Survival of Alpha and Intrinsically Photosensitive Retinal Ganglion Cells in NMDA-Induced Neurotoxicity and a Mouse Model of Normal Tension Glaucoma

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Purpose. We assess if α retinal ganglion cells (αRGCs) and intrinsically photosensitive retinal ganglion cells (ipRGCs) survive in mouse models of glaucoma.

Methods. Two microliters of N-methyl-D-aspartate (NMDA; 1 mM) or PBS were injected intraocularly 7 days before sacrifice. Immunohistochemical analyses of the retina were performed using antibodies against RNA-binding protein with multiple splicing (RBPMS), osteopontin, and melanopsin. Immunohistochemical analyses also were performed in adult mice with glutamate/aspartate transporter (GLAST) deletion (GLAST deletion (GLAST) knockout [KO] mice), a mouse model of normal tension glaucoma.

Results. NMDA-induced loss of RBPMS-positive total RGCs was 58.4% ± 0.4% compared to PBS-treated controls, whereas the loss of osteopontin-positive αRGCs was 5.0% ± 0.6% and that of melanopsin-positive ipRGCs was 7.6% ± 1.6%. In GLAST KO mice, the loss of total RGCs was 48.4% ± 0.9% compared to wild-type mice, whereas the loss of αRGCs and ipRGCs was 3.9% ± 0.4% and 9.3% ± 0.5%, respectively. The distribution of survived total RGCs, αRGCs, and ipRGCs was similar regardless of the location of the retina.

Conclusions. These results suggest that αRGC and ipRGC are highly tolerant to NMDA-induced neurotoxicity and NTG-like neurodegeneration in GLAST KO mice.

Keywords: αRGC, ipRGC, neurodegeneration, glaucoma, cell tolerance

Glaucoma is the second leading cause of blindness in the world.1,2 It is a neurodegenerative disease of the eye, which involves degeneration of retinal ganglion cells (RGCs) and optic nerve. In mammals, there are more than 40 different subtypes of RGCs that differ in soma size, morphology, dendritic arborization, and electrophysiologic functions.3–6 For example, one of the first subtypes to be characterized was αRGCs.7–9 αRGCs are identified by large cell bodies, wide and monosomatic dendritic fields, and they are rich in proteins, including SMI32, osteopontin (OPN; spp1), and voltage-gated potassium channel subunit Kv6.4 (kcnj6).10–13 On the other hand, intrinsically photosensitive RGCs (ipRGCs) are the more recently identified subtype and these cells have important roles in nonimage forming vision, such as pupillary light reflexes and recently identified subtype and these cells have important roles in nonimage forming vision, such as pupillary light reflexes and circadian rhythm.14,15 ipRGCs express the photopigment melanopsin and are identified by their dendritic arborizations stratifying in the sublaminae of the inner plexiform layer.16,17 Recent studies have shown that αRGCs and ipRGCs may be highly tolerant to cell death and may have the capacity to regenerate after optic nerve injury.11,18–20 Reports have indicated that function of ipRGCs is impaired in glaucoma patients.21 and the density of melanopsin-expressing RGCs is reduced in severely staged glaucoma patients.22 In rodents, brain derived neurotrophic factor (BDNF), which is a powerful neuroprotective agents for RGCs, rescues Brn3a-positive RGCs, but not melanopsin-positive RGCs in the ocular hypertension model.23 In addition, ipRGCs show high resistance to optic nerve transection or crush, glutamate neurotoxicity, and acute ocular hypertension, but not to chronic ocular hypertension, suggesting that ipRGCs respond differently among injuries.24–26

Glaucoma usually is associated with increased IOP, but a subset of glaucoma presents with statistically normal IOP, called normal tension glaucoma (NTG), suggesting the possibility that non-IOP-dependent factors may contribute to the disease progression.27,28 We previously reported that loss of glutamate/aspartate transporter (GLAST) in mice leads to progressive RGC loss and optic nerve degeneration while maintaining normal IOP, demonstrating key pathologic features of NTG.29–31 Retinal degeneration in GLAST knockout (KO) mice may occur due to various factors, such as aging, glutamate neurotoxicity, and oxidative stress, which are important risk factors in human glaucoma.32 Previous studies have reported that glaucoma patients show downregulation of glutamate transporters and glutathione levels,33,34 suggesting that GLAST KO mice are useful as animal models of NTG.

