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Atypical expression and activation of GluN2A and GluN2B-containing NMDA receptors at ganglion cells during retinal degeneration

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Abstract

Cellular communication through chemical synapses is determined by the nature of the neurotransmitter and the composition of postsynaptic receptors. In the excitatory synapse between bipolar and ganglion cells of the retina, postsynaptic AMPA receptors mediate resting activity. During evoked response, however, more abundant and sustained levels of glutamate also activate GluN2B-containing NMDA receptors (NMDARs). This phasic recruitment of distinct glutamate receptors is essential for visual discrimination; however, the fidelity of this basic mechanism under elevated glutamate levels due to aberrant activity, a common pathophysiology, is not known. Here, in both male and female mice with retinal degeneration (*rd10*), a condition associated with elevated synaptic activity, we reveal that changes in synaptic input to ganglion cells altered both composition and activation of NMDARs. We found that, in contrast to wildtype, the spontaneous activity of *rd10* cells was largely NMDAR-dependent. Surprisingly, this activity was driven primarily by atypical activation of GluN2A –containing NMDARs, not GluN2B-NMDARs. Indeed, immunohistochemical analyses and Western blot showed greater levels of the GluN2A-NMDAR subunit expression in *rd10* retina compared to wildtype. Overall, these results demonstrate how aberrant signaling leads to pathway-specific alterations in NMDAR expression and function.

Keywords

NMDA; receptors; retinal degeneration; subunit; remodeling

Introduction

Retinal degeneration (RD) encompasses a variety of diseases that lead to photoreceptor cell loss and functional changes to surviving tissue (Marc et al, 2003). Of particular importance

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is the increase in glutamatergic signaling observed across retinal cell classes (Borowska et al, 2011; Marc et al, 2007; Margolis et al, 2008; Stasheff, 2008; Ye & Goo, 2007; Yee et al, 2012). Bipolar cells and amacrine cells undergo anatomical and functional remodeling following disease onset (Chua et al, 2009; Strettoi & Pignatelli, 2000; Strettoi et al, 2002); however, it remains unclear whether remodeling occurs in retinal ganglion cells (RGCs), particularly given their relative morphological and functional stability following RD (Margolis et al, 2008; Mazzoni et al, 2008). This stability suggests the presence of a mechanism to mitigate damage to RGCs from the potentially excitotoxic conditions that result from neuronal hyperactivity in RD. Since RGCs are the only output of the retina, their proper function is critical for any retina-based repair strategy.

It is currently unknown how increased glutamatergic signaling affects RD RGCs. In the synapse between bipolar cells and ON RGCs, spontaneous excitatory postsynaptic currents (sEPSCs) are primarily mediated by AMPARs, while evoked currents have an additional NMDAR-mediated component (Fig. 1A)(Chen & Diamond, 2002). Elevations in synaptic glutamate concentration lead to the activation of GluN2B-containing NMDARs via glutamate escaping the immediate postsynaptic region, in a process known as spillover activation (Matsui et al, 1998). In a mouse mutant lacking the GABA_A receptor, in which RGC spontaneous activity is elevated, sEPSCs exhibit a significantly increased NMDAR component relative to *wt* sEPSCs (Sagdullaev et al, 2006). Based on these findings, we hypothesized that the increased spontaneous activity seen in RD RGCs recruits GluN2B-NMDAR during both spontaneous and evoked activity.

Using wholemount retina from *rd10* mice (Fig. 1B), a model of RD with a mutation in the *Pde6b* gene, a frequent cause of *Retinitis Pigmentosa* in human patients, we surprisingly found that spontaneous activity of *rd10* α -ON RGCs was not mediated by elevated activation of GluN2B-NMDAR. Instead, this spontaneous activity was driven by GluN2A-NMDARs, which were expressed atypically by *rd10* α -ON RGCs. The changes to NMDAR expression patterns in α -ON RGCs provide evidence that RGCs are not impervious to the retinal degenerative process, but rather undergo functional and structural remodeling.

Methods

Animals.

In all experimental procedures, animals were treated according to regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, in accordance with protocols approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine, and the NIH Guide for the Care and Use of Laboratory Animals. Thirty three wildtype (RRID:IMSR_JAX:000664) and 47 *rd10* (RRID:MGI_3581193) mice of either sex between 90–160 days old were obtained from the Jackson Laboratory (Bar Harbor, ME).

Experimental Design.

Preparation of retinal whole mounts. Methods for retinal wholemount preparation have been described in detail previously (Toychiev et al, 2013b). Briefly, after the animal was euthanized, its eyes were enucleated and placed in HEPES solution, constantly supplied with

oxygen. The cornea, iris and lens were removed. The retina was dissected into four equal quadrants. Quadrants were attached photoreceptor surface down to a modified Millicell Biopore filter (Millipore, Bedford, MA). This preparation was transferred to a recording chamber on the stage of an upright Nikon FN1 microscope equipped with Hoffman modulation contrast optics (Modulation Optics, Inc.; Greenvale, NY) and continuously perfused with bicarbonate-buffered Ames' medium supplemented with D-serine (100 μ M) (Sigma, St. Louis, MO) at a rate of 2 mL/min. Pharmacological agents were applied using an eight-channel superfusion system (Warner Instruments, Hamden, CT). All experiments were performed at a near physiological temperature of 32°C. All reagents were obtained from Sigma (St. Louis, MO) or Tocris (Minneapolis, MN).

Electrophysiology.

