Calcium is a vital intracellular signaling molecule. In the resting state, intracellular calcium concentration is low (<50 nM); however, during membrane depolarization or activation of receptors, there is an increase in concentration. Calcium is involved in neuronal processes such as membrane repolarization, neurotransmitter release, and gene transcription. Furthermore, evidence has shown that dysregulated or prolonged elevation of intracellular calcium can lead to cell death. Thus, evaluating changes in intracellular calcium may provide insight into pathologic events occurring prior to cell death.

A variety of optical methods for visualizing changes in intracellular calcium in neurons have been developed. Small-molecule calcium indicators, such as fluo-4 and fura-2, are fluorescent dyes that bind free cytosolic calcium, thereby altering their spectral properties. However, in tissue preparations, the specific labeling of cells of interest is a common challenge associated with the use of calcium indicator dyes. More recently, genetically encoded calcium indicators (GECIs) coupled to neuron-specific promoters have been used to generate transgenic mice with endogenous expression of calcium indicators such as GCaMP3. The Thy1-GCaMP3 transgenic mouse line has robust fluorophore expression in CNS neurons, and changes in GCaMP3 fluorescence accurately reflect intracellular calcium levels correlated with action potential firing. In this strain, Chen et al. demonstrated GCaMP3 expression also in retinal neurons; however, the specificity and functionality of GCaMP3 in the retina is not currently known.

Retinal ganglion cells (RGCs), the output neurons of the retina whose axons form the optic nerve, are lost in sight-threatening diseases such as glaucoma and diabetic retinopathy. Characterizing pathologic processes leading to RGC death is of interest for the understanding of disease mechanisms as well as for testing the efficacy of potential therapeutic strategies. Previous studies show well-characterized and progressive RGC loss after optic nerve transection (ONT).
Thy1-GCaMP3 Expression and Function in RGCs After ONT

impaired axonal transport\textsuperscript{14,15} and depression of the electroretinogram (ERG),\textsuperscript{17} have been documented; however, functional changes to individual RGCs after ONT, to our knowledge, have not been characterized. The objectives of the present study were to characterize the expression of GCaMP3 in the Thy1-GCaMP3 transgenic mouse and utilize ONT as a model to assess changes in intracellular calcium in RGCs after axonal injury.

METHODS

Animal Procedures and Tissue Preparation

All experiments were approved by the Dalhousie University Committee on Laboratory Animals and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Thy1-GCaMP3 mice (B6;CBA-Tg(Thy1-GCaMP3)6Gfng/J, stock no. 017893; The Jackson Laboratory, Bar Harbor, ME, USA) were housed in a standard 12 hour light–dark cycle and fed ad libitum. In a subset of animals, ONT was performed in the left eye 3, 5, or 7 days prior to sacrifice according to a protocol detailed elsewhere.\textsuperscript{18} In brief, mice were anesthetized (isoflurane, 2%–3%, 1.5 L O\textsubscript{2}/min; Baxter Corporation, Mississauga, ON, Canada) and the head stabilized in a stereotaxic device. The optic nerve was carefully exposed by blunt dissection and transected within the dural sheath approximately 1 mm behind the globe, keeping the vascular supply intact. Incisions were sutured, and animals recovered on a heating pad prior to their return to regular housing facilities.

Animals were sacrificed by injection of sodiumpentobarbitatal (100 mg/kg, Euthansol 240 mg/ml; CDMV, Saint-Hyacinthe, QC, Canada). Eyes were enucleated and placed in room temperature solution (Hibernate A; ThermoFisher Scientific, QC, Canada). Tiled images were obtained at 20X (AxioImager.M2, Plan-Apochromat 0.8 NA objective; Carl Zeiss, Oberkochen, Germany) with a fluorescence excitation light source (X-Cite 120Q; Excelitas Technologies, Waltham, MA, USA) and digital camera (AxioCam 506 camera; Carl Zeiss).