RGC death also are experimentally induced by intravitreal injection of toxins, such as N-methyl-D-aspartate (NMDA) or staurosporine.35 NMDA is a synthetic compound that selectively activates the NMDA subtype of glutamate receptors, and excessive activation of NMDA receptors induces a steep rise in intracellular calcium levels and causes excitotoxic cell death in neurons.36,37 Glutamate excitotoxicity is thought to have important roles in RGC death in many retinal diseases, such as glaucoma, diabetic retinopathy, optic nerve injury, and...
retinal ischemia. 32,39–42 Therefore, these experimentally-induced RGC death models are useful for exploring neuroprotective strategies that could lead to glaucoma therapy. 43,44 We examined RGCs and ipRGCs to determine if there are any subtype-specific or region-specific responses to NMDA treatment or to NTG-like neurodegeneration in GLAST KO mice.

MATERIALS AND METHODS

Mice
Experiments were performed using adult C57BL6 (wild type [WT]) and GLAST KO mice30 on a C57BL6 background in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal experiments were approved by the institutional animal care and use committee of the Tokyo Metropolitan Institute of Medical Science (approval number TMiMS: 18041).

NMDA Administration
WT mice were deeply anesthetized with isoflurane (Intervet, Tokyo, Japan) and then received intravitreal injection of PBS or NMDA under a microsurgical microscope (Olympus, Tokyo, Japan) using a glass microsyringe with a 33-gauge needle (Ito Corporation, Shizuoka, Japan). Eyes were punctured at the upper temporal limbus and a volume of 2 μL PBS or NMDA (1 mM in PBS; Sigma-Aldrich Corp., St. Louis, MO, USA) was injected.36,43 One week after injection, mice were sacrificed and the retinal samples were prepared for analysis.

Immunohistochemistry
Mice were sacrificed and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Eyes were enucleated, marked on the ear side so that they can be recognized as the temporal side of the retina. Eyes were postfixed in 4% paraformaldehyde for 2 hours at 4°C. Retinas then were isolated from the eye cup, incised radially into four radial pieces.

For immunolabeling with RNA-binding protein with multiple splicing (RBPMS) and OPN antibodies, the retinas were first incubated for 2 hours in a blocking solution containing 5% horse serum and 1% Triton X-100 in PBS (pH 7.4). The retinas then were incubated in a mixture of primary antibodies against RBPMS (1:1000; host, guinea-pig; ABN1376; Merck, Kenilworth, NJ, USA) and OPN (1:1000; host, goat; AF808-SP; R&D

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Immunostaining and quantification of total RGCs, αRGCs, and ipRGCs in the WT mouse retina. (A) Schematic illustration of the retinal area. (B, C) Immunohistochemical analysis of the flat-mounted WT mouse retina double-immunostained with anti-RBPMS antibody and anti-OPN antibodies (B), and with anti-RBPMS antibody and anti-melanopsin antibodies (C). These images were taken from the middle area. Scale bar: 200 μm. (D) Quantification of the RBPMS-, OPN-, and melanopsin-positive RGCs in (B, C). RGCs in the middle area of the dorsal, ventral, nasal, and temporal retina were counted and averaged. The data are represented as means ± SEM of six retinas. ***P < 0.001.
Systems, Minneapolis, MN, USA) at 4°C for 2 days in a blocking solution. After washing three times in PBS, the samples were incubated with the appropriate secondary antibodies (for RBPMS, 1:1000; donkey anti-guinea pig Alexa Fluor 488; AB_2340472; Jackson Immuno Research Laboratories, West Grove, PA, USA; and for OPN, 1:1000; donkey anti-goat Alexa Fluor 568; A11057; Invitrogen, Waltham, MA, USA) in a blocking solution for 1 day.