To compare RGCs of similar classes, α -ON RGCs were targeted based on large soma size and number of primary dendritic processes (Sun et al, 2002). In light-responsive cells, responses to brief illumination were recorded at the beginning of electrophysiological experiments. This was done to correlate the physiological identity of the cell to its dendritic stratification relative to the choline acetyltransferase (ChAT) ON-band during following morphometric analysis (see below). The identity of the cell was confirmed by staining for SMI-32 (Fig. 1B), a selective marker for α -like RGCs (Lin et al, 2004). Extracellular spiking activity was obtained from RGCs in a loose-patch mode using the electrode filled with HEPES-buffered extracellular Ringer's solution, containing the following (in mM): 137 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 Na-HEPES, 28 glucose, pH 7.4. Whole-cell recordings were made from RGCs using patch pipettes filled with either intracellular solution 1 or 2, depending on experimental protocol. Intracellular solution 1 contained (in mM): 120 Cs-gluconate, 10 tetraethylammonium chloride (TEA-Cl), 1.0 CaCl₂, 1.0 MgCl₂, 11 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 10 sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Na-HEPES), adjusted to pH 7.2 with CsOH. Intracellular solution 2 contained (in mM): 106 Cs-gluconate, 14 CsCl, 15 TEA-Cl, 1.0 CaCl₂, 1.0 MgCl₂, 11 EGTA, and 10 Na-HEPES, adjusted to pH 7.2 with cesium hydroxide. The calculated E_{Cl} for these intracellular solutions were approximately -60 and -35 mV, respectively. Here, spontaneous activity, both cell spiking and input currents, is defined as activity under constant conditions. Evoked activity is a deviation in cellular properties in response to light or other exogenous stimuli.

Following application of each pharmacological agent, activity was recorded for 10–15 minutes to allow for the cell to reach a stable state. Recording sessions lasted for approximately an hour. Electrodes were pulled from borosilicate glass (1B150F-4; WPI, Sarasota, FL) with a P-97 Flaming/Brown puller (Sutter Instruments, Novato, CA) and had a measured resistance of ~4–7 M Ω . All recordings were made with a MultiClamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Data were filtered at 5 kHz with a four-pole Bessel filter and were sampled at 15 kHz. Spiking activity and currents were analyzed using Signal (CED, Cambridge, UK). Individual sEPSC events were analyzed using Mini Analysis Program (RRID:SciRes_000143, Synaptosoft Inc., Decatur, GA). Each event was inspected and spurious noise peaks and overlapping events were rejected. The charge transfer (Q), an integral current measured as an area above the event curve, was

determined for each event, and the mean value (of 109–349 events) of each measure was calculated for each cell.

Cell identification.

During the physiological recordings, the pipette solution was supplemented with 0.05% sulforhodamine B or 50 μM Alexa Fluor 568 hydrazide to visualize targeted cell. Fluorescent images of the cell were first documented with a modified Nikon D5000 camera attached to the patch-clamp microscope. Next, the retina was fixed and immuno labeled for SMI-32, a marker of all α -RGCs and choline acetyltransferase (ChAT), a “landmarker” of the inner plexiform layer (IPL) to distinguish ON α -RGCs.

After the staining, the cell was found based on the original photograph taken under the Nikon microscope. The confocal z-stack of the entire cell was acquired with a Nikon C-1 microscope at 1 μm steps using a 20x water objective. Next, the preparation was imaged as confocal Z-stacks, taken at an area of $212 \times 212 \mu\text{m}^2$ and 0.6 μm step size with a 60x oil objective to achieve precise dendritic stratification relative to the ChAT-bands and to confirm the cell's immunoreactivity to SMI-32. The Alexa 568 filled cell, as well as ChAT and SMI-32 fluorescent images were taken. For vertical views, a narrow region of the Z-stack was rotated 90 degrees using ImageJ software (RRID:nif-0000-30467). The rotation of the stacks was adjusted based on the cholinergic bands, which formed thin, well-separated bands with the perfectly vertical orientation of the stack. RGCs whose dendrites stratified closer to the OFF-cholinergic band were classified as OFF-RGCs; the cells whose dendrites stratified closer to the ON-cholinergic band were classified as ON-RGCs.

Immunohistochemistry.

The distribution of NMDAR GluN2A subunit was quantified in retinal wholemounts prepared as previously described (Ivanova et al., 2013). Briefly, living retinas were isolated from the eye cups and mounted onto modified Biopore filter as described for patch-clamp recordings. The retinas attached to the filter were submersion-fixed in freshly prepared 4% carbodiimide in 0.1 M phosphate saline (PBS, pH = 7.3) for 15 minutes. Retinal wholemounts were blocked for 1 h in a PBS solution containing 5% Chemiblocker (membrane-blocking agent, Chemicon), 0.5% Triton X-100, and 0.05% sodium azide (Sigma). Primary antibodies were diluted in the same solution and applied for 48 h, followed by incubation for 24 h in the appropriate secondary antibody, conjugated to Alexa 488 (1:800; green fluorescence, Molecular Probes), Alexa 568 (1:800; red fluorescence, Molecular Probes), Alexa 647 (1:100; far red fluorescence, Molecular probes), or Cy5 (1:400; far red fluorescence, Jackson). In multilabeling experiments, sections were incubated in a mixture of primary antibodies, followed by a mixture of secondary antibodies. All steps were carried out at room temperature. After staining, the retina was flat mounted on a slide, ganglion cell layer up, and coverslipped using Vectashield mounting medium (H-1000, Vector Laboratories). The coverslip was sealed in place with nail polish. To avoid extensive squeezing and damage to the retina, small pieces of a broken glass cover slip (Number 1 size) were placed in space between the slide and the coverslip. The Alexa 568 fluorescence was sufficient to visualize the Alexa 568-filled neurons, without antibody enhancement. For the RGCs that were backfilled with sulforhodamine B, the dendritic processes of a RGC

were identified based on their appearance on the confocal reconstruction performed after the electrophysiological recording. The primary antibodies used in this study were the following: goat anti-ChAT (Millipore, Cat. AB144P, 1:2000, RRID:AB_2079751), rabbit anti-NMDAR GluN2A (Millipore, Cat. AB1555P, 1:400, RRID:AB_90770), and mouse anti-SMI-32 (Covance, Cat. SMI-32R, 1:2000, RRID:AB_509997).

