Involvement of AMPA Receptor and Its Flip and Flop Isoforms in Retinal Ganglion Cell Death Following Oxygen/Glucose Deprivation

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PURPOSE. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR) subunits can be posttranscriptionally modified by alternative splicing forming flip and flop isoforms. We determined if an ischemia-like insult to retinal ganglion cells (RGCs) increases AMPAR susceptibility to s-AMPA-mediated excitotoxicity through changes in posttranscriptional modified isoforms.

METHODS. Purified neonatal rat RGCs were subjected to either glucose deprivation (GD) or oxygen/glucose deprivation (OGD) conditions followed by treatment with either 100 μM s-AMPA or Kainic acid. A live–dead assay and caspase 3 assay was used to assess cell viability and apoptotic changes, respectively. We used JC-1 dye and dihydroethidium to measure mitochondria depolarization and reactive oxygen species (ROS), respectively. Calcium imaging with fura-2AM was used to determine intracellular calcium, while the fluorescently-labeled probe, Nanoprobe1, was used to detect calcium-permeable AMPARs. Quantitative PCR (qPCR) analysis was done to determine RNA editing sites AMPAR isoforms.

RESULTS. Glucose deprivation, as well as an OGD insult followed by AMPAR stimulation, produced a significant increase in RGC death. Retinal ganglion cell death was independent of caspase 3/7 activity, but was accompanied by increased mitochondrial depolarization and increased ROS production. This was associated with an elevated intracellular Ca2+ and calcium permeable-AMPARs. The mRNA expression of GLUA2 and GLUA3 flop isoform decreased significantly, while no appreciable changes were found in the corresponding flip isoforms. There were no changes in the Q/R editing of GLUA2, while R/G editing of GLUA2 flop declined under these conditions.

CONCLUSIONS. Following oxidative injury, RGCs become more susceptible to AMPAR-mediated excitotoxicity. RNA editing and changes in alternative spliced flip and flop isoforms of AMPAR subunits may contribute to increased RGC death.

Keywords: AMPA, flip/flop isoforms, cell death, RGCs, ADAR proteins

Glaucoma is a heterogeneous group of optic neuropathies associated commonly with elevated IOP that affects approximately 70 million people worldwide.1 It is the second leading cause of vision loss, and the number one leading cause of irreversible blindness.2 Glaucoma is characterized by the cupping of the optic disc and degeneration of the optic nerve, and is accompanied by slow and progressive death of retinal ganglion cells (RGCs), thus leading to the loss of the visual field.3,4 The etiologic mechanisms underlying the pathogenesis of glaucoma have yet to be elucidated.

Many cellular and molecular mechanisms have been proposed to account for the death of RGCs in glaucoma. Of these proposed mechanisms, ischemia and excitotoxicity appear to have a key role in glaucomatous pathogenesis.5 Increased immunohistochemical staining of hypoxia-inducible factor-1, a transcription factor induced by hypoxia, was observed in human glaucomatous retinas and optic nerve heads, providing the supporting evidence for the role of retinal ischemia in glaucoma.6 It is thought that tissue modeling at the optic nerve head, accompanying insufficient blood flow to the retina, exacerbates cupping of the optic nerve head, thereby compromising the retina’s access to oxygen, nutrients, and the ability to remove waste.7,8 In the retina, where metabolic demand is high, this could lead to depletion of ATP, causing the deregulation of mitochondrial bioenergetics, and provoking the increased production of reactive oxygen species (ROS), causing oxidative damage and eventual cell death, in particular of the RGCs.9,10

One of the main factors associating retinal ischemia with RGC death is the excitatory amino acid, glutamate. The neurotransmitter, glutamate, relays signals in the vertical pathway of the retina by the activation of ionotropic glutamate receptors (iGluRs), allowing the influx of monovalent and divalent cations, propagating action potentials.11,12 However, under conditions of retinal ischemia, abnormal concentrations of glutamate are released into the extracellular
milieu of the retina, causing a large influx of \([\text{Ca}^{2+}]\) through the activated iGluRs on RGCs, leading to deregulation of calcium-dependent cellular events and, therefore, mediating excitotoxicity in the RGCs.\textsuperscript{10,13–15} The iGluRs are composed of the N-methyl-D-aspartate receptors (NMDAR), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), and kainate receptors (KAR).\textsuperscript{34} All three receptors have been implicated in glutamate excitotoxicity in RGCs, with NMDAR receptors being the most widely studied receptor.\textsuperscript{5,17–19} However, recent findings are pinpointing AMPARs to have an equally large role in mediating excitotoxicity to RGCs.\textsuperscript{20–23}

The AMPARs-mediated excitotoxicity in RGCs is well established in the field evidenced by numerous publications demonstrating AMPAR-mediated damage to the ganglion cell layer.\textsuperscript{17,21,23–25} However, many of these studies were performed in total retina, in vivo, or mixed culture of retinal cells.\textsuperscript{17,24–26,27} Additionally, AMPARs-mediated excitotoxicity was conducted with either glutamate or kainic acid (KA), neither of which is specific for the AMPARs.\textsuperscript{24,28,29} These confounding factors make it hard to discern if AMPAR in RGCs are contributing to excitotoxicity directly, or as a secondary effect. In our previous studies,\textsuperscript{30} we demonstrated that stimulating AMPAR with \(\alpha\)-AMPA (a highly selective agonist) in a purified RGC culture, does not induce excitotoxicity, but instead, AMPAR RGC survival increases, suggesting that AMPAR isoforms play a role in excitotoxicity during disease conditions, in which the AMPARs-mediated excitotoxicity, like using cyclothiazide, induced RGC death and is the determinant for excitotoxicity. The AMPARs are hetero/homo tetrameric structures that are composed of 4 different subunits, GLUA1-4.\textsuperscript{31} Each subunit can be posttranscriptionally modified by alternative splicing in a region of the extracellular loop between TM3 and TM4, forming flip and flop isoforms.\textsuperscript{32} Flip and flop isoforms are expressed differently during development leading to a high level of expression of flip isoforms and a low expression of the flop isoforms. However, following development, the flop isoform increases, making the flip to flop isoform closer to a 50:50 ratio.\textsuperscript{33} Kinetically, these isoforms behave differently; typically, the flip isoforms have slower desensitization time, faster recovery time, producing a larger current amplitude and ion permeability, to \(\text{Ca}^{2+}\).\textsuperscript{33,35} Also, GLUA4 can be edited at the R/G site, where editing of the R/G site produces a faster desensitization and a faster recovery time from desensitization.\textsuperscript{56–58}

The complex heterogeneity of AMPARs isoforms may have a role in excitotoxicity during disease conditions, in which changes in AMPAR's posttranscriptional modifications (altering desensitization, recovery time, and ion permeability), may increase neuron susceptibility to AMPAR-mediated excitotoxicity. The involvement of the changes in AMPARs flip to flop ratios have been observed in clinical or experimental settings of neurodegenerative diseases and neuro trauma, such as amyotrophic lateral sclerosis (ALS), ischemia, retinitis pigmentosa, and Parkinson’s disease.\textsuperscript{39–42} In the current study, we found that purified RGC preconditioned in an ischemic-like injury including glucose deprivation (GD) and oxygen-glucose deprivation (OGD) are more susceptible to AMPAR-mediated excitotoxicity. Additionally, we characterized the posttranscriptional modification that occurs in the mRNA expression of GLUA1-4 flip and flop isoforms and the RNA editing enzymes, ADAR1-3, following GD and OGD injury.