For immunolabeling with RBPMS and melanopsin antibodies, the retinas were first incubated for overnight in a blocking solution containing 10% normal horse serum and 1% Triton X-100 in PBS (pH 7.4). The samples then were incubated with a mixture of primary antibodies against RBPMS (1:1000) and melanopsin (1:30000; host, rabbit; AB-N38; Advanced Targeting System, San Diego, CA, USA) at 4°C for 1 day in a blocking solution containing 1% normal horse serum and 1% Triton X-100 in PBS (pH 7.4). After washing three times in PBS, the samples were incubated in the appropriate secondary antibodies (for RBPMS, as described above, and for melanopsin, 1:1000; donkey anti-rabbit Alexa Fluor 647; ab150075; Abcam, Cambridge, UK) in a blocking solution containing 1% normal horse serum and 1% Triton X-100 in PBS (pH 7.4) for overnight 4°C.

### TABLE 1. Density of RBPMS-Positive Cells in PBS-Treated Control and 1 mM NMDA-Treated Retinas

<table>
<thead>
<tr>
<th>Region</th>
<th>Control (Cells/mm²)</th>
<th>NMDA (Cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>3692 ± 68</td>
<td>1391 ± 23*</td>
</tr>
<tr>
<td>Middle</td>
<td>2731 ± 44</td>
<td>1214 ± 36*</td>
</tr>
<tr>
<td>Peripheral</td>
<td>2243 ± 60</td>
<td>1016 ± 23*</td>
</tr>
<tr>
<td>Dorsal</td>
<td>3071 ± 95</td>
<td>1204 ± 16*</td>
</tr>
<tr>
<td>Ventral</td>
<td>2886 ± 72</td>
<td>1225 ± 9.0*</td>
</tr>
<tr>
<td>Nasal</td>
<td>2733 ± 63</td>
<td>1171 ± 16*</td>
</tr>
<tr>
<td>Temporal</td>
<td>2867 ± 42</td>
<td>1227 ± 4.1*</td>
</tr>
</tbody>
</table>

* P < 0.01.

### TABLE 2. Density of OPN-Positive Cells in PBS-Treated Control and 1 mM NMDA-Treated Retinas

<table>
<thead>
<tr>
<th>Region</th>
<th>Control (Cells/mm²)</th>
<th>NMDA (Cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>242 ± 11</td>
<td>238 ± 11*</td>
</tr>
<tr>
<td>Middle</td>
<td>265 ± 5.8</td>
<td>253 ± 8.3*</td>
</tr>
<tr>
<td>Peripheral</td>
<td>244 ± 14</td>
<td>234 ± 7.6*</td>
</tr>
<tr>
<td>Dorsal</td>
<td>256 ± 11</td>
<td>244 ± 2.6*</td>
</tr>
<tr>
<td>Ventral</td>
<td>253 ± 16</td>
<td>246 ± 6.9*</td>
</tr>
<tr>
<td>Nasal</td>
<td>254 ± 16</td>
<td>235 ± 8.1*</td>
</tr>
<tr>
<td>Temporal</td>
<td>250 ± 13</td>
<td>242 ± 2.2*</td>
</tr>
</tbody>
</table>

* Not statistically significant.
Histologic Analysis

The retinal whole mounts were examined with a fluorescence microscope (BZ-X800; Keyence, Osaka, Japan). The density of immunopositive RGCs was obtained from one central (0.1 mm from the optic disc), one middle (0.8 mm from the optic disc), and one peripheral (1.5 mm from the optic disc) areas (0.04 mm²) per quadrant (dorsal, ventral, nasal, and temporal parts) of each retina. RGCs are counted manually by three people,