GluN2A labeling was quantified in four *rd10* and *wt* retinal wholemounts triple labeled for GluN2A, choline acetyltransferase (ChAT, to distinguish ON and OFF sublaminae in the IPL), and SMI-32 (to visualize the ON α -subtype of RGCs). In contrast to cryostat sections, staining of a retinal wholemount permits tracing of the dendrites of the largest and brightest somas of α -cells. A series of confocal images was taken with 0.4 μ m Z-steps with 60x oil objective. The dendrites of each RGC were studied in single confocal sections at the cell's stratification plane. It has previously been shown that SMI-32 predominantly labels α -RGCs (Lin et al, 2004), although a subset of SMI-32-positive cells are also labeled (Coombs et al, 2006). To avoid these cells, which have smaller soma and dendritic tree diameters, we selected brightly-labeled RGCs that had larger soma diameter (> 21 μ m). Large SMI-32-positive cells were further classified as ON α -RGCs based on their dendritic stratification strictly below the ON-ChAT band. In each of four retinas, four short pieces of dendrites (~30 μ m in length) of 3 ON α -RGCs were analyzed (~300 puncta per single cell). The images were converted to inverted grayscale and the NMDAR GluN2A -positive puncta were evaluated using ImageMaster 2D Platinum 7 software.

Western blot analysis.

Western blot was performed as previously described, with minor modifications (Ma et al, 2010; Willis et al, 2005). Briefly, retina were dissected, rinsed in HEPES buffer and then lysed with RIPA buffer supplemented with phosphatase and proteinase inhibitors using 50 mg protein extraction beads in a Bioruptor Plus (5 cycles of 30 seconds on/30 seconds off; Diagenode). For experiments in which the photoreceptors were removed, *wt* retinas were attached to a filter with photoreceptor cells down, and 'shaved' with a blade. The tissue from the blade was collected for the Western blot analysis. Twenty micrograms of protein was resolved on NuPAGE Bis-Tris 4–12% gradient gels (Life Technologies) and transferred to polyvinylidene difluoride (PVDF). Blots were immunolabeled with antibodies against NMDAR GluN2A (1:500, Millipore, Cat. AB1555P RRID:AB_90770), NMDAR GluN2B (1:500, Millipore, Cat. AB1557 RRID:AB_2112907), RBPMS (1: 2000, Phosphosolutions, 1832- RBPMS RRID:AB_2395389), or β -actin (1:5000, Sigma, Cat. A5316 RRID:AB_476743) overnight at 4°C. In mouse, RBPMS (RNA-binding protein with multiple splicing) is a highly specific marker of all RGCs (Kwong et al, 2010; Rodriguez et al, 2014). Blots were washed and incubated at room temperature for 1 hour with IR-dye-conjugated antibodies against rabbit, mouse, or goat IgG (1:10000; LI-COR Biosciences) and then rinsed three times. Blots were imaged with Odyssey IR quantitative Western blot system (LI-COR Biosciences, Lincoln, NE). Densitometry was performed using ImageJ (RRID:nif-0000-30467) and values are expressed relative to β -actin or RBPMS. Results are graphed as mean $\pm$ SEM of three independent replicates. ANOVA and Tukey's post-test were used to determine significance ($p < 0.05$). Statistical analysis was performed using GraphPad Prism software (RRID:rid_000081).

Statistical analysis.

Statistical analysis was performed in either SPSS v. 22 (IBM, RRID:SCR_003210) or SigmaPlot v.11 (Systat, RRID:SCR_003210). Prior to parametric analysis, the data was tested for and found to have normal distributions and equal variances. For two group comparisons t-test was used. For multiple group comparisons, analysis of variance (ANOVA) or repeated measures ANOVA were used. Corrections were made using a post-hoc Tukey's test. The data are presented as mean $\pm$ standard deviation (SDM). The number of samples in parenthesis (n) indicates number of animals per group. To avoid introduction of non-independent data into statistical analysis, first, multiple samples from an individual animal were averaged within the subject, then the data between animals were compared (Aarts, Verhage et al. 2014).

Results

Retinal degeneration leads to elevated aberrant activity in the inner retina, which increases glutamatergic input to RGCs. To assess the effects of this elevated activity, we (i) determined the contribution of NMDAR versus non-NMDAR input to the elevated spontaneous activity in *rd10* α -ON RGCs, (ii) examined the relative contribution of distinct type of NMDARs in identified α -ON RGCs, and (iii) used Western blot and immunohistochemistry to determine the expression and distribution of distinct NMDARs in the retina.