Immunohistochemistry and Imaging

For flat-mount preparations, isolated retinas were fixed in paraformaldehyde (PFA, 4% in PBS, 1 hour, room temperature). Sagittal retinal sections were obtained from whole-fixed eyes (PFA, 4% in PBS, overnight, 4°C; 20% sucrose, overnight, then frozen at –80°C). Both flat-mount retinas and retinal sections were treated with blocking solution (10% normal serum in PBS, 017-000-121, 005-000-121; Jackson Laboratory, Bar Harbor, ME, USA) overnight at 4°C prior to incubation with primary antibodies (3–5 days at 4°C) and the percentages of colabeled cells (GFP+RBPMS+, GCaMP3 expresser). Retinas were washed in PBS and incubated overnight with the appropriate secondary antibodies: 4°C, diluted 1:1000 in PBS with 0.3% Triton X-100: anti-RBPMS; RGC marker, 1:100, 1832-RBPMS; PhosphoSolutions, Aurora, CO, USA), anti-ChAT (cholinergic amacrine cell marker, 1:100, AB144P; Millipore, Billerica, MA, USA, or 315-ChAT; PhosphoSolutions), anti-syntxin (amacrine cell marker, 1:100, 1990-STX; PhosphoSolutions), anti-GAD67 (GABAergic amacrine cell marker, 1:100, MAB5406; Millipore), anti-GABA (GABAergic amacrine cell marker, 1:1000, A2052; Sigma-Aldrich Corp., Oakville, ON, Canada), anti-green fluorescent protein (anti-GFP) AlexaFluor 488 conjugate (binds to the GFP region of GCaMP3, 1:400, A21311; Thermofisher). Retinas were washed in PBS and incubated overnight with the appropriate secondary antibodies: 4°C, diluted 1:1000 in PBS with 0.3% Triton X-100: Cy3 (706-165-148, 106-165-003; Jackson ImmunoResearch), AlexaFluor 633 (A21082; ThermoFisher), AlexaFluor 647 (A21236; Thermofisher; 711-605-152; Jackson ImmunoResearch). Retinas were mounted on microscope slides with antifade media (VectaShield; Vector Laboratories, Burlington, ON, Canada). Tiled images were obtained at 20X (Axio-

Calcium Imaging

Retinas were dissected and mounted on black filter paper (Millipore) with the RGC side facing up and maintained in Hibernate A solution for 30 min after sacrifice to ensure calcium imaging experiments. Mounted retinas were then placed in a microscope-mounted chamber and superfused with oxygenated Hank’s balanced salt solution (HBSS) throughout the experiment (Sigma-Aldrich Corp.) with 10 mM HEPES, pH 7.4, 100% O\textsubscript{2}, ~2 mL/min. GCaMP3 was excited with a xenon lamp (Lambda LS with Lambda 10-2 shutter controller; Sutter Instruments, Novato, CA, USA; 488/516-nm excitation/emission filters; Omega Optical, Brattleboro, VT, USA) and emitted fluorescence captured with a charge-coupled device (CCD) camera (Sensicam PCO; Kelheim, Germany) connected to an upright microscope (Axioskop, Achromplan 40× water immersion objective, 0.80 W NA; Carl Zeiss). Images were captured at 320 × 256 pixels (4 × 4 binning, 213 × 171-μm field of view) and recorded continuously at 0.05 to 0.2 Hz (Axon Imaging Workbench 4; Molecular Devices, Sunnyvale, CA, USA) before, during, and after kainic acid (KA; R&D Systems, Minneapolis, MN, USA) applications. Escalating concentrations of KA (10, 50, 100 μM in HBSS) were applied by superfusion for 30 seconds, followed by 15-minute washout periods before the next application.

Data Analysis

To determine the expression rate and specificity of Thy1-GCaMP3 in RGCs, six (500 × 500 μm) sample areas, two each from the central, midperipheral, and peripheral retina (approximately 0.5, 1, and 1.5 mm from the optic nerve head, respectively), were selected from each flat-mounted retina stained with anti-GFP, anti-RBPMS, and anti-ChAT antibodies. The number of GFP-immunoreactive (GFP+; GCaMP3 expressing) and RBPMs-immunoreactive (RBPMs+) cells were counted (Zen2Lite; Carl Zeiss) in each region, and cell densities (cells/mm\textsuperscript{2}) were computed from the averages of the three retinal areas. The number of GFP+ cells that were colabeled with RBPMs or ChAT were also quantified (Zen2Lite; Carl Zeiss), and the percentages of colabeled cells (GFP+/RBPMs+ and GFP+/ChAT+) calculated.