### TABLE 3. Density of RBPMS-Positive Cells in WT and GLAST KO Retinas

<table>
<thead>
<tr>
<th>Region</th>
<th>WT (Cells/mm²)</th>
<th>GLAST KO (Cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>3691 ± 67</td>
<td>1898 ± 51*</td>
</tr>
<tr>
<td>Middle</td>
<td>2725 ± 43</td>
<td>1418 ± 64*</td>
</tr>
<tr>
<td>Peripheral</td>
<td>2246 ± 62</td>
<td>1163 ± 84*</td>
</tr>
<tr>
<td>Dorsal</td>
<td>2878 ± 71</td>
<td>1413 ± 167*</td>
</tr>
<tr>
<td>Ventral</td>
<td>3068 ± 93</td>
<td>1472 ± 78*</td>
</tr>
<tr>
<td>Nasal</td>
<td>2881 ± 36</td>
<td>1625 ± 82*</td>
</tr>
<tr>
<td>Temporal</td>
<td>2722 ± 63</td>
<td>1461 ± 71*</td>
</tr>
</tbody>
</table>

* *P < 0.01.

### TABLE 4. Density of OPN-Positive Cells in WT and GLAST KO Retinas

<table>
<thead>
<tr>
<th>Region</th>
<th>WT (Cells/mm²)</th>
<th>GLAST KO (Cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>265 ± 3.8</td>
<td>247 ± 6.9*</td>
</tr>
<tr>
<td>Middle</td>
<td>241 ± 11</td>
<td>238 ± 3.6*</td>
</tr>
<tr>
<td>Peripheral</td>
<td>257 ± 13</td>
<td>246 ± 4.5*</td>
</tr>
<tr>
<td>Dorsal</td>
<td>253 ± 14</td>
<td>256 ± 6.0*</td>
</tr>
<tr>
<td>Ventral</td>
<td>257 ± 12</td>
<td>242 ± 11*</td>
</tr>
<tr>
<td>Nasal</td>
<td>250 ± 14</td>
<td>232 ± 5.9*</td>
</tr>
<tr>
<td>Temporal</td>
<td>257 ± 16</td>
<td>244 ± 12*</td>
</tr>
</tbody>
</table>

* Not statistically significant.

**Figure 3.** RGCs survive after NMDA-induced neurotoxicity in mice regardless of the location of the retina. (A) Immunostaining of total RGCs and αRGCs in the middle areas from the dorsal, ventral, nasal, and temporal areas of the retina in WT mice 7 days after intraocular injection of PBS or 1 mM NMDA. Scale bar: 200 μm. (B, C) Quantification of the RBPMS-positive (B) and OPN-positive (C) cell density in each area. The data are averaged cell density of the central, middle and peripheral regions, and represented as means ± SEM of six retinas for each experiment. **P < 0.01.
blind to the treatments. The average density of RGCs per square millimeter was calculated.

**Statistics**

Data are represented as mean ± SEM. When statistical analyses were performed, the 1-way ANOVA followed by the Tukey’s post hoc test was used. $P < 0.05$ was regarded as statistically significant. JMP version 13.1.0 (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses.

### RESULTS

#### Expression of OPN and Melanopsin in Mouse RGCs

To detect OPN-positive αRGCs and melanopsin-positive ipRGCs, we performed immunohistochemical analysis of the flat-mounted WT mouse retina with anti-RBPMS, anti-OPN, and anti-melanopsin antibodies. We divided the areas of the retina as shown in Figure 1A. Total RGCs were labeled with an anti-RBPMS antibody and some of them were double-labeled with an anti-OPN antibody (Fig. 1B). Melanopsin-positive ipRGCs