NMDARs contribute to spontaneous activity in *rd10* RGCs

In wildtype α -ON RGCs, spontaneous excitatory activity is mediated primarily by synaptic AMPARs, while evoked responses have a significant NMDAR component (Fig. 1C, D), reflecting the recruitment of NMDARs (Chen & Diamond, 2002; Cohen, 2000; Sagdullaev et al, 2006). During RD, RGCs exhibit spontaneous excitatory events with magnitudes similar to light-evoked currents (Toychiev et al., 2013). Therefore, we hypothesized that the spontaneous activity of RD RGCs has an NMDAR component. To test this, we recorded spiking activity following the application of D-AP5 (50 μ M) and CNQX (5 μ M), selective antagonists of NMDARs and non-NMDA glutamate receptors, respectively. In *rd10* α -ON RGCs, D-AP5 eliminated rhythmicity and significantly reduced the resting spike rate (18.1 ± 3.26 control to 7.81 ± 1.70 D-AP5; $n = 7$ (in 5 mice); $p = 0.01$, repeated measures ANOVA, Fig. 1E). Subsequent application of CNQX further reduced spike rate (2.94 ± 0.92 D-AP5+CNQX, $n = 7$ (5); $p = 0.01$). This effect of NMDAR antagonist in *rd10* retina was different from its action on *wt* RGCs. In particular, in *wt* α -ON RGC spontaneous spiking activity is not affected by D-AP5 (5.75 ± 3.72 control to 5.27 ± 2.10 D-AP5; $n = 9$ (5); $p = 0.38$, repeated measures ANOVA, Fig. 1E). This is consistent with the fact that in *wt* α -ON RGCs NMDARs are activated only during evoked responses (Fig. 1D).

Next, to determine synaptic mechanisms leading to atypical effect of NMDAR blockers on spiking activity, we measured the effect of pharmacological agents on sEPSCs that mediate this spiking activity of *rd10* α -ON RGCs. At a holding potential of -60 mV, the reversal for chloride, D-AP5 reduced the number and amplitude of sEPSCs (Fig. 2B); however, holding the cell at -60 mV also induces voltage-dependent magnesium block of NMDARs, which prevents the full recruitment of NMDARs (Nowak et al, 1984). This was corrected by

increasing the chloride concentration in the patch pipette's intracellular solution and holding the cell at -35 mV, the chloride reversal, while preserving physiological levels of Mg^{2+} and Ca^{2+} in extracellular solution (Intracellular solution 2 in Methods). This approach also avoids the necessity of blocking inhibitory transmission, which would lead to network effects on bipolar cell output (Eggers & Lukasiewicz, 2006; Sagdullaev et al, 2006; Zhang et al, 1997), including the emergence of spontaneous compound excitatory events (Yee et al, 2012). The effect of D-AP5 was more pronounced under these conditions. Overall, glutamate receptor antagonists had similar effects on currents and spike rate, with a significant effect of both D-AP5 and CNQX (-0.081 ± 0.01 control to -0.030 ± 0.005 D-AP5 to 0.00039 ± 0.0065 D-AP5+CNQX, $n=7(4)$; $p = 0.01$, repeated measures ANOVA, Fig. 2C). Larger contribution of non-NMDARs at -60 mV vs -35 mV (Fig. 2B, left and right panels in presence of D-AP5) is consistent with an increase in driving force for the cation current at holding potential further away from their reversal (0 mV). Consistent with earlier findings, in *wt* α -ON RGCs application of D-AP5 did not affect sEPSCs (-0.017 ± 0.008 control to -0.016 ± 0.009 , $n=7(4)$; $p = 0.29$, repeated measures ANOVA, Fig. 2A).

The origin of the NMDAR-dependent component of *rd10* RGC spontaneous activity is unclear, as NMDARs localize on RGC dendrites as well as on presynaptic cells (Haverkamp & Wässle, 2000, Kalloniatis et al., 2004). In particular, blocking NMDARs on presynaptic amacrine cells could modify bipolar cell output to RGCs. To separate the contribution of direct stimulation of RGC NMDARs from that of the NMDARs of the presynaptic network, we applied intracellular MK-801 (1 mM), infused through the patch pipette, to block only the NMDARs of the recorded RGC (Fig. 3A, top). sEPSCs were monitored for 15 minutes under each condition in the following progression: (1) immediately following cell break-in (control), (2) infusion of MK-801 into the recorded cell, and (3) bath application of D-AP5 (50 μ M) alone and (4) in combination with CNQX (5 μ M) (Fig. 3A, traces). In pilot experiments, prior to bath application of D-AP5, the complete infusion of intracellular solution from the recording pipette was verified by including sulforhodamine in the pipette solution. Visualizing the sulforhodamine under fluorescent illumination indicated that the pipette solution completely filled the cell. In these experiments, we also determined that MK-801 has reached its full effect on RGC activity within 10–15 minutes from the onset of the whole cell configuration (Fig. 3B).

In α -ON RGCs, MK-801 significantly reduced sEPSC charge transfer (-0.0877 ± 0.00291 to $-0.0323 \pm 2.21e-3$ Q/s, $n = 9(5)$; $p = 0.001$, repeated-measures ANOVA; Fig. 3C). The subsequent addition of D-AP5 to block remaining NMDARs did not significantly affect charge transfer (-0.0323 ± 0.00221 to -0.0231 ± 0.00262 Q/s, $n = 9(5)$; $p = 0.103$, repeated measures ANOVA), establishing that the NMDAR component of *rd10* RGC activity is primarily mediated by NMDARs localized on RGC dendrites, and not in the presynaptic network.

Receptors containing the GluN2A subunit primarily mediate the NMDAR component of *rd10* ON RGC sEPSCs

In *wt* ON RGCs, spontaneous glutamate release activates synaptic AMPA glutamate receptors, while elevations in glutamate concentration activate GluN2B-NMDA receptors (Chen & Diamond, 2002; Sagdullaev et al, 2006). Given the increased glutamate receptor activation in RD RGCs (Marc et al., 2007), we hypothesized that the increased NMDAR component of *rd10* RGC spontaneous activity is primarily mediated by these receptors. RGCs have been shown to contain synaptic and perisynaptic NMDARs, and specific subunits are associated with both RGC type and localization; the NMDARs of ON RGCs are predominantly perisynaptic, and express the GluN2B subunit (Zhang & Diamond, 2009). Therefore, we monitored the spiking activity of *rd10* ON RGCs during the application of a series of glutamate receptor antagonists, starting with antagonists of the GluN2B subunit.