After calcium imaging experiments, GCaMP3+ cells were selected as regions of interest (ROIs) and the fluorescence intensity overtime for each ROI was computed (Axon Imaging Workbench 4; Molecular Devices). Changes in GCaMP3 fluorescence during each KA application were quantified and calcium transient amplitudes (∆F/F\textsubscript{0}) were defined as the difference between peak and baseline fluorescence (ΔF) divided by baseline fluorescence (F\textsubscript{0}; ClampFit 10.4; Molecular Devices). GCaMP3+ cell densities (cells/mm\textsuperscript{2}) and the proportion of responding cells (%) in control and ONT groups were calculated from each field of view during calcium imaging experiments. Calcium transient data are presented as mean (SD), unless otherwise noted, and were analyzed by 1-way ANOVA followed by Tukey’s multiple comparisons test when applicable (Prism6; GraphPad, La Jolla, CA, USA). For the post hoc analysis of only those cells that were considered to have responded to KA, we considered only cells that had responses greater than 5% of baseline fluorescence (the average baseline fluctuation in fluorescence). Because only a negligible number of RGCs responded to 10 μM KA, data corresponding to this KA concentration were not reanalyzed.
RESULTS

Characterization of GCaMP3 Expression in the Thy1-GCaMP3 Transgenic Mouse Retina

Uniform expression of GCaMP3 was observed across all sectors of the retina. Figure 1A shows GCaMP3 expression with GFP immunoreactivity in a fixed whole retinal flatmount from a control Thy1-GCaMP3 mouse. Axon bundles were observed in some areas (Figs. 1A, 1B), but these did not impede visualization of individual cell bodies. GFP immunoreactivity was mostly excluded from nuclei (Figs. 1B, 1C), and in some cases axons and dendrites of individual cells (Figs. 1B, 1C; arrows) were observed. GFP immunoreactivity colocalized extensively with the RGC-specific marker RBPMS, but not the cholinergic amacrine cell marker, ChAT (Figs. 1C–1E). The mean (SD) density of GFP-immunoreactive (GFP+) cells in control retinas was 2609 (481) cells/mm², of which, 85% (6%) of GFP+ cells were also RBPMS+. The mean (SD) density of RBPMS+ cells was 3462 (557), and the mean percentage of RBPMS+ cells expressing GCaMP3 was 64% (4%).

After ONT, RGC density decreased; however, the specificity of GCaMP3 expression to RGCs did not change. As shown in Figure 2, no colocalization of GFP and ChAT immunoreactivity was found in flatmounts from control (Fig. 2A) or ONT groups (7 days post ONT, Fig. 2B). The RGC population was reduced 7 days post ONT (GFP+: 58.5%; RBPMS+: 57.5%; decrease versus control cell densities, n = 3–4 retinas per group), while the populations of ChAT immunoreactive and GFP+/RBPMS– cells remained unchanged. Quantification of GFP+/ChAT+ cells showed limited colocalization in retinas from the control and ONT groups (percent GFP+/ChAT+: control: 0.08% [0.08%]; ONT: 0.03% [0.04%]). Furthermore, there was no quantifiable colocalization between GFP immunoreactivity and amacrine cell markers (ChAT, GABA, GAD67, or syntaxin) in control retina sections (Supplementary Fig. S1).
Calcium Transients of RGCs Expressing GCaMP3 Were Reduced After ONT

In cells of the control retina group, GCaMP3 fluorescence increased in a concentration-dependent manner after the application of KA and returned to baseline levels upon washout of KA (Figs. 3A, 3B). There was a modest increase in intracellular calcium levels in response to $10 \mu\text{M KA}$ in some, but not all cells tested (Fig. 3C, Table; $\Delta F/F_0$ [SD]: control: 0.15 [0.22]). Higher concentrations of KA ($50$ and $100 \mu\text{M}$) evoked large calcium transients in nearly all cells tested (Fig. 3C, Table; $\Delta F/F_0$ [SD]: $50 \mu\text{M}: 0.99$ [0.67], $100 \mu\text{M}: 1.29$ [0.57]; $n = 504-2975$ cells/group from 14 retinas).