**METHODS**

**Purified RGCs Isolation and Culture**

All animal procedures were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) policy for the Use of Animals in Ophthalmic and Vision Research, and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center. Purified neonatal RGCs were isolated using a double immunopanning technique as published previously.\textsuperscript{30,43} Time-pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA, USA), and retinas were dissected from euthanized postnatal (days 4–6) rat pups. Collected retinas were dissociated in papain solution (4.5 units/mL, #3125; Worthington, Lakewood, NJ, USA). Dissociated cell suspension were incubated with rabbit antimacrophage antibody (#CLAD51240; Cedarlane Laboratories, Ontario, Canada) and then plated twice to a 150-mm petri dish coated with goat anti-rabbit IgG (H+L chain) antibody (#111-005-003; Jackson ImmunoResearch, West Grove, PA, USA), to remove microglia from the cell suspension. Subsequently, nonadherent cells were transferred to a 100-mm petri dish coated with Thy1.1 antibody (from hybridoma T11D7; American Type Culture Collection, Rockville, MD, USA), a selective RGC marker. Following 1 hour of incubation with intermittent shaking of the plate (every 10 minutes), the 100-mm petri dishes were washed with Dulbecco’s phosphate-buffered saline (DPBS) multiple times (#14287080; Invitrogen, Carlsbad, CA, USA), removing nonadherent cells and leaving behind RGCs. The RGCs then were incubated with trypsin (1250 units/mL) (#T9935; Sigma-Aldrich Corp., St. Louis, MO, USA) in a 37°C incubator for 5 minutes and successively mechanically triturated using a pipette, to dissociate the cells from the 100-mm petri dish. The RGCs were seeded onto plates coated with poly-D-lysine (#P6407; Sigma-Aldrich Corp.) and mouse-fibrinogen-1 (#540001001; Trevigen, Inc., Gaithersburg, MD, USA). The RGC cultures obtained had a purity of 99.7% ± 0.3% at 0 days in vitro (DIV). The RGCs were cultured in serum-free defined medium containing Dulbecco’s modified Eagle’s medium (DMEM, #11960; Invitrogen), forskolin (5 ng/mL, #F6886; Sigma-Aldrich Corp.), and two trophic factors: brain-derived neurotrophic factor (BDNF, 50 ng/mL, #450-02; Peprotech, Rocky Hill, NJ, USA) and ciliary neurotrophic factor (CNTF, 10 ng/mL, #450-15; Peprotech).\textsuperscript{30,44} This culture medium is designated as “RGC medium” throughout the manuscript. Cultures of RGC were maintained in a 37°C, humidified incubator containing 10% CO\(_2\). Every 3 days, half of the RGC medium in the wells were replaced with fresh RGC medium. All RGCs were cultured for 7 to 10 DIV before performing experiments.

**Oxygen and/or GD Induction**

We used RGC Medium as the control medium. To simulate ischemia-like conditions, normoxic and hypoxic (0.5% oxygen) conditions were generated by culturing RGCs in phenol-free/glucose-free DMEM (A1443001; Life Technologies, Carlsbad, CA, USA) lacking nutrients and trophic factors. Normoxic glucose-deprived DMEM (GD) was maintained in a 37°C humidified incubator containing 10% CO\(_2\), while hypoxic glucose-deprived DMEM (OGD) was kept in 37°C humidified hypoxia chamber (InviVO2 Hypoxia Workstation; Baker Ruskinn, Sanford, ME, USA) with 10% CO\(_2\), 0.5% O\(_2\), and 89.5% N\(_2\). Before running all experiments, all the media were kept in their respective conditions overnight to prequilibrate the medium to their proper gas conditions.
Table 1. List of Nomenclatures for Different Treatments Applied on Purified RGCs

<table>
<thead>
<tr>
<th>Medium</th>
<th>Normoxia or Hypoxia</th>
<th>Drug Treatment 8 h</th>
<th>Preconditioning</th>
<th>Drug Treatment, 4 h Following Preconditioning</th>
<th>Treatment Nomenclatures</th>
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<tr>
<td>RGC medium</td>
<td>Normoxia</td>
<td>s-AMPA, 100 μM; KA, 100 μM; CFM-2, 100 μM; H2O2, 100 μM; FCCP, 100 nM</td>
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<td>H-RGC Medium</td>
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<td>Glucose deprivation</td>
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<td>-</td>
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<td>-</td>
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<td>GD-Precon-s-AMPA</td>
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<tr>
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### Treatments

Treatments were performed in either GD or OGD conditions for the total treatment duration of 8 hours with the addition/combination of AMPAR agonists: s-AMPA (100 μM, #0254; Tocris, Bristol, UK) and KA (100 μM, #0222; Tocris), AMPAR antagonist: CFM-2 (100 μM, #1082; Tocris), H2O2 (100 μM, #H1009; Sigma-Aldrich Corp.), or the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 100 nM; #C2920; Sigma-Aldrich Corp.). Certain treatments required the preconditioning (Precon) of either GD or OGD for 4 hours (producing an ischemic or hypoxic and ischemic injury), which then were followed by the addition of either s-AMPA (100 μM) or KA (100 μM) for another 4 hours. For the simplicity of nomenclature, the different treatment nomenclatures are listed in Table 1.

### Live/Dead Assay

Purified RGCs were seeded in a black-walled, clear-bottom 96-well plate (#655909; Greiner Bio One, Monroe, NC, USA) at an approximate density of 10,000 cells per well. Retinal ganglion cells seeded in the wells of the 96-well plate were washed three times with DPBS and subsequently were treated in either N-RGC Medium, H-RGC Medium, GD, OGD, GD-s-AMPA, OGD-s-AMPA, GD-Precon-s-AMPA, OGD-Precon-s-AMPA, GD-Precon-KA, or OGD-Precon-KA. To determine RGC survival, the LIVE/DEAD Viability/Cytotoxicity Kit (#L3224; Invitrogen) containing calcein-acetomethoxy (calcein-AM) and ethidium homodimer-1 (EthD-1) dyes was used as described by the manufacturer. Cells were incubated in either normoxic or hypoxic DPBS containing 2 μM calcein-AM and 1 μM EthD-1 for 30 minutes at 37°C. Cells then were washed with normoxic or hypoxic DPBS 3 times and fluorescent images at ×10 were immediately imaged using the Cytation 5 Cell Imaging Multi-Mode Reader (Bio-Tek, Winooski, VT, USA). Gas concentrations and temperature were maintained while being imaged in the Cytation 5 Reader. The RGCs treated with ice-cold methanol for 10 minutes were used as positive control for dead cells. A total of nine images were acquired per well in a fixed 3 × 3 grid and were averaged for each individual “n” value. Retinal ganglion cell viability was quantified in a masked manner and living cells were determined by green fluorescence (calcein-AM). Retinal ganglion cells containing any red fluorescence by EthD-1 staining DNA were considered as dead or dying cells; n = 10 to 19.

### Caspase 3/7 Activity

Approximately 10,000 purified RGCs were seeded into the wells of 96-well plates (#553072; BD Falcon, Franklin Lakes, NJ, USA). Retinal ganglion cells were subjected to 50 μl treatments of N-RGC Medium, H-RGC Medium, GD, OGD, GD-s-AMPA, OGD-s-AMPA, GD-Precon-s-AMPA, OGD-Precon-s-AMPA, GD-Precon-KA, OGD-Precon-KA, OGD-CFM-2 Precon-s-AMPA, or OGD-CFM-2-Precon-s-AMPA. Another set of RGCs subjected to 1 μM staurosporine (ab120056; Abcam, Cambridge, MA, USA) for 24 hours were used as a positive control for increased caspase 3/7 activities. The activities of caspase 3/7 following experimental treatments were detected with the addition of 100 μl luciferase-lysis solution (Caspase-Glo 3/7 Luciferase assay, #G8091; Promega, Madison, WI, USA) into each well. The 96-well plates were shaken on an orbital shaker for 30 seconds, following which the cell lysate was allowed to incubate in the well for 1 hour at room temperature. The cell lysates were transferred onto a white-walled 96-well plate (#353072; BD Falcon). The luminescence signals from the cell lysate solution were determined on a plate reader (Cytation 5 Cell Imaging Multi-Mode Reader; Bio-Tek). A 2-way ANOVA analysis was performed where *P < 0.05, **P < 0.01, and ***P < 0.001. All experiments were performed in triplicates, n = 4.
Mitochondrial Membrane Potential