Surprisingly, Ro 25–6981 (5 μ M), a highly selective blocker of NMDARs containing the GluN2B subunit (Fischer et al, 1997; Manookin et al, 2010; Zhang & Diamond, 2009), only marginally reduced spike rate ($p = 0.04$), while subsequent additions of D-AP5 ($p = 0.001$) and CNQX ($p = 0.01$) significantly reduced spike rate to a larger extent (Fig. 4A, $n = 8(5)$, control: 16.37 ± 1.023 , Ro 25–6981: 12.72 ± 1.162 , D-AP5: 5.27 ± 0.738 , CNQX: 1.804 ± 0.1876). We confirmed this in a separate experiment, in which ifenprodil (10 μ M), another selective antagonist of NMDARs containing the GluN2B subunit (Manookin et al, 2010; Williams, 1993), also did not greatly affect spike rate (Fig. 4B, $n = 7(4)$; $p = 0.043$, repeated measures ANOVA). Subsequent additions of D-AP5 ($p = 0.001$) and CNQX ($p = 0.001$) both significantly reduced spike rate (control: 17.63 ± 0.711 , ifenprodil: 15.00 ± 0.738 , D-AP5: 8.33 ± 0.463 , CNQX: 2.21 ± 0.985). To make sure that the effect of these agents did not arise from altering responses of AMPARs, we recorded sEPSCs, first applying CNQX to isolate the NMDAR component (Fig. 4C). CNQX did not significantly affect charge transfer ($p = 0.402$), nor did ifenprodil ($p = 0.899$). Subsequent addition of D-AP5 abolished sEPSCs ($p = 0.01$) (Fig. 4C, $n = 10(5)$, control: -0.0850 ± 0.0135 , CNQX: -0.0692 ± 0.0150 , ifenprodil: -0.072 ± 0.016 , D-AP5: -0.0012 ± 0.001). Together, these data suggest that *rd10* α -ON RGCs have increased expression NMDARs containing the GluN2A subunit.

Atypical spontaneous activation of GluN2A-containing NMDARs suggests that *rd10* ON RGCs utilize a different mechanism than *wt* cells in response to elevated levels of glutamate. To test this, we applied TBOA (5 μ M), a selective antagonist of excitatory amino acid transporters, to elevate synaptic glutamate concentration and increase the charge transfer of *wt* sEPSCs (Fig. 5). TBOA concentration was chosen to match *wt* sEPSC magnitude to that of individual (non-compound) *rd10* sEPSCs. The average charge transfer of *wt* sEPSCs following TBOA application (*wt*+TBOA) was used as a baseline value for normalization in statistical analysis. Next, we compared the effects of specific glutamate receptor antagonists on the activity of α -ON RGCs. Application of ifenprodil to block GluN2B-NMDARs significantly decreased charge transfer of *wt* sEPSCs (ifenprodil: $67.3 \pm 14.7\%$ of baseline, $n = 7(4)$ cells, $p = 0.024$, repeated measures ANOVA), and did not differ from control (prior to TBOA) levels ($54.5 \pm 4.93\%$ of baseline, $n = 7(4)$, $p = 0.525$ vs. ifenprodil, repeated measures ANOVA). Subsequent application of D-AP5 to block remaining NMDARs did not

additionally affect *wt* sEPSCs ($57.9 \pm 8.97\%$, $n = 5(4)$, $p = 1.00$ vs ifenprodil, repeated measures ANOVA). In contrast, ifenprodil did not significantly affect *rd10* sEPSCs (control: $104 \pm 11.6\%$, ifenprodil: $107 \pm 4.87\%$, $n = 5(4)$, $p = 1.00$, repeated measures ANOVA), while D-AP5 reduced sEPSCs ($63.6 \pm 5.12\%$, $n = 5$, $p = 0.001$ vs ifenprodil) to *wt* control levels ($p = 0.28$ vs *wt* control). This suggests that GluN2A-NMDARs are the primary mediators of the elevated glutamate concentration in *rd10* α -ON RGCs, in contrast to *wt* α -ON RGCs, which rely on GluN2B-NMDARs.

GluN2A subunit expression is increased in the *rd10* ON sublamina

It has previously been shown that bipolar and amacrine cells have modified glutamate receptor expression following photoreceptor degeneration (Chua et al, 2009; Strettoi & Pignatelli, 2000). The electrophysiological results described above suggest an increased role of GluN2A-NMDARs in *rd10* α -ON RGCs. To address this, Western blot analysis was performed to compare levels of GluN2A and GluN2B between *wt* and *rd10* retinas (Fig. 6). Retinas from both eyes of *wt* ($n = 6$ animals) and *rd10* ($n = 6$ animals) were used in the analysis. Indeed, GluN2A levels in *rd10* retina were significantly greater than in *wt* ($61 \pm 31.12\%$ increase over *wt*, $p = 0.0271$, t-test), while GluN2B levels did not differ ($9.0 \pm 23.1\%$ increase over *wt*, $p = 0.7750$, t-test, Fig. 6 A,B). However, these data do not account for the presence of photoreceptors in *wt* potentially contributing to altered levels of β -actin, a protein commonly used as a reference in Western blot. To eliminate this possibility we first compared the levels of GluN2A in *rd10* to *wt* retina in which the photoreceptor layer has been removed. In these experiments, *wt* retinas were attached to a Millicell Biopore filter (Millipore, Bedford, MA) with the photoreceptor cells facing the filter tissue using gentle suction, and scraped or 'shaved' with a blade (insert in Fig. 6C). The tissue from the blade was collected for the Western blot analysis. Second, the levels of GluN2A were normalized to RNA-binding protein with multiple splicing (RBPMS). In mouse, RBPMS is a highly specific marker of all RGCs (Kwong et al., 2010; Rodriguez et al., 2014). The advantage of this approach is that, in contrast to β -actin, RBPMS is present only in RGCs, which are believed to be the least affected neurons in RD. Consistent with the data from whole retinas using β -actin as reference, we found greater levels of GluN2A in *rd10* compared to *wt* whole retina ($p = 0.0151$, ANOVA) and *wt*-shaved retina ($p = 0.0211$, ANOVA), when normalized to RBPMS (Fig. 6D). There was no significant difference between *wt*-whole and *wt*-shaved retinas ($p = 0.1827$, ANOVA).