In retinas of the ONT group, there was no change in baseline fluorescence levels compared to the control group (Figs. 4A–4D; fluorescence units [SD]: control: 45 [10], day 3: 40 [1], day 5: 40 [3], day 7: 37 [2]); however KA-evoked calcium transients in cells from the ONT group were significantly smaller than those from retinas of the control group (Fig. 4E; $\Delta F/F_0$ [SD]: $10 \mu\text{M}$ control: 0.15 [0.22], day 3: 0.05 [0.10], day 5: 0.03 [0.03], day 7: 0.01 [0.02], $50 \mu\text{M}$ control: 0.99 [0.67], day 3: 0.51 [0.36], day 5: 0.28 [0.26], day 7: 0.29 [0.34]; $100 \mu\text{M}$ control: 1.29 [0.57], day 3: 0.53 [0.33], day 5: 0.50 [0.36], day 7: 0.47 [0.33]; $P < 0.05$; $n = 74-2975$ cells/group from four retinas/ONT group and 14 retinas in the control group). There was also a progressive decrease in the number of GCaMP3+ cells after ONT (Fig. 4F; cells/mm² [SD]: control: 2198 [453], day 3: 2224 [643], day 5: 1383 [375], $P < 0.05$; day 7: 913 [178], $P < 0.05$; $n = 4-14$ retinas/group).

The Proportion of GCaMP3+ Cells Responding to KA Decreased After ONT

In retinas from the control group, the proportion of cells responding to KA increased in a concentration-dependent manner (Table); however, the proportion of remaining GCaMP3+ cells responding to KA was reduced as early as day 3 post ONT (Table). When only cells demonstrating an appreciable response to KA (>5% $\Delta F/F_0$) were considered, mean calcium transient amplitudes in cells from the ONT group were significantly reduced compared to those from the control group (Fig. 5, $\Delta F/F_0$ [SD]: $50 \mu\text{M}$ control: 1.00 [0.67], day 3: 0.50 [0.35], day 5: 0.31 [0.28], day 7: 0.35 [0.36]; $100 \mu\text{M}$ control: 1.23 [0.57], day 3: 0.53 [0.33], day 5: 0.50 [0.36], day 7: 0.47 [0.33]; $P < 0.05$; $210-2953$ cells/group from 4-14 retinas/group).

**DISCUSSION**

In the retina, the Thy1 promoter is known to drive selective expression of fluorescent reporter proteins in most RGCs. However, some studies have demonstrated Thy1-driven expression of reporters also in other types of retinal neurons or changes in reporter expression after injury. Results of the present study showed that GCaMP3 was expressed in the majority (64%) of RGCs across the entire retina of Thy1-GCaMP3 transgenic mice. Previous studies using the Thy1-cyan fluorescent protein (CFP) line have shown greater, yet still incomplete, expression of CFP in the RGC population quantified using retrograde tracers. It is well documented that expression of Thy1-XFPs in the various transgenic lines described by Feng et al. demonstrate differences in the proportion of RGCs expressing fluorescent proteins. For example, the Thy1-yellow fluorescent protein (YFP) line H mouse retina exhibits YFP

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**TABLE. Proportion of GCaMP3+ Cells Responding to KA in the Absence and Presence of ONT**

