression and D-serine content are developmentally regulated in neuronal ganglion cells of the retina. *J.Neurochem.* 104, 970-978

74. Kalbaugh, T. L., Zhang, J., Diamond, J. S. (2009) Coagonist release modulates NMDA receptor subtype contributions at synaptic inputs to retinal ganglion cells. *J.Neurosci.* 29, 1469-1479

75. De Miranda, J., Panizzutti, R., Foltyn, V. N., Wolosker, H. (2002) Cofactors of serine racemase that physiologically stimulate the synthesis of the N-methyl-D-aspartate (NMDA) receptor coagonist D-serine. *Proc.Natl.Acad.Sci.U.S.A* 99, 14542-14547

76. Wolosker, H., Blackshaw, S., Snyder, S. H. (1999) Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proc.Natl.Acad.Sci.U.S.A* 96, 13409-13414

77. Basu, A. C., Tsai, G. E., Ma, C. L., Ehmsen, J. T., Mustafa, A. K., Han, L., Jiang, Z. I., Benneyworth, M. A., Froimowitz, M. P., Lange, N., Snyder, S. H., Bergeron, R., Coyle, J. T. (2009) Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior. *Mol.Psychiatry* 14, 719-727

78. DeVito, L. M., Balu, D. T., Kanter, B. R., Lykken, C., Basu, A. C., Coyle, J. T., Eichenbaum, H. (2011) Serine racemase deletion disrupts memory for order and alters cortical dendritic morphology. *Genes Brain Behav.* 10, 210-222

79. Schell, M. J., Brady, R. O., Jr., Molliver, M. E., Snyder, S. H. (1997) D-serine as a neuromodulator: regional and developmental localizations in rat brain glia resemble NMDA receptors. *J.Neurosci.* 17, 1604-1615

80. Miller, R. F. (2004) D-Serine as a glial modulator of nerve cells. *Glia* 43, 275-283

81. Stevens, E. R., Gustafson, E. C., Sullivan, S. J., Esguerra, M., Miller, R. F. (2010) Light-evoked NMDA receptor-mediated currents are reduced by blocking D-serine synthesis in the salamander retina. *Neuroreport* 21, 239-244

82. Sullivan, S. J., Miller, R. F. (2010) AMPA receptor mediated D-serine release from retinal glial cells. *J.Neurochem.* 115, 1681-1689

83. Cahill, H., Nathans, J. (2008) The optokinetic reflex as a tool for quantitative analyses of nervous system function in mice: application to genetic and drug-induced variation. *PLoS.One.* 3, e2055

84. Hamase, K., Konno, R., Morikawa, A., Zaitsu, K. (2005) Sensitive determination of D-amino acids in mammals and the effect of D-amino-acid oxidase activity on their amounts. *Biol.Pharm.Bull.* 28, 1578-1584

85. Reed, B. T., Sullivan, S. J., Tsai, G., Coyle, J. T., Esguerra, M., Miller, R. F. (2009) The glycine transporter GlyT1 controls N-methyl-D-aspartic acid receptor coagonist occupancy in the mouse retina. *Eur.J.Neurosci.* 30, 2308-2317

86. Durand, G. M., Kovalchuk, Y., Konnerth, A. (1996) Long-term potentiation and functional synapse induction in developing hippocampus. *Nature* 381, 71-75

87. Balu, D. T., Coyle, J. T. (2011) Glutamate receptor composition of the post-synaptic density is altered in genetic mouse models of NMDA receptor hypo- and hyperfunction. *Brain Res.* 1392, 1-7

88. Diaz, C. M., Macnab, L. T., Williams, S. M., Sullivan, R. K., Pow, D. V. (2007) EAAT1 and D-serine expression are early features of human retinal development. *Exp.Eye Res.* 84, 876-885

89. Somohano, F., Roberts, P. J., Lopez-Colome, A. M. (1988) Maturational changes in retinal excitatory amino acid receptors. *Brain Res.* 470, 59-67

90. Abdeljalil, J., Hamid, M., Abdel-Mouttalib, O., Stephane, R., Raymond, R., Johan, A., Jose, S., Pierre, C., Serge, P. (2005) The optomotor response: a robust first-line visual screening method for mice. *Vision Res.* 45, 1439-1446