Next, to provide further anatomical support for the functional change observed in the physiological data, we immunolabeled *wt* and *rd10* retinal wholemounts with an antibody that recognizes the GluN2A-NMDAR subunit ($n = 3$ mice each). To insure that the differences in GluN2A expression is specific to α -ON RGCs, we utilized SMI-32, a neurofilament antibody that has been shown to predominantly label α -RGCs (Coombs et al, 2006; Lin et al, 2004; Nixon et al, 1989). Since many RGC subtypes exist, this approach allows for the comparison of the same cell type across animal models. Brightly-labeled cells with large somas were targeted in retinal whole mounts, and were classified as α -ON RGCs according to the inner stratification of their dendritic arbors relative to the ON ChAT band (Fig. 1B, see Methods). Co-staining for the GluN2A subunit allowed for analyses of punctal density and size along segments of RGC dendrites (Fig. 7A). We found that GluN2A density

of *rd10* α -ON RGC dendrites was significantly greater than *wt* (Fig. 7B, 0.73 ± 0.08 in *rd10* vs. 0.48 ± 0.02 in *wt*; $p = 0.005$, $n = 3$ mice, t-test). Finally, the size of GluN2A puncta was compared. The GluN2A puncta size was significantly higher in *rd10* vs. *wt* α -ON RGC dendrites (Fig. 7C, 0.21 ± 0.01 in *rd10* vs. 0.17 ± 0.01 in *wt*; $n = 3$ mice, $p = 0.005$, t-test). These results support that the elevated glutamatergic activity in *rd10* leads to an upregulation of the synaptic GluN2A subunit in α -ON RGCs. Together, these results extend the physiological data, indicating increased GluN2A expression by *rd10* α -ON RGCs.

Discussion

Here, we have described a link between aberrant activity and functional changes in glutamate receptor expression in *rd10* mouse retina. The elevated spontaneous activity of *rd10* α -ON RGCs has a significant NMDAR component (Figs. 1 and 2), in contrast to *wt*. Direct glutamatergic input to RGCs mediates this additional component, rather than auxiliary network effects (Fig. 3). This activity was not eliminated by a selective block of the GluN2B-NMDARs, suggesting that *rd10* α -ON RGCs have increased expression NMDARs containing the GluN2A subunit (Figs. 4–5). This was further confirmed by Western blot and immunohistochemical analyses (Fig. 6–7). Below, we will discuss the events that may lead to altered NMDAR subunit expression and potential role for this change as a protective mechanism for RGC survival in RD.

Changes in neuronal activity in RD alter glutamate receptor expression in RGCs

It has previously been shown that RD can lead to aberrant expression of glutamate receptors in bipolar cells of *rd1* (Chua et al, 2009; Strettoi & Pignatelli, 2000; Strettoi et al, 2002) and *rd10* mice (Barhoum et al, 2008; Puthussery et al, 2009), which suggests that modifications to glutamatergic input can lead to changes to neuronal function. While it has been shown that glutamatergic drive to RGCs is increased during RD (Marc et al, 2007), it is unclear whether this is associated with changes in receptor expression. The magnitude of individual spontaneous events observed in RD RGCs resemble light responses (Toychiev et al, 2013a; Yee et al, 2012), suggesting that NMDARs may partially mediate resting activity, and/or that glutamate receptor expression is altered in these cells. Indeed, there is an increased NMDAR component to the spontaneous activity of *rd10* α -ON RGCs (Fig. 4), and a significantly greater expression of the GluN2A subunit in the ON sublamina of the IPL (Figs. 6–7). GluN2A subunit is associated with RGC synapses, while the GluN2B subunit is located perisynaptically (Zhang & Diamond, 2009). It is important to note that our experiments do not provide the spatial resolution to distinguish between synaptic and perisynaptic receptors, despite the respective localizations established in *wt* retina. Nevertheless, the data indicate an increase in GluN2A expression in α -ON RGCs, and its potential role in RD.

Why would there be an increase in GluN2A-NMDARs, but not GluN2B-NMDARs? In *wt* ON RGCs, spontaneous activity predominantly engages AMPARs, while evoked responses additionally recruit perisynaptic NMDA receptors, due to the saturation of synaptic receptors and glutamate spillover (Sagdullaev et al, 2006; Zhang & Diamond, 2006). Given the significant increase in spontaneous activity in *rd10* RGCs, an increased contribution of

GluN2B-NMDARs was expected. Surprisingly, we instead observed an upregulation of the GluN2A subunit, and no overall change in expression of the GluN2B subunit (Figs. 6, 7). This suggests the importance of the ratio of GluN2A to GluN2B expression in maintaining synaptic connections (Yashiro et al 2008). An increase in GluN2A expression has also been noted in transgenic *rdta* mice, in which rod photoreceptors are genetically ablated (Liu et al, 2001). There was an accompanying increase in GluN2B expression, but this was attributed to the early onset of rod degeneration during development, in contrast to *rd10* mice, in which rods develop fully and do not begin to degenerate until after eye-opening.