Purified RGCs were seeded into black-walled, clear bottom 96-well plates (655090; Greiner Bio One) at a density of 10,000 cells per well. Before experimental treatments, RGCs were stained with 1 μM JC-1 dye (ab113950; Abcam) in DPBS for 30 minutes at 37°C. Then, RGCs were washed two times with warm DPBS and immediately treated with either N-RGC Medium, H-RGC Medium, GD, OGD, GD-sAMPA, OGD-sAMPA, GD-Precon-sAMPA, OGD-Precon-sAMPA, GD-Precon-KA, OGD-Precon-KA, GD-FCCP, or OGD-FCCP for a total of 8 hours at 37°C. The Cytation 5 reader maintained the temperature and gas concentration throughout the experiments. Retinal ganglion cells treated with FCCP were used as positive control for mitochondrial depolarization. Retinal ganglion cell mitochondrial membrane potentials (ΔΨm) were determine by the Cytation 5 plate reader, taking the fluorescent intensity of the red JC-1 monomers (exitation wavelength 510 nm, emission wavelength 590 nm) every 15 minutes at 37°C. The 4.5-hour time point (30 minutes following the treatments after 4-hour preconditioning) was used to determine AMPA receptor mitochondrial depolarization following 4 hours of injury. All experiments were conducted in triplicates (n = 10). A 2-tailed t-test was performed and *P < 0.05 and **P < 0.01 were considered significant.

ROS Assay

Dihydroethidium (DHE, #D11347; Thermo Fisher Scientific, Waltham, MA, USA) was used to detect superoxide generated from AMPA receptor stimulation following GD and OGD conditions. Approximately 10,000 purified RGCs were seeded per well in black-walled, clear-bottom 96-well plates. The RGCs were treated with DHE (2 μM) and were subjected to various treatments. Fluorescent intensities (ex, 510 nm; em, 590 nm) were measured every 5 minutes up to 8 hours by the Cytation 5 plate reader. Experimental plates were maintained at 37°C in either normoxic or hypoxic conditions. In between (4 hours) the experiments, an additional 100 μL secondary treatments were added to the appropriate wells (final volume 200 μL). At the 5-hour time point (1 hour after the secondary treatments), fluorescent images (×10) of oxidized DHE were taken with the Cytation 5 plate reader in a 3 × 3 grid. Fluorescent intensities from oxidized DHE products were quantified in each individual RGC with the Gen5 Software (Bio-Tek), where the integral fluorescence of a cell was divided by the total area of the cell.

[Ca2+]j Measurement

Purified RGCs were seeded on 35-mm glass bottom (MatTek cat# P35G-0-7-C; MatTek, Ashland, MA, USA) dishes at a density of 10,000 cells per dish. Retinal ganglion cells were treated in conditions of RGC medium, GD, or OGD for 4 hours followed by the incubation of fura-2-AM (3 μM; #F1221; Invitrogen) for 30 minutes at 37°C. Retinal ganglion cells then were washed with 37°C normoxic or hypoxic Krebs–Ringer buffer solution (115 mM NaCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 24 mM NaHCO3, 5 mM KCl, 25 mM HEPES, and 5 mM glucose, pH 7.4) over the 17 nm) over the 15 nm) every 15 minutes. The 4.5-hour time point (30 minutes following the treatments after 4-hour preconditioning) was used to determine AMPA receptor mitochondrial depolarization following 4 hours of injury. All experiments were conducted in triplicates (n = 10). A 2-tailed t-test was performed and *P < 0.05 and **P < 0.01 were considered significant.

Detection of Calcium-Permeable AMPA Receptor

Purified RGCs were seeded at a density of 10,000 cells per well. Retinal ganglion cells were treated in either RGC medium, GD, or OGD conditions for 4 hours. The detection of calcium-permeable AMPA receptor was performed with Nanoprobe 1 (EUM001; Kerafast, Boston, MA, USA), a fluorescent ligand-directed probe. Following the treatments, RGCs were then coincubated with Nanoprobe1 (600 nM) and sAMPA (100 μM), in either normoxic or hypoxic extracellular buffer (138 mM NaCl, 1.5 mM KCl, 1.2 mM MgCl2, 5 mM HEPES, 2.5 mM CaCl2, and 14 mM glucose at pH 7.4) for 5 minutes. Cells then were washed twice to remove nonbound Nanoprobe 1. Retinal ganglion cells were imaged at 37°C under normoxic or hypoxic conditions, using the Cytation 5 plate reader (x60; ex, 534 nm; em, 566 nm). CEF-2 (100 μM) was used to block AMPA receptor’s activation. Some RGCs were treated with D-Mannitol (200 mM) for 30 minutes to test the possibility that cell hypertonic shrinkage may be contributor to fluorescent labeling by the nanoprobe. The experiment was performed four times.

Real-Time PCR

Purified RGCs were seeded at a density of 300,000 cells per well in 6 well plates. Retinal ganglion cells were treated either in RGC, GD, or OGD medium for various time points (3, 4, 6, and 8 hours), and subsequently total cellular RNA was extracted with Trizol (#A00741; Life Technologies) following the manufacturer’s protocol. Total RNA was reverse transcribed to cDNA with the iScript Reverse Transcription Supermix for RT-qPCR (#170-8841; Bio-Rad, Hercules, CA, USA). Quantitative expression of genes of interest (primers are listed in Table 2) was performed and the Cytation 5 plate reader (x60; ex, 534 nm; em, 566 nm).CFM-2 (100 μM) was used to block AMPA receptor’s activation. Some RGCs were treated with D-Mannitol (200 mM) for 30 minutes to test the possibility that cell hypertonic shrinkage may be contributor to fluorescent labeling by the nanoprobe. The experiment was performed four times.
forward primers and verified by BLAST searches. Quantification of a RNA editing of a single nucleotide was determined by measuring the peak heights of the nucleotides of interest (edited/[unedited + edited]).

Statistical Analysis

SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA) was used to perform our statistical analysis. Two-tailed t-tests were performed to compare two groups, while 1-way ANOVAs followed by the Dunnett’s post hoc test were used to compare multiple groups to a control group. The 1-way ANOVAs followed by the Tukey post hoc test were used for multiple comparisons between groups. A 2-way ANOVA was performed to determine the differences within and between groups. Statistical significance of the experimental data was described to determine the differences within and between groups. A 2-way ANOVA was performed to compare two groups, while 1-way ANOVAs followed by the Dunnett’s post hoc test were used to compare multiple groups to a control group. The 1-way ANOVAs followed by the Tukey post hoc test were used for multiple comparisons between groups. A 2-way ANOVA was performed to determine the differences within and between groups. Statistical significance of the experimental data was described as *P < 0.05; **P < 0.01; ***P < 0.001 within groups and #P < 0.05; ##P < 0.01; ###P < 0.001 between groups. Data are presented as mean ± SEM.

RESULTS

Oxygen/Glucose Deprivation Preconditioning

Induced Injury and RGC Susceptibility to s-AMP A

To determine if ischemic-like injury could make RGCs more susceptible to cell death by AMPAR stimulation, purified RGCs were preconditioned in glucose-free DMEM in either normoxic or hypoxic (0.5% O2) conditions for 4 hours. Retinal ganglion cells then were treated with 100 μM s-AMP A in their respective medium for an additional 4 hours. At the end of 8 hours, Live/Dead images (Fig. 1A) were taken to determine RGC survival. Retinal ganglion cells treated with methanol were used as positive controls for dead cells while RGCs incubated with N-RGC Medium were used as the positive control for live cells. Quantification of RGC survival (Fig. 1B) showed no difference in cell survival between the normoxic (87 ± 1.4%) and hypoxic (85 ± 2.3%) groups of RGC Medium–treated groups. However, a significant decrease was observed in the GD group (66 ± 4.4%, P < 0.05) not of the OGD group (85 ± 1.5%), when compared to N-RGC Medium group. As observed in our previous findings,28 the GD–s-AMP A (86 ± 2.2%) or OGD–s-AMP A (85 ± 2.3%) did not decrease RGC viability. Interestingly, though, RGCs incubated in GD-Precon-s-AMP A (52 ± 6.4%; P < 0.001) and OGD-Precon-s-AMP A (22 ± 5.1%; P < 0.001) treatments saw an appreciable decrease in RGC survival when compared to N-RGC Medium treatment group. Similarly, GD-Precon–KA (9 ± 4.4%; P < 0.001) or OGD-Precon–Ka (16 ± 5.3%; P < 0.001) produced a drastic decrease in cell survival.