91. Martina, M., Krasteniakov, N. V., Bergeron, R. (2003) D-Serine differently modulates NMDA receptor function in rat CA1 hippocampal pyramidal cells and interneurons. *J.Physiol* 548, 411-423

92. Chen, L., Muhlhauser, M., Yang, C. R. (2003) Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. *J.Neurophysiol.* 89, 691-703

93. Sullivan SJ, C. J. E. M. M. R. Light-evoked NMDA receptor currents in retinal ganglion cells are attenuated in mice lacking functional serine racemase. 641.9. 2010. Society for Neuroscience.

94. Fletcher, E. L., Hack, I., Brandstatter, J. H., Wassle, H. (2000) Synaptic localization of NMDA receptor subunits in the rat retina. *J.Comp Neurol.* 420, 98-112

95. Daniels, B. A., Baldridge, W. H. (2009) D-Serine enhancement of NMDA receptor-mediated calcium increases in rat retinal ganglion cells. *J.Neurochem.*

96. Haverkamp, S., Wassle, H. (2004) Characterization of an amacrine cell type of the mammalian retina immunoreactive for vesicular glutamate transporter 3. *J.Comp Neurol.* 468, 251-263

97. Hardingham, G. E., Fukunaga, Y., Bading, H. (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat.Neurosci.* 5, 405-414

98. Bordji, K., Becerril-Ortega, J., Nicole, O., Buisson, A. (2010) Activation of extrasynaptic, but not synaptic, NMDA receptors modifies amyloid precursor protein expression pattern and increases amyloid-ss production. *J.Neurosci.* 30, 15927-15942

99. Duffy, S., Labrie, V., Roder, J. C. (2008) D-serine augments NMDA-NR2B receptor-dependent hippocampal long-term depression and spatial reversal learning. *Neuropsychopharmacology* 33, 1004-1018

100. Verrall, L., Burnet, P. W., Betts, J. F., Harrison, P. J. (2010) The neurobiology of D-amino acid oxidase and its involvement in schizophrenia. *Mol.Psychiatry* 15, 122-137

101. Labrie, V., Fukumura, R., Rastogi, A., Fick, L. J., Wang, W., Boutros, P. C., Kennedy, J. L., Semeralul, M. O., Lee, F. H., Baker, G. B., Belsham, D. D., Barger, S. W., Gondo, Y., Wong, A. H., Roder, J. C. (2009) Serine racemase is

associated with schizophrenia susceptibility in humans and in a mouse model. *Hum.Mol.Genet.* 18, 3227-3243

102. Rosenberg, D., Kartvelishvily, E., Shleper, M., Klinker, C. M., Bowser, M. T., Wolosker, H. (2010) Neuronal release of D-serine: a physiological pathway controlling extracellular D-serine concentration. *FASEB J.*

103. O'Brien, K. B., Esguerra, M., Miller, R. F., Bowser, M. T. (2004) Monitoring neurotransmitter release from isolated retinas using online microdialysis-capillary electrophoresis. *Anal.Chem.* 76, 5069-5074

104. Newman, E. A., Zahs, K. R. (1998) Modulation of neuronal activity by glial cells in the retina. *J.Neurosci.* 18, 4022-4028

105. Zhang, W., Robert, A., Vogensen, S. B., Howe, J. R. (2006) The relationship between agonist potency and AMPA receptor kinetics. *Biophys.J.* 91, 1336-1346

106. Tsubokawa, H., Oguro, K., Masuzawa, T., Nakaima, T., Kawai, N. (1995) Effects of a spider toxin and its analogue on glutamate-activated currents in the hippocampal CA1 neuron after ischemia. *J.Neurophysiol.* 74, 218-225

107. Vorwerk, C. K., Naskar, R., Schuettauf, F., Quinto, K., Zurakowski, D., Gochenauer, G., Robinson, M. B., Mackler, S. A., Dreyer, E. B. (2000) Depression of retinal glutamate transporter function leads to elevated intravitreal glutamate levels and ganglion cell death. *Invest Ophthalmol.Vis.Sci.* 41, 3615-3621

