AMPA receptor mediated D-serine release from retinal glial cells

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Abstract

The 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. D-serine appears to be the major coagonist acting on retinal ganglion cell NMDA receptors, but the cell type from which it originates and whether its release can be modulated by activity are unknown. In this study, we utilized a mutant mouse line with elevated D-serine to investigate this question. Direct measurements of extracellular D-serine using capillary electrophoresis demonstrate that D-serine can be released from the intact mouse retina through an AMPA receptor dependent mechanism. AMPA-evoked D-serine release persisted in the presence of a cocktail of neural inhibitors but was abolished after administration of a glial toxin. These findings provide the first evidence that extracellular D-serine levels in the retina can be modulated, and that such modulation is contingent upon glial cell activity.

Keywords

Retina; D-serine; NMDA receptor; AMPA receptor; capillary electrophoresis; glia

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 (Johnson and Ascher 1987;Kleckner and Dingledine 1988). 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 (Hashimoto *et al*. 1992), especially in regions rich in NMDARs (Schell *et al*. 1995). 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 (Wolosker *et al*. 1999). D-serine has since been implicated in numerous mechanisms ascribed to NMDARs, including neuroplasticity (Basu *et al*. 2009;Panatier *et al*. 2006) and learning and memory (Duffy *et al*. 2008). Furthermore, abnormalities in D-serine regulation have been implicated in the NMDAR hypofunction theory of schizophrenia (Verrall *et al*. 2010;Labrie *et al*. 2009).

Early immunohistochemical and ultrastructural studies localized D-serine to astrocytes adjacent to neurons expressing NMDARs (Schell *et al*. 1997). A similar result was later confirmed in the retina, where D-serine was found in astrocytes and Müller glia (Stevens *et al*. 2003). However, the recent discovery of D-serine in neurons throughout the brain (Kartvelishvily *et al*. 2006) and serine racemase mRNA in retinal neurons (Takayasu *et al*. 2008) brings into question the idea that D-serine originates solely from glia in the CNS.

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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 (Gustafson *et al.* 2007;Stevens *et al.* 2003) 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 (Kim *et al.* 2005) and release of D-serine (Mothet *et al.* 2005). However, cultured cortical neurons (Kartvelishvily *et al.* 2006) and, more recently, neurons from cortical slice have displayed similar AMPA-evoked D-serine release (Rosenberg *et al.* 2010). 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 (O'Brien *et al.* 2004), 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 (Konno and Yasumura 1983). 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 (O'Brien *et al.* 2005). 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 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.

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 (Newman and Zahs 1998). 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 (Hamase *et al*. 2005). 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).

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 (Zhang *et al*. 2006)) 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 (Tsubokawa *et al*. 1995), 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 (Vorwerk *et al*. 2000). We treated the retinas with TFB-TBOA, a high affinity EAAT1 and EAAT2 blocker (Shimamoto *et al*. 2004), which dramatically elevated extracellular glutamate over the course of 50 minutes (Figure 3A, 3B).

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 (O'Brien *et al*. 2005;Dun *et al*. 2007).

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.

For possibility (3) to hold true, AMPA must have some direct effect on glia. Müller cells appear to express AMPA receptors (Vitanova 2007) and are responsive to AMPA in isolation (Wakakura and Yamamoto 1994). 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 (Newman 2005), the purenurgic (P2) antagonist suramin was added. Whole-mount retinas were incubated in Fluo-4 AM over time periods favoring glial loading (Newman and Zahs 1998). 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.

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 (Kato *et al*. 1993), and eliminates retinal Müller cell function, while retaining the retina's responsiveness to light (Wang *et al*. 2009;Wang and Kefalov 2009). 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 (Bringmann *et al*. 2009), 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.

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 (Mothet *et al*. 2005). Because the coagonist site is not saturated on RGC NMDARs (Gustafson *et al*. 2007;Kalbaugh *et al*. 2009;Daniels and Baldridge 2009;Stevens *et al*. 2003), 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 (Hamase *et al*. 2005). 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 (Zeevalk and Nicklas 1990), optic nerve crush (Vorwerk *et al*. 2004;Schuettauf *et al*. 2000), and models of glaucoma (Schuettauf *et al*. 2002). 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 (Kirschner *et al*. 2009) and serine racemase knockout mice are protected against ischemia (Mustafa *et al*. 2010), while the addition of coagonist exaggerates NMDA excitotoxicity in the retina and D-serine removal attenuates it (Hama *et al*. 2006).

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 (O'Brien *et al*. 2005), expressed primarily by glial cells (Dun *et al*. 2007). 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 (Kalbaugh *et al*. 2009). 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 (Gustafson *et al*. 2007), implying that released glycine was not reaching the coagonist site.