Caspase 3/7 Activity Is Not Exacerbated by AMPA Receptor Stimulation Following Oxygen and/or Glucose Deprivation Preconditioning

Neurons die generally through the apoptotic pathway in chronic neurodegenerative diseases.45 In RGCs, AMPAR mediated excitotoxicity has been implicated in cell apoptosis.46 To evaluate if the increased in cell death through the activation of AMPAR following OGD preconditioning is mediated through the apoptotic pathway, we measured caspase 3/7 (well-known mediators of the apoptosis pathway) activity through a luciferase enzymatic assay (Fig. 2). As expected, RGCs in N-RGC Medium (15,876 ± 750 relative fluorescence units [RFU], P < 0.05) and all treatments including the GD and OGD (P < 0.01) conditions had a significant increase in caspase 3/7 activity compared to RGCs in N-RGC Medium (11,667 ± 448 RFU). There were no significant differences in caspase 3/7 activities between GD (35,115 ± 2145 RFU) to GD–s-AMP A (30,713 ± 1385 RFU) or to GD-Precon–s-AMP A (30,017 ± 1895 RFU). However, RGC’s caspase 3/7 activities significantly decreased compared to the OGD (35,839 ± 2491 RFU) group when RGCs were incubated in the treatment groups of OGD–s-AMP A (26,134 ± 839 RFU; P < 0.001) or OGD-Precon–s-AMP A (26,272 ± 232 RFU; P < 0.001). In the OGD–s-AMP A and OGD-Precon–s-AMP A treatment groups, there were significant decreases in caspase 3/7 activity compared to GD–s-AMP A (30,714 ± 1385 RFU) and GD-Precon–s-AMP A (30,017 ± 1895 RFU) treatment groups, respectively. The AMPAR antagonism following the addition of the selective noncompetitive antagonist, CFM-2 (P < 0.001), resulted in a significantly decreased caspase activity compared to the GD and OGD treatments groups. Glucose deprivation–Precon–KA (25,541 ± 915 RFU) and OGD-Precon–Ka (27,121 ± 1032 RFU) had similar decreases in caspase 3/7 activity as that when OGD-Precon–Ka was compared to the GD and OGD groups (P < 0.001), suggesting that following GD/OGD preconditioning injury, AMPAR stimulation, through s-AMP A, may activate through similar pathways that KA may act upon.

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that results in RGC death. Staurosporine, a protein kinase inhibitor known to induce apoptosis through the activation of caspase 3,47 was used as our positive control. Retinal ganglion cells incubated in staurosporine (1 μM) for 24 hours, demonstrated a 5.5-fold increase in caspase 3/7 activity when compared to N-RGC Medium (P < 0.001).

**AMPAR Stimulation Decreases Mitochondrial Membrane Potential in RGCs Following Oxygen and/or GD Preconditioning**

No increase in caspase 3/7 activities were observed when RGCs were either treated with s-AMPA or KA following 4 hours of OGD. An AMPAR-mediated RGC death may be influenced by different pathways independent from those activating caspases. Depolarization of mitochondria have been observed in caspase-independent cell death (CICD).48,49 Therefore, JC-1 staining was conducted to determine if OGD preconditioning followed by s-AMPA treatment could further depolarize the mitochondria in RGCs. The mitochondria of healthy cells are hyperpolarized, which is indicated by accumulation of JC-1 (a catatonic dye) in the mitochondria forming aggregates (fluoresces red). When the cell is stimulated by a noxious agent or is unhealthy, the mitochondria depolarizes causing the release of JC-1 from the mitochondrial matrix; thus, JC-1 moves into the cytosol as a monomer (fluoresces green). In an 8-hour time course (Fig. 3A), N-RGC Medium and H-RGC Medium decrease in the fluorescent ratio between aggregate/monomer of the JC-1 dye. Both GD and OGD conditions can further depolarize RGC mitochondria. We used FCCP (a potent mitochondrial uncoupler of oxidative phosphorylation)50 as a positive control for mitochondria depolarization. At the 4-hour time point (right before treatment) for GD and OGD preconditioning, RGCs’ mitochondria are not fully depolarized and could be depolarized further. In normoxic (Fig. 3B) and hypoxic (Fig. 3C) treatments, the “before treatment” group refers to the 4-hour time point during the 8-hour experiment (before adding s-AMPA or KA), while “after treatment” group refers to 30 minutes following the treatments (4.5-hour time point). In the normoxic group, there was a significant depolarization of the mitochondria in the GD-Precon–s-AMPA (P < 0.05) and GD-Precon–KA (P < 0.05) groups. However, in hypoxic conditions, only the OGD-Precon–s-AMPA (P < 0.01) treatment group showed significant further depolarization of the mitochondria in RGCs.
ROS Increases in RGCs Following AMPAR-Mediated Depolarization in Oxygen and/or Glucose Deprivation Preconditioning

Mitochondria are the main source for ROS produced as natural byproducts. However, under deregulation, mitochondria can exhibit an increase in ROS production. Reactive oxygen species that are not regulated become harmful to cells, causing oxidative damage to DNA, lipids, and proteins. To determine if ROS contributes to RGC death mediated by AMPAR stimulation following GD or OGD injury, DHE was used to detect ROS production. Dihydroethidium selectively binds to superoxide, which results in the oxidation of DHE to form 2-hydroxyethidium which intercalates with double-stranded DNA and produces a red fluorescence that can be measured. In a time course of 8 hours, a gradual accumulation of ROS in RGCs was observed during GD (Fig. 4A) incubation, where it reached a fluorescent intensity plateau approximately 7 to 8 hours (≈1000 RFU). Hydrogen peroxide (H2O2) treatment of RGCs was used to increase ROS production as a positive control, which gave a slightly steeper slope and higher plateau of ROS accumulation compared to GD-treated RGCs (Fig. 4A). Even in the presence of minimal (0.5%) O2 (OGD group), ROS accumulation was observed but with a lower slope of that of the GD group (Fig. 4A). The OGD groups’ ROS accumulation reached a plateau at approximately the 5- to 6-hour time point (≈550 RFU). At the 5-hour time point, the normoxic treated groups (Figs. 4B, 4C), ROS production significantly increased in RGCs subjected to either GD (1.26 ± 0.01-fold, n = 1857, P < 0.001), or other treatment conditions including GD-s-AMPA (1.40 ± 0.01, n = 1539, P < 0.001), and in the GD-Precon–s-AMPA (1.54 ± 0.01, n = 1497, P < 0.001) compared to the N-RGC Medium (control) group (1.00 ± 0.01, n = 785). Increase in fluorescent staining of the oxidized DHE products accumulated in the nucleus of the cell (brightfield images as reference, Fig. 4B). Although treatment of RGCs with GD-s-AMPA did not result in a decrease in RGC survival (Fig. 1), ROS increased significantly (P < 0.001) over the GD group. However, the treatment of RGCs with GD-Precon–s-AMPA showed a significant induction of more ROS than the RGCs treated with GD (P < 0.001) or GD-s-AMPA (P < 0.05). Comparably, the hypoxic treatment groups saw a similar trend in ROS accumulation at the 5-hour time point. A significant increase in ROS accumulation was observed in the RGCs subjected to various conditions, including OGD (1.51 ± 0.02, n = 1582; P < 0.001), OGD-s-AMPA (1.56 ± 0.2, n = 1365; P < 0.001), OGD-Precon-s-AMPA (1.67 ± 0.02, n = 1163; P < 0.001) compared to the RGCs treated with H-RGC Medium (1.00 ± 0.01, n = 375; Figs. 4B, 4D). Additionally, the OGD-Precon-s-AMPA (P < 0.001) group had significantly more ROS than the OGD and OGD-s-AMPA groups. In normoxic (P < 0.001) and hypoxic conditions (P < 0.001; Figs. 4C, 4D), H2O2 (100 μM) significantly increased ROS in RGCs compared to the RGCs in N-RGC Medium and H-RGC Medium. Although there was an increase in ROS in the positive control H2O2 treatment groups, it was not higher than the GD-Precon-s-AMPA or the OGD-Precon-s-AMPA groups. Much of the fluorescent staining of the H2O2 treatment groups were more highly localized in the nucleus and less in the RGC somas as in the other treatment groups (Fig. 4B). Additionally, the fluorescent intensity in the nucleus was decreased in the H2O2 groups. This may be attributed to membrane permeabi-
lization of dead and dying cells, where the oxidized product of DHE is released extracellularly and binds to other dead RGC's DNA; therefore, diffusing the fluorescent signal. Figure 4E demonstrates that high concentration of H$_2$O$_2$ (1 mM) increased DHE's fluorescent signal dramatically, but then shows a decline in fluorescence as presumably due to the decline in the viability of the RGCs.