108. Shimamoto, K., Sakai, R., Takaoka, K., Yumoto, N., Nakajima, T., Amara, S. G., Shigeri, Y. (2004) Characterization of novel L-threo-beta-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. *Mol.Pharmacol.* 65, 1008-1015

109. Vitanova, L. (2007) Non-NMDA receptors in frog retina: an immunocytochemical study. *Acta Histochem.* 109, 154-163

110. Wakakura, M., Yamamoto, N. (1994) Cytosolic calcium transient increase through the AMPA/kainate receptor in cultured Muller cells. *Vision Res.* 34, 1105-1109

111. Newman, E. A. (2005) Calcium increases in retinal glial cells evoked by light-induced neuronal activity. *J.Neurosci.* 25, 5502-5510

112. Kato, S., Ishita, S., Sugawara, K., Mawatari, K. (1993) Cystine/glutamate antiporter expression in retinal Muller glial cells: implications for DL-alpha-aminoadipate toxicity. *Neuroscience* 57, 473-482

113. Wang, J. S., Estevez, M. E., Cornwall, M. C., Kefalov, V. J. (2009) Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat.Neurosci.* 12, 295-302

114. Wang, J. S., Kefalov, V. J. (2009) An alternative pathway mediates the mouse and human cone visual cycle. *Curr.Biol.* 19, 1665-1669

115. Bringmann, A., Pannicke, T., Biedermann, B., Francke, M., Iandiev, I., Grosche, J., Wiedemann, P., Albrecht, J., Reichenbach, A. (2009) Role of retinal glial cells in neurotransmitter uptake and metabolism. *Neurochem.Int.* 54, 143-160

116. Zeevalk, G. D., Nicklas, W. J. (1990) Chemically induced hypoglycemia and anoxia: relationship to glutamate receptor-mediated toxicity in retina. *J.Pharmacol.Exp.Ther.* 253, 1285-1292

117. Vorwerk, C. K., Zurakowski, D., McDermott, L. M., Mawrin, C., Dreyer, E. B. (2004) Effects of axonal injury on ganglion cell survival and glutamate homeostasis. *Brain Res.Bull.* 62, 485-490

118. Schuettauf, F., Naskar, R., Vorwerk, C. K., Zurakowski, D., Dreyer, E. B. (2000) Ganglion cell loss after optic nerve crush mediated through AMPA-kainate and NMDA receptors. *Invest Ophthalmol.Vis.Sci.* 41, 4313-4316

119. Schuettauf, F., Quinto, K., Naskar, R., Zurakowski, D. (2002) Effects of anti-glaucoma medications on ganglion cell survival: the DBA/2J mouse model. *Vision Res.* 42, 2333-2337

120. Kirschner, D. L., Wilson, A. L., Drew, K. L., Green, T. K. (2009) Simultaneous efflux of endogenous D-ser and L-glu from single acute hippocampus slices during oxygen glucose deprivation. *J.Neurosci.Res.* 87, 2812-2820

121. Mustafa, A. K., Ahmad, A. S., Zeynalov, E., Gazi, S. K., Sikka, G., Ehmsen, J. T., Barrow, R. K., Coyle, J. T., Snyder, S. H., Dore, S. (2010) Serine racemase deletion protects against cerebral ischemia and excitotoxicity. *J.Neurosci.* 30, 1413-1416

122. Hama, Y., Katsuki, H., Tochikawa, Y., Suminaka, C., Kume, T., Akaike, A. (2006) Contribution of endogenous glycine site NMDA agonists to excitotoxic retinal damage in vivo. *Neurosci.Res.* 56, 279-285

123. Pow, D. V., Hendrickson, A. E. (2000) Expression of glycine and the glycine transporter Glyt-1 in the developing rat retina. *Vis.Neurosci.* 17, 1R-9R

124. Lee, S. C., Zhong, Y. M., Yang, X. L. (2005) Expression of glycine receptor and transporter on bullfrog retinal Muller cells. *Neurosci.Lett.* 387, 75-79

125. Supplisson, S., Bergman, C. (1997) Control of NMDA receptor activation by a glycine transporter co-expressed in Xenopus oocytes. *J.Neurosci.* 17, 4580-4590