Our results suggest that ON RGCs alter NMDAR expression in response to elevated spontaneous glutamate transmission, by increasing both the total expression and the density of the GluN2A subunit (Fig. 7). This may take advantage of an existing mechanism for synaptogenesis in RGCs. The GluN2A subunit has been associated with synaptogenesis and long-term potentiation in the superior colliculus and visual cortex (Cho et al, 2009; de Marchena et al, 2008; Zhao & Constantine-Paton, 2007), with an obligatory role for metaplasticity in the latter (Philpot et al, 2007). Meanwhile, GluN2B has been associated with long-term depression, and is obligatory under certain circumstances (Collingridge et al, 2010). NMDARs in visual cortex undergo a switch from containing GluN2B to GluN2A following visual experience (Quinlan et al, 1999a; Quinlan et al, 1999b), and it has recently been shown, using interneurons derived from the medial ganglionic eminence, that this switch can be driven acutely by repetitive synaptic activation (Matta et al, 2013). Thus, the hyperactivity that occurs during RD may stimulate a mechanism, mediated by GluN2A, responsible for preserving synaptic connections to RGCs. The increased expression of GluN2A may reduce activation of the GluN2B subunit, which is auxiliary or detrimental to this preservation.

Physiological role for altered NMDAR expression in RD

Elevated activity in retinal disease increases endogenous glutamatergic signaling to RGCs (Marc et al, 2007). It has long been established that excitotoxicity from excess glutamate can have adverse effects on the retina (Lucas & Newhouse, 1957), particularly from NMDAR activation (Seki & Lipton, 2008); however, it has also been shown that RGCs within intact retina are particularly resilient to glutamate excitotoxicity (Ullian et al, 2004). Indeed, RGCs in *rd10* show few morphological changes and survive until late stages of degeneration (Mazzoni et al, 2008; Stasheff et al, 2011). In contrast to RGCs, amacrine cells are highly vulnerable to glutamate toxicity (Ullian et al, 2004). As shown in the *rd1* mouse, functional activation of amacrine cell NMDARs declines steadily during RD (Chua et al, 2009). Our findings suggest that the increased expression of the GluN2A subunit may contribute to the survival of RGCs. Indeed, GluN2A- and GluN2B-NMDAR subunits have opposing influences on cell survival (reviewed by Brassai et al, 2014). In forebrain neurons, acute expression of GluN2B-NMDARs *in vitro* and *in vivo* enhanced NMDA-induced neurotoxicity compared to GluN2B-NMDARs (Martel et al., 2012). In retinal cell cultures exposed to NMDA-induced excitotoxic conditions, curcumin, a neuroprotective compound with a yet unknown mechanism of action, reduces cell death and induces an increase in GluN2A expression (Matteucci et al, 2011; Matteucci et al, 2005). Conversely, pharmacological inhibition or cre-mediated ablation of the GluN2B subunit results in

increased RGC survival under excitotoxic conditions (Bai et al, 2013). It is thus possible that the increased expression of the GluN2A subunit in the *rd10* mouse reflects a survival mechanism of ON RGCs, to promote synaptic NMDAR activation and reduce extrasynaptic activation.

In earlier studies, we and others have revealed that ON and OFF RGCs have different expression and activation patterns of GluN2A and GluN2B-containing NMDARs (Sagdullaev et al., 2006; Zhang and Diamond, 2009). This poses an intriguing possibility for distinct pathway specific changes in NMDAR activation during RD, a focus of an ongoing investigation in our laboratory. Although the primary deficit of RD is photoreceptor death, the aberrant activity that occurs afterward can introduce secondary complications, such as the alteration of NMDAR expression described here. Previously, we have shown that aberrant activity can be treated pharmacologically to increase the fidelity of signal transmission in degenerated retina (Toychiev et al., 2013b). Our current data suggest that the hyperactivity that occurs during RD induces stress on RGCs, triggering an upregulation of GluN2A expression in α -ON RGCs, which may represent a potential survival mechanism. It is unclear whether these changes affect visual signal processing, however, and this should be explored in future studies. These results emphasize the importance of addressing complications of RD, along with the primary loss of photoreceptors, when developing treatment strategies to restore vision.

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Highlights

- Phasic recruitment of distinct glutamate receptors is essential for visual discrimination.
- The fidelity of this fundamental mechanism under aberrant activity, a common pathophysiology, is not known.
- Aberrant activity leads to pathway-specific alterations in NMDA receptor subunit composition and function.

Figure 1. NMDARs significantly contribute to spontaneous spiking activity in *rd10* α -ON RGCs. **A**, Diagram illustrating distinct distribution and roles for glutamate receptors in ON RGCs. In *wt*, spontaneously released glutamate activates AMPARs, while perisynaptic NMDARs are not activated. In *rd10*, the effect of increased synaptic glutamate is unknown. **B**, Confocal reconstruction of a representative α -ON RGC in retinal wholemount. The neuron was backfilled with the Alexa568 following electrophysiological recording. The stratification depth and the identity of the α -ON RGCs were consequently confirmed by immunostaining for choline acetyl transferase (ChAT) and SMI-32, respectively. An arrowhead points to the α -ON RGC axon. **C**, Spontaneous action potentials decrease with the sequential application of D-AP5 (50 μ M) and CNQX (5 μ M) to block NMDARs and non-NMDA iGluRs, respectively. **D**, In *wt* α -ON RGCs, NMDARs are activated exclusively during light-evoked activity. **E**, Average spike rates under different experimental conditions. AP/s – action potentials per second. Error bars indicate SDM. ** $p < 0.001$.