Oxygen GD but Not GD Preconditioning Increased Intracellular Calcium Concentration in Purified RGC Following AMPAR Stimulation

During ischemic events of the retina, increased calcium in RGGs increases the activation of Ca$^{2+}$-dependent proteases, which may produce deregulation of mitochondria and upregulation of ROS and other programmed cell death mediators. The influx of calcium during ischemia may be mediated by several different types of receptors, such as the ionotropic glutamate receptors, voltage gated calcium channels, the modulation of metabotropic glutamate receptors, or the reversal of the Na$^+$/Ca$^{2+}$ exchanger.$^{35}$ In this study we have observed an increase in ROS, depolarization of the mitochondria, and an increase in cell death when RGCs are treated with s-AMPA following 4 hours of GD/OGD preconditioning (ischemic injury). Preconditioning of GD/OGD may cause an increase in AMPAR-mediated intracellular calcium resulting in excitotoxicity to RGCs that do not occur in RGCs that are not preconditioned. To evaluate if AMPAR stimulation-mediated influx of intracellular calcium concentration is altered after injury, we performed calcium imaging on RGCs incubated in N-RGC Medium, GD, and OGD medium for 4 hours, in which they subsequently were stimulated with 100 $\mu$M s-AMPA.
Following the incubation of N-RGC Medium for 4 hours, the intracellular calcium concentration in RGCs following s-AMPA (100 µM) stimulation was 764 ± 672 nM (n = 74). No significant changes of intracellular calcium concentrations were observed in RGCs stimulated by s-AMPA following 4 hours of treatment of GD (840 ± 85 nM, n = 66). However, RGCs incubated in OGD (1085 ± 97 nM, n = 77, P < 0.05) for 4 hours and treated with s-AMPA, significantly increased intracellular calcium compared to RGCs in N-RGC Medium group. These data suggest OGD is altering AMPARs in RGCs to increase intracellular calcium.

FIGURE 4. To detect ROS levels, DHE dye was incubated with purified RGCs (A) ROS increases in GD and OGD conditions over an 8-hour time course. Treatment of RGCs to 100 µM H2O2 generated increased levels of ROS (positive control). (B) Images of DHE fluorescent overlaid with brightfield images revealed a substantial increase in fluorescence in RGCs treated in GD-Precon-s-AMPA (normoxia condition) and OGD-Precon-s-AMPA (hypoxia condition). (C, D) Quantification of normoxic and hypoxic DHE fluorescent images 1 hour following treatments (5-hour time point) showed that all treatments in either GD (n = 1857) or OGD (n = 1582) conditions significantly (P < 0.001) increased ROS in RGCs compared to N-RGC Mediumcontrol group, n = 785) and H-RGC Medium control group, n = 375). (C) Glucose deprivation-s-AMPA and GD-Precon-s-AMPA ROS fluorescence significantly increased by 1.4 ± 0.01-fold (n = 1559, P < 0.001) and 1.45 ± 0.01-fold (n = 1497, P < 0.001), respectively, compared to the control group (N-RGC Medium). Reactive oxygen species levels following the GD-Precon-s-AMPA treatment also were significantly (P < 0.05) higher than GD-s-AMPA. In the hypoxic condition (D), OGD-Precon-s-AMPA (n = 1163, P < 0.001) treatment increased ROS in RGCs by 1.67 ± 0.02-fold compared to H-RGC Medium treatments. Additionally, OGD-Precon-s-AMPA (P < 0.001) significantly increased ROS in RGCs compared to OGD and OGD-s-AMPA (n = 1365). Hydrogen peroxide significantly increased ROS in RGCs incubated in either (C) normoxia (n = 1120, P < 0.001) or (D) hypoxia (n = 1104, P < 0.001) conditions compared to the controls. Over a 60-minute period, purified RGCs treated with 1 mM H2O2, (E) increased the intensity of DHE fluorescence within the first 40 minutes. Conversely, a decline in DHE fluorescence in the last 20 minutes occurred, suggesting a diffusion of the DHE-oxidized products through decline in membrane integrity of dead or dying cells, as observed in the fluorescent images of RGCs treated with 100 µM H2O2 in normoxic and hypoxic conditions. Scale bars: 200 µm. Error bars: mean ± SEM. *P < 0.05, ***P < 0.001.
Figure 5. Changes in [Ca\(^{2+}\)](i) in purified RGCs mediated by \(s\)-AMPA (100 \(\mu\)M) following 4 hours of GD or OGD conditions were determined by using fura-2-AM (3 \(\mu\)M) dye. Purified RGCs incubated in GD (840 ± 85 nM, \(n = 66\)) for 4 hours did not increase [Ca\(^{2+}\)], when compared to N-RGC Medium (control; 764 ± 72 nM, \(n = 74\)). However, RGCs incubated in OGD conditions for 4 hours significantly increase [Ca\(^{2+}\)], (1085 ± 97 nM, \(n = 77\), *\(P < 0.05\)). Error bars: mean ± SEM.

GD or OGD injury, a novel ligand targeted photocleavable fluorescent probe was used to tag cp-AMPARS.\(^ {57}\) This probe contains a polyamine ligand that is similar to 1-naphthothiacetyl spermine (NASPM) or Joro spider toxin that targets cp-AMPAR pore when the receptor is activated by an AMPAR agonist, such as glutamate or \(s\)-AMPA, thus blocking the receptor.\(^ {57}\) An electrophilic moiety on the probe, which contains the Cy3 fluorophore, forms a covalent bond with the receptor, in which the ligand probe can be photocleaved, freeing up the receptor to allow it to be functionally active.\(^ {57,58}\)

In our current study, in the normoxia and hypoxia conditions there was a small fluorescent staining of cp-AMPARS in the RGCs maintained in RGC medium at 4 hours (Fig. 6A). However, there is an increased expression of cp-AMPAR in RGC, incubated in GD for 4 hours. RGCs in OGD conditions for 4 hours resulted in a considerable increase in cp-AMPAR expression. The coinubcation with CFM-2 (AMPA noncompetitive antagonist) and \(s\)-AMPA, was able to block the activation of AMPAR, thus inhibiting Nanoprobe 1 from linking with cp-AMPARS. In the present experiment, Nanoprobe 1 was not photocleaved and, therefore, cp-AMPARs became endocytosed in the RGCs (Fig. 6A), due to blockade of the AMPAR channel pore,\(^ {58}\) which contributed some staining inside the cytosol. In this current study, AMPAR trafficking was not studied, just the expression of cp-AMPARs at the time following 4 hours of GD and OGD incubation. Notably, shrunken dead cells (determined by brightfield images and denoted by arrows) that were attached to the well bottom (due to poly-D-lysine and mouse Laminin-1) contained fluorescently tagged cp-AMPARs (Fig. 6A). To see if cell volume change may have influenced the measurements the addition of 200 mM D-Mannitol for 30 minutes was performed to induce hyperosmolarity conditions and to demonstrate if RGCs shrink in size (Fig. 6B, denoted in arrows). However, there was a lack of expression of cp-AMPARs on RGCs incubated in 200 mM D-Mannitol, suggesting that the staining with cp-AMPAR was not an artifact of cell shrinkage.