Glycine serves as a major inhibitory neurotransmitter in the retina (Miller *et al*. 1977). 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 (Pow and Hendrickson 2000)

and Müller (Lee *et al*. 2005) 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 (Supplisson and Bergman 1997), despite the fact that glycine and D-serine have comparable potencies in expression systems void of amino acid transporters (McBain *et al*. 1989). Similarly, mutant mice with reduced expression of the GlyT1 transporter display greater coagonist site occupancy in the retina (Reed *et al*. 2009). 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 (O'Brien *et al*. 2005;Dun *et al*. 2007), unlike the cortex where the high affinity transport ASC-1 is also expressed (Rutter *et al*. 2007). 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.

Acknowledgments

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Abbreviations

AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
AAA	α-aminoadipic acid
ASCT2	alanine-serine-cysteine transporter
cyclo	cylothiazide
DAO-	D-amino acid oxidase mutant
DsDa	D-serine deaminase
EAAT	excitatory amino acid transporter
GlyT1	Glycine transporter 1
GYKI 52466	4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride
NAS	1-naphthyl acetyl spermine
NBDF	4-fluoro-7-nitrobenz-2-oxa-1,3-diazole
NMDAR	N-methyl-D-aspartate receptor
TFB-TBOA	(3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid
TTX	tetrodotoxin
wt	wild type
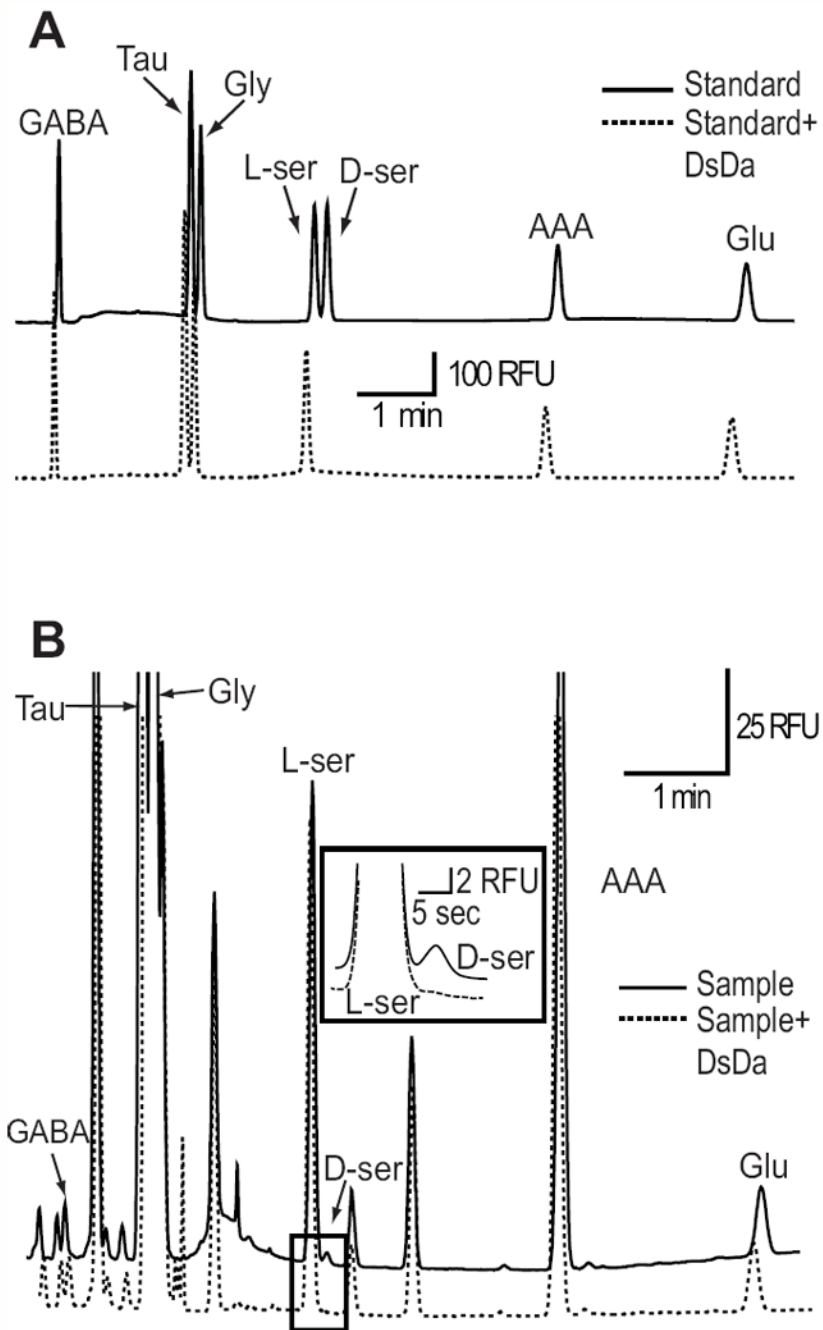
Figure 1.
Identification of D-serine via capillary electrophoresis. **(A)** Electropherogram showing the separation of D-serine (D-ser), from a series of amino acid standards; Gamma-aminobutyric acid (GABA), Taurine (Tau), Glycine (Gly), L-serine (L-ser), α-aminoadipic acid (AAA), and Glutamate (Glu). 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.
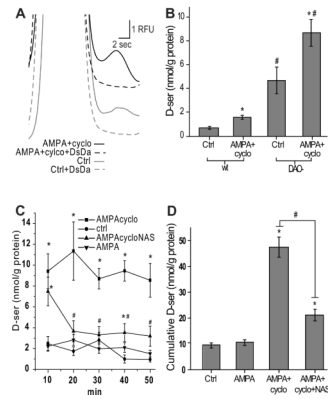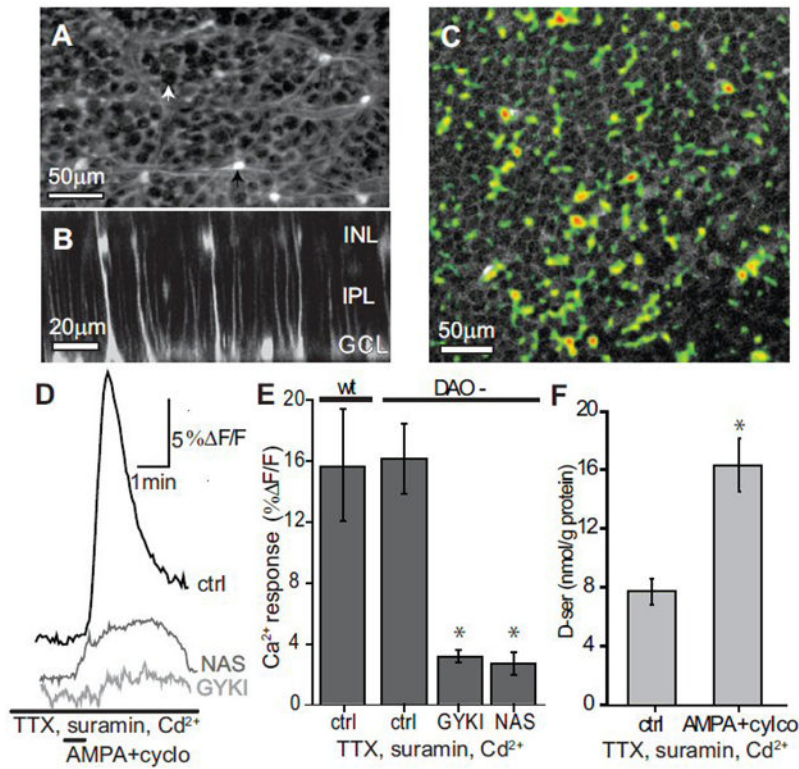
Figure 2.