#### Table 5. Density of Melanopsin-Positive Cells in PBS-Treated Control and 1 mM NMDA-Treated Retinas

<table>
<thead>
<tr>
<th>Region</th>
<th>Control (Cells/mm²)</th>
<th>NMDA (Cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>27 ± 1.8</td>
<td>24 ± 1.5*</td>
</tr>
<tr>
<td>Middle</td>
<td>27 ± 2.4</td>
<td>26 ± 1.1*</td>
</tr>
<tr>
<td>Peripheral</td>
<td>29 ± 0.9</td>
<td>26 ± 1.4*</td>
</tr>
<tr>
<td>Dorsal</td>
<td>29 ± 1.0</td>
<td>28 ± 1.8*</td>
</tr>
<tr>
<td>Ventral</td>
<td>27 ± 1.3</td>
<td>26 ± 1.8*</td>
</tr>
<tr>
<td>Nasal</td>
<td>28 ± 2.2</td>
<td>23 ± 1.5*</td>
</tr>
<tr>
<td>Temporal</td>
<td>27 ± 1.4</td>
<td>26 ± 1.9*</td>
</tr>
</tbody>
</table>

* Not statistically significant.

#### Table 6. Density of Melanopsin-Positive Cells in WT and GLAST KO Retinas

<table>
<thead>
<tr>
<th>Region</th>
<th>WT (Cells/mm²)</th>
<th>GLAST KO (Cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>27 ± 2.4</td>
<td>24 ± 1.1*</td>
</tr>
<tr>
<td>Middle</td>
<td>28 ± 1.3</td>
<td>24 ± 1.1*</td>
</tr>
<tr>
<td>Peripheral</td>
<td>30 ± 1.0</td>
<td>26 ± 1.3*</td>
</tr>
<tr>
<td>Dorsal</td>
<td>30 ± 1.4</td>
<td>28 ± 1.6*</td>
</tr>
<tr>
<td>Ventral</td>
<td>27 ± 1.6</td>
<td>25 ± 1.2*</td>
</tr>
<tr>
<td>Nasal</td>
<td>28 ± 1.8</td>
<td>25 ± 1.6*</td>
</tr>
<tr>
<td>Temporal</td>
<td>28 ± 1.6</td>
<td>23 ± 1.0*</td>
</tr>
</tbody>
</table>

* Not statistically significant.
also were detected in some RBPMS-positive cells (Fig. 1C). Quantitative analysis in the middle areas of the dorsal, ventral, nasal, and temporal retina showed that the OPN- and melanopsin-positive RGCs were 8.9% ± 0.4% and 1.9% ± 0.4% of the RBPMS-positive cells, respectively (Fig. 1D). In our study, we did not detect many cells that were positive for OPN and melanopsin (Supplementary Fig. S1).

**OPN-Positive zRGCs Survive After NMDA-Induced Neurotoxicity**

To examine the survival rate of zRGCs following insult, we injected PBS or 1 mM of NMDA intraocularly to adult WT mice (6.1 ± 0.2 and 6.0 ± 0.3 months old, respectively) 7 days before sacrifice, and performed immunohistochemical analysis on the whole retina with anti-RBPMS and anti-OPN antibodies. As shown in representative images (Fig. 2A), NMDA significantly decreased the density of total RGCs in the central, middle, and peripheral areas (Fig. 2B). In contrast, the density of OPN-positive zRGCs was not decreased in the central, middle, or peripheral areas (Fig. 2C). We then performed similar quantitative analysis in four quadrants. As shown in representative images from the middle areas in each quadrant (Fig. 3A), NMDA significantly decreased the density in the dorsal, ventral, nasal, and temporal areas (Fig. 3B). In contrast, the density of OPN-positive zRGCs was not decreased in the dorsal, ventral, nasal, or temporal areas (Fig. 3C). The numeric data for the effects of NMDA on total RGCs and OPN-positive zRGCs are summarized in Tables 1 and 2.