Oxygen GD Downregulated the mRNA Expression of Gria2 Flop and Gria3 Flop

Each of the AMPAR subunits can be alternatively spliced in the extracellular ligand-binding domain into two variants: flop and flop.\(^ {52}\) The alternatively splice variants differ in their kinetics, where the flop variant desensitizes slower than the flop variant and has an enhanced steady-state, allowing for a greater current amplitude.\(^ {32}\) To determine if OGD injury to RGCs alters the expression of AMPAR’s subunit alternative spliced isoforms, the mRNA expressions of Gria1-4’s (GLUA1-4 gene) flop and flop isoforms were evaluated by qPCR and compared to RGCs treated to N-RGC medium (control), and following the incubation of purified RGCs in either GD or OGD medium for 3, 4, 6, and 8 hours (Supplementary Fig. S2A). The relative expression of total Gria2, 3, and 4 compared to Gria1 (1.1 ± 0.2) of the control RGC group were 1.0 ± 0.1-fold, 0.2 ± 0.02-fold (\(P < 0.01\)), and 0.5 ± 0.1-fold (\(P < 0.01\)), respectively. (Supplementary Fig. S2B) Retinal ganglion cells maintained in N-RGC Medium had an even expression of flop and flop isoforms for Gria1, 2, and 4. However, Gria3 flop expression (0.3 ± 0.3-fold, \(P < 0.001\)) was significantly lower than Gria3 flip expression (1.0 ± 0.1) (Supplementary Fig. S2B). Total Gria1 expression following RGC injury (Fig. 7A) did not change in OGD conditions; however, RGCs incubated in GD conditions significantly decreased total Gria1 expression at the 4-hour (0.14 ± 0.4-fold, \(P < 0.001\)) and 8-hour (0.37 ± 0.01, \(P < 0.05\) at all time points. All Gria3 flip mRNA expression significantly decreased by at least 50% at all time points (\(P < 0.01\)) when RGCs were incubated in OGD, though at 8 hours Gria1 flip in GD conditions increased by 19 ± 9.35-fold. Gria1 flip’s mRNA expressions were similar to Gria1 flip’s expression in OGD, whereas Gria1 flop expression was significantly decreased compared to RGC Medium at the 4- (0.37 ± 0.05-fold, \(P < 0.001\)), 6- (0.61 ± 0.02-fold, \(P < 0.05\)), and 8-hour (0.08 ± 0.02-fold, \(P < 0.01\)) time points. There also was a significant (\(P < 0.01\)) decrease of Gria1 flop expression of RGCs in 4 hours GD medium by more than 80%. Gria2 total mRNA expression decreased significantly (40%) at the 4-hour time point for the GD and OGD conditions (\(P < 0.001\)) and by at least 50% at the 8-hour time point for the OGD condition (\(P < 0.01\); Fig. 7B). No significant changes were observed in the Gria2 flip expression over the 8-hour time points; however, Gria2 flip expression significantly decreased in a similar manner as Gria2 total mRNA expressions. This effect was seen at greater than a 60% decrease at the 4-hour (\(P < 0.001\)) time point for the GD and OGD conditions and a 70% decrease in expression (\(P < 0.001\)) following 8 hours of incubation in OGD conditions. Only the 6-hour incubation of OGD condition significantly decreased Gria3 total expression, whereas at the other time points there was a significant effect on mRNA expression (Fig. 7C). No changes of Gria3 flip mRNA expression were observed, but Gria3 flop mRNA expression significantly decreased in OGD conditions at 4 (0.37 ± 0.03-fold, \(P < 0.001\)), 6 (0.41 ± 0.11-fold, \(P < 0.001\)), and 8 (0.25 ± 0.13-fold) hours. Lastly, Gria4 total (Fig. 7D) expression significantly increased by 39.50 ± 19.24-fold (\(P < 0.01\)) during 8 hours of GD exposure, whereas 3- (\(P < 0.01\)), 4- (\(P < 0.01\)), and 8-hour (\(P < 0.05\)) exposures of OGD significantly decreased Gria4 flip mRNA expression levels by more than 35%. Gria4 flop also decreased significantly at 4 hours GD (0.25 ± 0.08-fold; \(P < 0.01\)) incubation and 8 hours OGD (0.30 ± 0.01-fold, \(P < 0.01\)) incubation. A summary of the effects of normoxia and hypoxia on flip and flop isoforms over the 8-hour time points is presented in Table 3.

RNA Editing of Gria2 Q/R Site and Gria2-4 R/G Sites

Editing of the Q/R site is efficient in neurons where 99% of all Gria2 mRNA are edited.\(^ {59,60}\) Similarly, in the RGC control group (RGC Medium), 94 ± 2.1% of Gria2 mRNA were edited at the Q/R site of RGCs. At all time points in either GD or OGD conditions, there were no significant changes in Q/R editing (Fig. 8A). There also were no changes in R/G editing of either Gria2 flip and Gria3 flip following GD or OGD treatment up to
Calcium-permeable AMPARs in purified RGCs were tagged by the activation of receptors with \( \alpha \text{-AMPA} \) opening the receptor allowing a ligand-targeted fluorescent probe (Nanoprobe 1) to covalently bind to the receptor. Retinal ganglion cells then were imaged at \( \times60 \), exciting the probe fluorophore at 534 nm and recording the emission at 566 nm and overlaid onto brightfield images. (A) Calcium-permeable AMPARs were detected in RGCs incubated in either N-RGC Medium or H-RGC Medium for 4 hours. Retinal ganglion cells incubated in GD conditions for 4 hours increased in cp-AMPARs. Intense staining of cp-AMPARs expression was observed from RGCs conditioned in OGD for 4 hours. AMPA antagonist, CFM-2 prevented \( \alpha \text{-AMPA} \) from binding AMPARs, leaving the receptor closed, and, therefore, prevented Nanoprobe1 from targeting cp-AMPARs, as shown by lack of fluorescence. Calcium-permeable AMPARs also are prominently observed in shrunken cell bodies of dead or dying RGCs (determined by brightfield images), as denoted by the arrows (A). (B) Retinal ganglion cells were subjected to hyperosmolarity conditions by treatment with 200 mM D-Mannitol to determine if the reduction of RGCs cell body size causes nonspecific fluorescence from Nanoprobe1 binding. As indicated by the arrows, cell bodies that were reduced in size due to hyperosmolarity conditions did not exhibit fluorescence. Scale bars: 20 \( \mu \text{m} \).
8 hours. The percent of pre-mRNA that are edited at the R/G site for the control groups of Gria2 flip and Gria3 flip were at 86.6 ± 1.7% and 61.6 ± 6.9%, respectively (Fig. 8B). R/G editing of Gria4 flip could not be discerned in this current study due to an inability to get clear sequencing data from our sample. A significant decrease of roughly 20% reduction in the R/G editing of Gria2 flop occurred with GD (55.6 ± 4.6%, P < 0.01) and OGD (56 ± 5.0%, P < 0.01) conditions at 4 hours, compared to the control group, which had an R/G editing efficiency of 76 ± 2.0%. Gria3 flop and Gria4 flop control group R/G editing efficiency were at 68 ± 4.1% and 66 ± 2.6%, respectively. No changes in Q/R editing were observed in either GD or OGD conditions up to 8 hours.