Figure 2. NMDARs significantly contribute to spontaneous EPSCs in *rd10* α -ON RGCs. To reduce Mg^{2+} block of NMDARs, spontaneous currents were recorded using a modified intracellular solution with an increased chloride concentration at a holding potential of -35 mV, the reversal for Cl^{-} in both wt and rd10 α -ON RGCs. **A**, In wt, NMDARs do not contribute to sEPSCs. **B**, In contrast, NMDARs significantly contribute to sEPSCs in *rd10*, even when whole-cell sEPSCs were obtained with the pipette filled with standard intracellular solution recorded at a holding potential of -60 mV; however, their role is underestimated due to Mg^{2+} block. **C**, Average sEPSCs charge transfers acquired at -35 mV, under various experimental conditions. Error bars indicate SDM. $^{**}p < 0.001$.

Figure 3. The NMDAR-mediated component of spontaneous activity is due to a direct input to RGCs, not a network effect.

A, Representative sEPSCs were recorded in whole cell configuration from morphologically identified α -ON RGCs, using a pipette filled with intracellular solution containing MK-801 (1 mM) to block NMDARs only within the recorded α -ON RGC without disrupting NMDAR transmission in the retinal network. Illustrations depict intracellular application of MK-801 and subsequent bath application of D-AP5 (50 μ M) and CNQX (5 μ M). **B**, The time course of MK-801 infusion and blockade of NMDARs within the α -ON RGCs. **C**, Average sEPSCs charge transfers under different experimental conditions in *rd10* α -ON RGCs. Error bars indicate SDM. ** $p < 0.001$.

Figure 4. Spontaneous excitatory activity in *rd10* α -ON RGCs is driven by AMPA and synaptic GluN2A-NMDA receptors.

A, Traces show spontaneous spiking activity of a representative *rd10* α -ON RGC. Graph shows average frequency of spiking activity across different conditions. The addition of Ro 25–6891 (5 μ M), a selective blocker of NMDARs containing the GluN2B subunit, has small effect on spontaneous spike rate. Subsequent additions of D-AP5 (50 μ M), a broad spectrum NMDAR antagonist, and CNQX (5 μ M) an antagonist of non-NMDA glutamate receptors, both significantly reduced spike rate to a much larger extent compared to Ro 25–6891. **B**, Effect of ifenprodil (10 μ M), a selective blocker of NMDARs containing the GluN2B subunit on α -ON RGC spontaneous spiking activity ($n = 7$). Subsequent additions of D-AP5 and CNQX both significantly reduced spike rate. **C**, Traces show sEPSCs recorded at Cl[−] reversal potential of −35 mV with high chloride intracellular solution, as in previous figures. The NMDAR component was isolated following application of CNQX (10 μ M). Graphs

show average charge transfer during sEPSCs across different conditions. Error bars indicate SDM. * $p < 0.01$, ** $p < 0.001$.

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Figure 5. Elevated glutamate concentration activates different NMDARs in *wt* α -ON RGCs than in *rd10*.

A, Representative sEPSC recordings from *wt* and *rd10* at $V_h = -35$ mV under control conditions and following the application of pharmacological agents. **B**, Representative averaged sEPSCs recorded under different pharmacological conditions from morphologically identified *wt* (P110, $n = 349$ events from $n = 7$ cells) and *rd10* (P96, $n = 194$ events from $n = 7$) α -ON RGCs. In *rd10*, only individual events were selected for the analysis, and not those occurring as compound overlapping events. The number next to each reagent indicates the order of application. **C**, Average sEPSCs charge transfers of *wt* and *rd10* α -ON RGC under various conditions, normalized to *wt* following TBOA application (*wt*+TBOA), indicated by dotted line. Ifenprodil returned *wt* sEPSCs to control levels, but did not significantly affect *rd10* sEPSCs. Additional application of D-AP5 decreased *rd10* to *wt* control levels. Error bars indicate SDM. * $p < 0.01$, ** $p < 0.001$.

Figure 6. Western blot analysis GluN2A and GluN2B subunits expression in retinas from *wt* and *rd10* mice.

A, GluN2A and GluN2B expression levels in individual animals. **B**, Densitometry shows greater levels of GluN2A in *rd10* relative to *wt* ($p = 0.0271$). Measurements were normalized to β-actin. **C**, GluN2A levels in *wt* (whole retinas and shaved) and *rd10* mice. **D**, Densitometry shows greater levels of GluN2A in *rd10* compared to *wt*-whole retina ($p = 0.0151$) and *wt*-shaved retina ($p = 0.0211$). There is no significant difference between *wt* whole and shaved ($p = 0.1827$). Insert illustrates photoreceptor shaving procedure in *wt* retina. Measurements were normalized to RNA-binding protein with multiple splicing (RBPMS). Error bars indicate SDM.

Figure 7. Expression of GluN2A-NMDAR subunit in α -ON RGCs in *rd10* mice.

A, High magnification images of dendrites from *wt* and *rd10* α -ON RGCs identified with selective marker SMI-32 (green) and co-labeled for NMDAR GluN2A subunit (red). Only the dendrites from identified α -ON RGCs which stratified proximal of the ON-ChAT band were selected (see Fig. 1B). Dashed boxes show regions of interest magnified in middle and lower panels. Only GluN2A puncta associated with the dendrites of the RGCs are shown. GluN2A subunit density (**B**) and puncta size (**C**) were both significantly higher in *rd10* α -ON RGCs compared to *wt*. * $p < 0.01$. Error bars indicate SDM.