In summary, NMDA-induced loss of RBPMS-positive total RGCs was 58.4% ± 0.4% compared to that of PBS-treated controls, whereas the loss of OPN-positive zRGCs was only 5.0% ± 0.6%. These results suggested that NMDA induces RGC death throughout the retina and zRGCs survive after NMDA-induced neurotoxicity regardless of the location of the retina.
RGCs Survive NTG-Like Retinal Degeneration in GLAST KO Mice

To examine the survival rate of RGCs during NTG-like retinal degeneration, we performed immunohistochemical analysis with anti-RBPMS and anti-OPN antibodies in adult GLAST KO mice (7.1 ± 0.5 months old). As shown in representative images (Fig. 4A), the density of total RGCs in GLAST KO mice was significantly decreased in the central, middle, and peripheral areas compared to that of age-matched WT mice (6.5 ± 0.2 months old; Fig. 4B). In contrast, the density of OPN-positive RGCs was not decreased in the central, middle, or peripheral areas (Fig. 4C). We then performed similar quantitative analysis in four quadrants. As shown in representative images from the middle areas in each quadrant (Fig. 5A), the density of total RGCs in GLAST KO mice was significantly decreased in the dorsal, ventral, nasal, and temporal regions compared to WT mice (Fig. 5B). In contrast, the density of OPN-positive RGCs was not decreased in these four areas (Fig. 5C). The numeric data for the total RGCs and OPN-positive RGCs in GLAST KO mice are summarized in Tables 3 and 4.

In summary, in GLAST KO mice, the loss of total RGCs was 48.4 ± 0.9% compared to that of WT mice, whereas the loss of OPN-positive RGCs was only 3.9 ± 0.4%. These results suggested that RGCs survive NTG-like retinal degeneration in GLAST KO mice regardless of the location of the retina.

Melanopsin-Positive ipRGCs Survive After NMDA-Induced Neurotoxicity and in GLAST KO Mice

We next examined the survival rate of melanopsin-positive ipRGCs after NMDA injection and in GLAST KO mice. The density of ipRGCs after NMDA injection was not significantly decreased compared to that of PBS-treated control mice in the central, middle, and peripheral areas (Fig. 6). Similarly, the
The density of ipRGCs after NMDA injection was not decreased compared to that of control mice in the dorsal, ventral, nasal, and temporal areas (Fig. 7). In GLAST KO mice, the density of ipRGCs in the central, middle, and peripheral areas was not significantly decreased compared to WT mice (Fig. 8). In addition, the density of ipRGCs in the dorsal, ventral, nasal, and temporal areas in GLAST KO mice was not decreased compared to WT mice (Fig. 9). The numeric data for the effects of NMDA on melanopsin-positive ipRGCs are summarized in Table 5, and for melanopsin-positive ipRGCs in GLAST KO mice are summarized in Table 6.

In summary, loss of melanopsin-positive ipRGCs was 7.6% ± 1.6% after NMDA neurotoxicity and 9.3% ± 0.5% in GLAST KO mice. The results suggested that ipRGCs survive after NMDA neurotoxicity and during NTG-like retinal degeneration in GLAST KO mice regardless of the location of the retina.

**DISCUSSION**

We showed that zRGCs and ipRGCs survive after NMDA-induced neurotoxicity and during NTG-like retinal degeneration in GLAST KO mice. We also examined whether there was any lesion specificity for the RGC loss, and found that zRGCs and ipRGCs survive regardless of the location of the retina. These results suggested that zRGCs and ipRGCs have high tolerance to IOP-independent pathogenic factors that may be involved in the progress of glaucoma.

We showed that zRGCs and ipRGCs are tolerant to glutamate neurotoxicity. Our findings are supported by a recent study demonstrating that susceptibility of different types of genetically identified RGCs to NMDA excitotoxicity varies significantly, and that zRGCs are the most resistant RGCs to NMDA excitotoxicity. Currently, the mechanisms that explain their high survival rate under the toxic environment in...
our models are unknown; although, some studies suggest a role of dopaminergic amacrine cells for protection of ipRGCs in NMDA-induced retinal injury.46–48 Other studies have reported that aRGCs and ipRGCs are tolerant to optic nerve injury, and one explanation for this may be their high mTOR activity.11,18,49,50 Therefore, future studies may focus on identifying the survival mechanisms in these two RGC subtypes. Furthermore, overexpression of OPN or melanopsin in RGCs boosts mTOR signaling to promote axon regeneration.11,51 These data suggest that understanding the neuroprotective and neuroregenerative mechanisms in aRGCs and ipRGCs and overexpression of relevant key genes to non-aRGCs and non-ipRGCs may be an effective therapeutic strategy for neurodegenerative diseases, such as glaucoma.