Increased mRNA Expressions of Adar and Adarb2 Following GD and OGD Conditions

AMPAR RNA editing is processed by the RNA editing enzymes called adenosine deaminases that act on RNA (ADAR1, 2, and 3). Downregulation of ADAR2 in a mouse model of

FIGURE 7. Retinal ganglion cells AMPAR subunits genes, Gria1-4, total, flip isoform, and flop isoform expression following 3, 4, 6, and 8 hours of GD and OGD conditions. Changes in gene mRNA expression levels were detected by qPCR, using cDNA template reverse transcribed from total RNA, isolated from RGCs maintained in either RGC Medium, GD, or OGD conditions. (A) Expression of Gria1 Total decreased at 4 hours in GD by more than 7.1-fold (P < 0.001) and at 8 hours in GD by 2.7-fold (P < 0.01). Gria1 flip expression was attenuated at 3, 4, 6, and 8 hours in OGD by 2.3-fold (P < 0.01), 2.7-fold (P < 0.001), 2.1-fold (P < 0.01), and 2.8-fold (P < 0.001), respectively. However, 8 hours of GD increased Gria1 flip expression by 19-fold (P < 0.05). Gria1 flop expression decreased at 4 hours in GD by 5.4-fold (P < 0.01). Similarly, OGD reduced Gria1 flop expression at 4 hours (2.7-fold, P < 0.001), 6 hours (1.7-fold, P < 0.05), and 8 hours (12.2-fold, P < 0.001). (B) Gria2 total expression was significantly reduced at 4 hours GD (4.4-fold, P < 0.001), 4 hours OGD (2.5-fold, P < 0.001), and 8 hours OGD (2.2-fold, P < 0.01). No changes were observed in Gria2 flip, but a decrease in Gria2 flop expression occurred at 4 hours in GD (3.7-fold, P < 0.001), 4 hours in OGD (2.8-fold, P < 0.001), and 8 hours in OGD (3.5-fold, P < 0.001). (C) There was reduction in expression of Gria3 total at 6 hours OGD (1.9-fold, P < 0.05), but no changes in expression occurred for Gria3 flip over the 8 hours course. However, Gria3 flop expression was attenuated at 4 hours (1.8-fold, P < 0.01), 6 hours (2.5-fold, P < 0.001), and 8 hours (4.1-fold, P < 0.001) in OGD. (D) Significantly increased expression of Gria4 total occurred at 8 hours GD (39.5-fold, P < 0.01). Downregulation of Gria4 flip expression occurred at 3 hours (2.2-fold, P < 0.01), 4 hours (2.7-fold, P < 0.01), and 8 hours (1.8-fold, P < 0.05) in OGD but not in any time points in GD conditions. Lastly, Gria4 flop expressions declined following 4 hours in GD (1.8-fold, P < 0.01) and 8 hours in OGD (3.4-fold, P < 0.01). Gene expressions were normalized to Actb expression (internal control) and values were compared to RGCs treated in RGC Medium in normoxic conditions. Statistical analysis was performed using a 1-way ANOVA, followed by the Dunnett’s post hoc test, comparing multiple groups to a control group (RGC Medium). Significance changes (* P < 0.05, **P < 0.01, ***P < 0.001) were found following comparison of averages from technical triplicates. Error bars: mean ± SEM, n = 3 to 6.
TABLE 3. The Summary of the Overall Effects of Normoxia and Hypoxia Conditions on Purified RGCs Total, Flips, and Flop mRNA Expressions of Gria1–4 Over an 8-Hour Time Period

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total and Alternative Spliced Isoforms</th>
<th>GD Time, h</th>
<th>OGD Time, h</th>
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<td>3  4  6  8</td>
<td>3  4  6  8</td>
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<tr>
<td>Gria1</td>
<td>Total</td>
<td>–  –  –  ↑</td>
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<td></td>
<td>Flip</td>
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<td></td>
<td>Flop</td>
<td>–  –  –  –</td>
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<tr>
<td>Gria2</td>
<td>Total</td>
<td>–  –  –  –</td>
<td>–  –  –  –</td>
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<tr>
<td></td>
<td>Flip</td>
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<td></td>
<td>Flop</td>
<td>–  –  –  –</td>
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</tr>
<tr>
<td>Gria3</td>
<td>Total</td>
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<td>–  –  –  –</td>
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<td>Flip</td>
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<td>Gria4</td>
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<td>Flop</td>
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</tbody>
</table>

Expression of mRNA of each isoform at the end of 3, 4, 6, and 8 hours was compared to RGCs in N-RGC Medium. Symbols ↑, ↓, and – denote significantly increased, significantly decreased, and no difference, respectively.

FIGURE 8. RNA editing efficiency of the Q/R site of Gria2 and the R/G site of Gria2–4 flip and flop isoforms were determined by sequencing the qPCR amplicons. Nucleotide sequences were examined on an electropherogram, where the peak differences in height of nucleotide A and G amplitudes were determined. (A) Gria2 had no significant changes in Q/R editing. (B) Additionally, no changes in R/G editing efficiency occurred in Gria2 flip and Gria3 flip mRNA. A reduction in R/G editing occurred at 4 hours of GD and 4 hours of OGD by 21% (P < 0.01) and 19.7% (P < 0.01), respectively. No significant changes in editing efficiencies at the R/G site of Gria3 flop and Gria4 flop were found up to the 8-hour time point under GD and OGD conditions. Statistical analysis was performed using a 1-way ANOVA followed by the Dunnett’s post hoc test comparing multiple groups to a control group (RGC Medium). Significance was defined by **P < 0.01. Error bars: mean ± SEM; n = 3 to 6. Some treatment time points were not assessed, which were not calculated due to sequencing limitations causing data point to be excluded. ND, not determined.
glaucoma has been shown to promote RGC cell death. However, ADARs acting upon R/G editing during injury has not been characterized in purified RGCs (Supplementary Fig S2C). Adar (ADAR2 gene) expression (15 ± 4.5-fold, P < 0.01) was significantly higher than Adarb1 (ADAR1 gene; 1.9 ± 0.5-fold) and Adarb2 (ADAR3 gene; 0.5 ± 0.4-fold) expressions in RGCs maintained in N-RGC Medium conditions. (Fig. 9A) A significant increase in the level of expression of Adar mRNA occurred at 3 hours (4.5 ± 0.1-fold, P < 0.001) and 6 hours (3.6 ± 0.9-fold; P < 0.01) during GD conditions compared to RGCs incubated in NGM Medium. Additionally, OGD conditions for 3 hours were able to significantly (P < 0.05) increase Adar mRNA levels by 2.3 ± 0.04-fold (Fig. 9A). However, the mRNA expression of Adarb1, Adarb2, (whose enzymatic function is unclear) significantly increased in expression at 4 and 8 hours following OGD condition by 6.1 ± 0.8-fold and 4.5 ± 0.4-fold, respectively. At the 8-hour time point following GD conditions, Adarb2 mRNA expression significantly increased by 210 ± 143-fold (Fig. 9C).

**DISCUSSION**

Retinal ganglion cells are the output neurons that relay visual signals to the brain through action potentials. The generation of action potentials is crucial for the survival of RGCs. In the context of glaucoma, the activation of AMPARs by the endogenous agonist, glutamate, produce a detrimental effect on RGC survival. However, subjecting RGCs to an ischemia-like injury for 4 hours in either GD or OGD conditions, followed by the reperfusion for 4 hours in either GD or OGD conditions, did not alter over the 8-hour time period in either GD or OGD conditions, compared to RGC Medium group. (C) Expression of Adarb2 mRNA, however, increased by 6.1-fold at 4 hours OGD (n = 3) and 4.5-fold at 8 hours OGD (n = 3). Lastly, GD for 8 hours (n = 5) increased RGC’s Adarb2 expression by 209.5-fold (P < 0.01). Gene of interest expressions were normalized to Actb expression (internal control) and values were compared to those of RGCs treated in RGC Medium in normoxic conditions. One-way ANOVAs, followed by the Dunnett's post hoc test, comparing multiple groups to a control group were performed. Values of statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001) are depicted in the histograms. All samples were performed in triplicates and averaged. Error bars: mean ± SEM.