AMPA+cyclothiazide evokes D-serine release in wt and DAO- retinas. **(A)** Extracellular D-serine in DAO- retinas incubated for 50 minutes (Ctrl) or with 50 μM AMPA plus 50 μM cyclothiazide (AMPA+cylco). Both peaks were eliminated by DsDa. **(B)** Comparison of extracellular D-serine between 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.
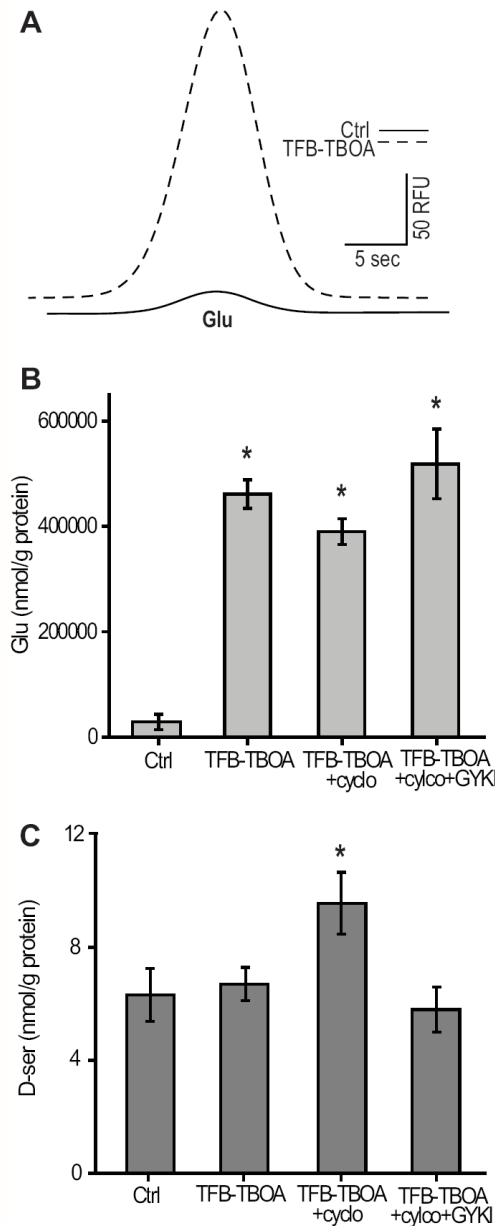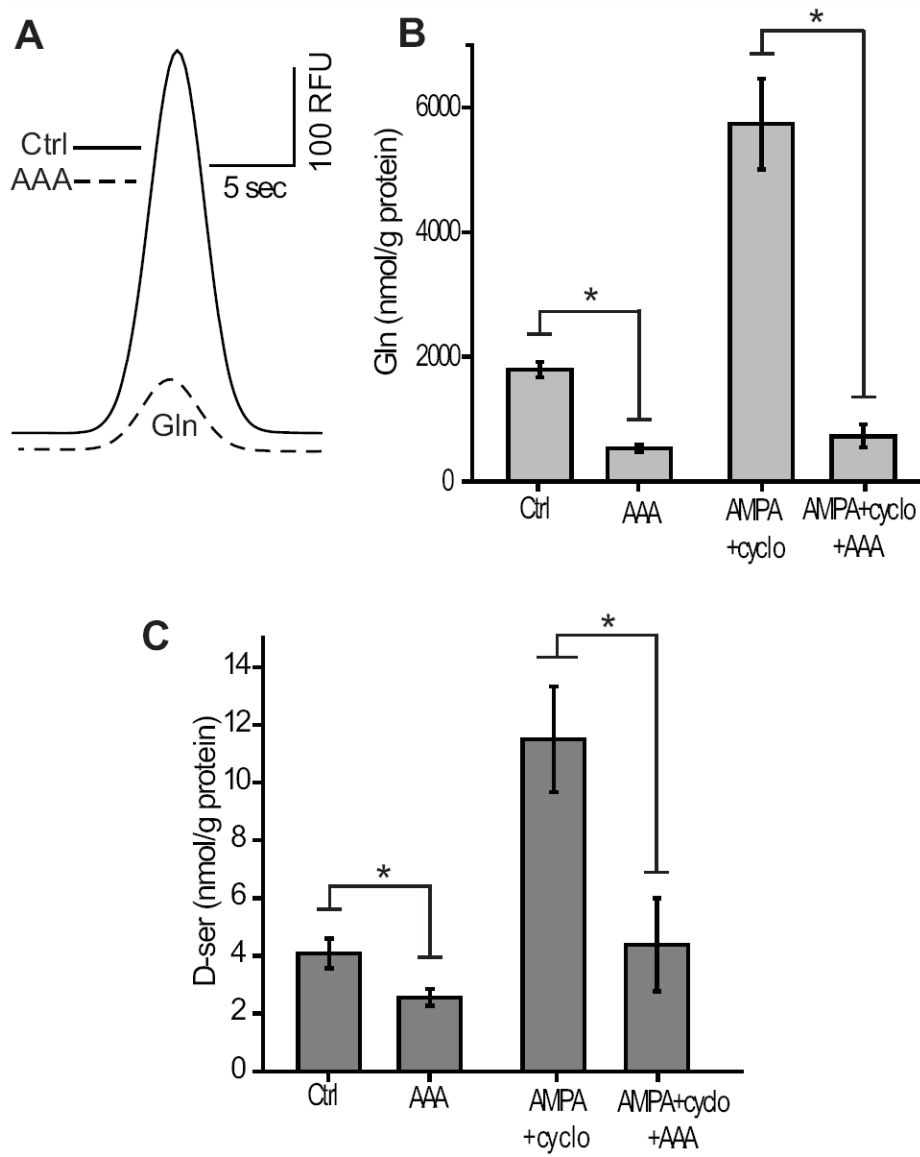
Figure 3.
Blocking Glutamate uptake elevates extracellular D-serine by acting through AMPA receptors. **(A)** Raw trace showing glutamate (Glu) increase following a 50 minute incubation in 10 μM TFB-TBOA. **(B)** TFB-TBOA significantly elevated Glu in all conditions tested. There was no difference in 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).

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+cylco still released D-serine in the combined presence of
TTX, suramin, and Cd^{2+}. (*, statistically different from control, p<0.05, n=4-5).

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).

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.

	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

*
statistically different from wt at p<0.05. n=5.