In our study, the distribution of aRGCs and ipRGCs was similar, and the survival rate of these RGCs also was similar regardless of the location of the retina. Our results are in agreement with previous studies showing that under normal conditions, cells positive for SMI-32 (aRGCs) and the melanopsin fluorescent reporter (ipRGCs) were distributed evenly throughout the mouse retina,52 and that the ratios of ipRGC in the peripheral, para-central, and central retinal regions were consistent in the fully-developed Sprague Dawley rat retina, in which immunotoxin induced uniform cell lesioning across the entire retina.53 However, some reports indicate that ipRGC distribution is asymmetrical and that they are more abundant in the superior or dorsal retina.15,54,55 We cannot explain the reason for this discrepancy, but it is possible that the use of low concentration of the melanopsin antibody may have contributed to the difference. In our study, we did not detect any statistically significant differences in the surviving ipRGCs as well as aRGCs in each retinal region in the NMDA-treated and GLAST KO mouse retina. It would be interesting to find if aRGCs and ipRGCs survive in the human glaucoma retina and if their localization shows regional specificity.

Fascinatingly, ipRGC responses and function are impaired in glaucoma patients causing smaller post-illumination pupil response (PIPR) and reduced sleep quality.56–59 One study suggests that glaucoma patients show “disturbed synaptic function and altered interaction between photoreceptors,
RGCs, and ipRGCs. Together with our data that demonstrate the high survival rate of ipRGCs in glaucoma models, one may speculate that the cells are not dead, but synaptic connections are disturbed in glaucoma patients. In such case, in which retraction of dendrites and/or axons is partly causing visual impairment in addition to RGC death, promoting dendrite or axon regeneration and reforming synapses could restore neuronal connections and lead to partial recovery of sight. If this is possible, there is a hope for glaucoma patients to improve their vision while preventing or slowing down disease progression with conventional therapy. Future studies will include investigations into the synaptic connections in glaucoma models.

We used GLAST KO mice as an animal model of NTG. This model closely mimics pathology of NTG, including RGC loss, optic nerve atrophy, and visual impairment while maintaining normal IOP. However, some glaucomatous features are not observed in GLAST KO mice. For example, the RGC loss in these mice is not restricted to specific regions as seen in human glaucoma, and the onset of retinal degeneration occurs not in aged mice, but in young mice (3 weeks of age). The early onset of disease may be an advantage as this allows obtaining experimental results speedily and GLAST KO mice have been providing useful information regarding NTG therapy.

Survival of RGCs and ipRGCs in GLAST KO mice may provide an experimental platform for examining selective vulnerability of RGCs in NTG-like degeneration, which could lead to a new therapy.

In summary, our findings suggested that RGC and ipRGC are highly tolerant to NMDA neurotoxicity and glaucoma-like retinal degeneration in GLAST KO mice. Although the roles of RGCs and ipRGCs in glaucoma have not been fully elucidated yet, further studies to elucidate the reasons for this high tolerance and the detailed survival mechanisms of RGCs and ipRGCs, for example, determination of factors that are common to resilient RGCs, may lead to devising a new therapeutic approach for glaucoma.

**Figure 9.** ipRGCs survive in GLAST KO mice regardless of the location of the retina. (A) Immunostaining of total RGCs and ipRGCs in the middle areas from the dorsal, ventral, nasal, and temporal areas of the retina in WT and GLAST KO mice. Scale bar: 200 μm. (B, C) Quantification of the RBPMS-positive (B) and melanopsin-positive (C) cell density in each area. The data are averaged cell density of the central, middle and peripheral regions, and are represented as means ± SEM of six retinas for each experiment.
Subtype-Specific RGC Tolerance in Glaucoma Models

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