AMPAR Receptor Involvement in RGC Death

How does the activation of AMPAR by the endogenous agonist, glutamate, produce a detrimental effect on RGC survival during the disease state of glaucoma? If RGCs AMPARs are constantly stimulated by endogenous glutamate, then the overstimulation of the receptors cannot produce RGC death in-vitro, then some type of alterations to the AMPAR must be occurring during the disease state. This suggests the possibility that upregulation of the receptors’ expression, differences in receptor kinetics, or changes in cellular signaling to induce RGC death occur during pathologic conditions. In the current study, purified neonatal RGCs treated with s-AMPA throughout duration of the 8-hour experiment were not able to produce an exacerbation of RGC death, as observed previously. However, subjecting RGCs to an ischemia-like injury for 4 hours in either GD or OGD conditions, followed by the treatment of s-AMPA or KA agonist, increased RGCs’ susceptibility to AMPAR-mediated excitotoxicity. An increase of GluA1-4 subunit expression during injury also has been suggested to induce excitotoxicity by increasing intracellular calcium concentrations in RGCs. Normally, in rats, the GluA1-3 subunits are abundant throughout the CNS, whereas GluA4 expression is significantly lower in most places of the brain excluding the reticular thalamic nuclei and the cerebellum, where the subunits are highly expressed. In the adult...
AMPA Receptor Involvement in RGC Death

Editors' Summary

In ischemic or traumatic injury, glutamate is increased and binds to AMPA receptors (AMPARs). This results in increased intracellular Ca$^{2+}$ and Na$^{+}$, which can be lethal to neurons. The AMPA subunits can be edited at the Q/R site, which affects the amino acid inserted and influences AMPAR functions. ADAR1 and ADAR2 have been shown to edit the Q/R site of GLUA2, GLUA3, and GLUA4. The ratio of the flip to the flop R/G transcript is significant in determining AMPAR function. Studies have shown that a decrease in the Q/R site ratio increases AMPAR function, which may be protective in injury.

RGC death through excitotoxicity can be reduced by manipulating Q/R editing. In the current study, Wang et al. demonstrate that decreased Q/R editing increased AMPAR function and protected RGCs from OGD. These findings suggest that manipulating Q/R editing in vivo may be a strategy for protecting RGCs during ischemic injury.

References


Wang et al.'s study suggests that manipulating Q/R editing in vivo may be a strategy for protecting RGCs during ischemic injury.
over the flop. Similarly, Jakobs et al. found that RGCs of postnatal day 5 expressed a 1:1 flip and flop ratio in GLUA1 and GLUA2 subunits. GLUA3 subunits highly expressed the flip isoform over the flop isoforms, while the opposite was true for the GLUA4 subunits. In adult mouse RGCs, the flop isoform was highly expressed compared to the flip isoform. Following an ischemia-like injury to our RGC culture, the downregulation of the mRNA expression level of flip and flop isoforms of Gria1 and Gria4 occurred. No significant changes were observed in Gria2 and Gria3 flip expressions; however, there were significant decreases in the Gria2 and Gria3 flop expressions (Figs. 7B, 7C, highlighted in the green box). Although no changes occurred in mRNA expression of the flip isoforms of Gria2 and 3, the flop isoforms decreased in expression; therefore, increasing the flip to flop ratio of both Gria2 and 3. Similar increases in mRNA expression of the flip to flop ratio of AMPAR subunits have been identified and associated with neuronal cell death in ALS, in a mouse model of retinitis pigmentosa, following a retinal lesion of chick optic tectum, and in kainic-induced epilepsy. Additionally, the transgenic mouse model and the overexpression of GLUA2, 3, or 4 flip isoforms in a homomeric or heteromeric receptor form have been demonstrated to induce cell death, suggesting that the increase mRNA expression of GLUA2 and 3’s flip to flop ratios observed in our current study may be associated with RGC cell death induced by AMPAR stimulation following an ischemic-like injury.

Glutamate excitotoxicity has been implicated to induce apoptosis, where the excessive influx of Ca\(^{2+}\) causes dysregulation of the cellular Ca\(^{2+}\) homeostasis. High concentrations of intracellular Ca\(^{2+}\) can further increase the production of ROS by altering the permeability transition pore of the mitochondria or activating enzymes, such as nitric oxide synthase, ultimately resulting in damage to DNA, lipids, and proteins. Damage to the mitochondria causes mitochondrial outer membrane permeabilization, thus releasing apoptotic inducing factors, such as cytochrome c, resulting in the activation of caspases. Following ischemic-like injury in our purified RGC culture, the activation of AMPARs was able to increase ROS and cause mitochondrial depolarization, which induced RGC death that was independent of caspase activation. In other studies, CICD of RGCs has been observed in vitro and in vivo. In the in vitro study, the inhibition of caspase activity provided early protection of RGCs, but was not able to stop the death of RGC that was mediated by TNF-\(\alpha\) or hypoxia. Furthermore, mitochondria depolarization occurred and the production of ROS increased in purified RGC resulting in apoptotic and necrotic cells. Additionally, the inhibition of caspase, was not able to protect RGCs in vivo, following ablation of the superior colliculus, where minimal cleaved caspase 3 immunolabeled cells correlated with TUNEL stained RGCs. Caspase-independent cell death may result from Ca\(^{2+}\)-dependent enzymes, such as calpain1. In one study, NMDAR-mediated in excitotoxicity to hippocampal neurons resulted in mitochondrial depolarization and release of cytochrome c. However, the activation of calpain1 inhibited the released cytochrome c from cleaving and activating the caspase cascade, yet cell death occurred.

Along with apoptosis, AMPAR-mediated excitotoxicity has been shown to induce necrosis of RGCs. Recently, parapaptosis has been suggested to be another mechanism of programmed cell death of RGCs during retinal ischemia/reperfusion injury and optic nerve crush. Similar to apoptosis, parapaptosis requires gene transcription; however, it does not require the activation of caspases. Additionally, unlike apoptosis, parapaptosis is characterized by vacuolization of the cytoplasm, no nuclear fragmentation, and the swelling of the mitochondria and endoplasmic reticulum. While in our study a caspase-independent cell death occurred, the role of parapaptosis in this process is unclear.

Similarly seen in our current in vitro study, different models of ocular hypertension have shown increased expression of AMPARs in RGC somas and ectopically at the optic nerve head. The application of an AMPAR antagonist or blocker was able to prevent the loss of RGCs in these models; however, it is not known if these neurons and other retina cells containing AMPARs are impaired; thus, possibly affecting vision acuity and function. Additionally, it is uncertain if changes in posttranscriptional modification of the AMPARs occurred in these models as observed in our investigation. In the study of Cueva Vargas et al., only the expression of the GLUA2 subunit and its Q/R site was determined in their model, whereas the expression of the other AMPAR subunits and their posttranscriptionally modified isoforms were not reported. Using a nonspecific blocker of AMPARs or cp-AMPARs would inhibit all AMPAR isoforms, which would inhibit both the deleterious and beneficial effects of AMPAR stimulation in the retina. Currently, there are no specific or selective antagonists against each of the posttranscriptionally modified AMPAR isoforms. Future investigation modulating the expression of each of the posttranscriptionally-modified AMPAR isoforms could determine which of the isoforms to target for better neuroprotection without inhibiting the benefits of AMPAR stimulation.

Taken together, the current study provides insight into the complexity of AMPAR involvement in RGC excitotoxic death. To the best of our knowledge, this is the first study in RGCs that characterizes changes in AMPAR posttranscriptional modification flip and flop isoforms and in the GLUA2-4 R/G editing site following an ischemia like injury. Alterations in these posttranscriptional modifications of receptors gives rise to heterogeneous type of AMPARs that differ in their kinetics, thus affecting the ion conductance and current amplitude. Changes in these posttranscriptionally modified isoforms may explain why certain RGCs are more susceptible to excitotoxicity than others. In addition, changes occurring at the posttranscriptional level for these modified AMPAR isoforms and their subtle differences in kinetics, could help explain glutamate excitotoxicity in the chronic and progressive pathogenesis of neurodegeneration in glaucoma.

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