<table>
<thead>
<tr>
<th>KA Concentration, $\mu\text{M}$</th>
<th>Proportion of Cells Responding, %</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>10</td>
<td>38</td>
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expression in less than 0.5% of RGCs, whereas nearly 100% of RGCs in the Thy1-YFP line are YFP\(^+\) and approximately 80% of RGCs are CFP\(^+\) in the Thy1-CFP line. Thus, it is clear that transgenic expression of fluorophores under the control of the Thy1 promoter in the retina does not label 100% of RGCs. Furthermore, previous reports have demonstrated varied fluorescence intensity of cells (dim versus bright) within Thy1-CFP\(^+\) and Thy1-YFP line \(\text{H}\)\(^+\) retinas, which was also observed in the present study of Thy1-GCaMP3 retinas. These findings suggest that there is a range of reporter expression levels within the RGC population in Thy1-XFP retinas; thus, cells with low reporter expression levels may exhibit dim fluorescence and fall below the level of detection, depending on the methods used to quantify RGC populations.

In the present study, Thy1-GCaMP3 retinas of the control group stained with RBPMS, an RGC-specific marker, revealed RGC densities similar to previous reports in C57Bl/6 background mice. The majority of GFP\(^+\), GCaMP3-expressing cells (85%) colocalized with RBPMS; however, there were some instances of GFP\(^+\)/RBPMS\(^-\) cells. These results were somewhat surprising as the characterization of the RBPMS antibody by Rodriguez et al. did not find a population of murine RGCs that were not labeled by anti-RBPMS. However, earlier studies using a different anti-RBPMS antibody found a small population of RGCs, confirmed by retrograde labeling, that lacked RBPMS.

**Figure 3.** KA-evoked calcium transients increased in a concentration-dependent manner. (A) Pseudocolor photomicrographs taken from the calcium imaging experiments in a retina from the control group showing the peak change in GCaMP3 fluorescence (middle) from baseline (left), indicating elevated intracellular calcium induced by bath application of 50 \(\mu\)M KA, returning to baseline (right) after KA wash out. Scale bar: 50 \(\mu\)m. (B) Example traces (50 ROIs selected from one experiment) illustrate a concentration-dependent increase of GCaMP3 fluorescence in response to increasing KA concentrations. Arrows indicate timing of 30-second KA application (left to right: 10, 50, 100 \(\mu\)M) with 15-minute washout periods between KA applications. (C) Mean data show a concentration-dependent increase in calcium transient amplitudes (\(n = 50\)–2973 cells/group from 14 retinas; \(*P < 0.05, 1\)-way ANOVA).
Indeed, we believe that the GFP⁺/RBPMS⁺/C0 cells observed in the present study are, in fact, RGCs, as these cells often had clear axons and processes labeled with GFP and did not colocalize with amacrine cell markers. However, due to the low number of these cells in each experiment, it is difficult to definitively confirm their identity. It is possible that these GFP⁺/RBPMS⁻ cells were amacrine cells that were not adequately labeled by the markers chosen in this study or perhaps glial cells that had phagocytosed GCaMP3 from dying RGCs.

Results of the present study showed a decrease in RGC density 7 days post ONT. The rate of cell loss observed in retinas of the Thy1-GCaMP3 mouse line (GFP⁺, 58.5%, and RBPMS⁺, 57.5%, decreases compared to control) is within the ranges previously reported in C57/BL6 (~70% loss) and Thy1-CFP (~55% loss) strains 7 days after ONT.²⁰,²⁶ It is important to note that the present study demonstrates that GCaMP3 expression is restricted to RGCs and is not expressed in a significant population of displaced amacrine cells, even after ONT. These results differ from previous reports with other...
related transgenic strains such as Thy1-CFP. Raymond et al.\textsuperscript{21} examined retinas from Thy1-CFP transgenic mice with immunohistochemistry and showed that many CFP-positive cells in that mouse line also expressed amacrine cell markers (ChAT and GAD67), suggesting that the Thy1-driven expression of CFP is not specific for RGCs. However, results of the present study showed colocalization between GFP immunoreactivity and amacrine cell markers in a negligible proportion of cells, which did not change after ONT. Wang et al.\textsuperscript{18} used the Thy1-CFP mouse to study cell loss after ONT and showed that CFP and the retrograde tracer, FluoroGold, were found in microglia after axonal injury. The authors concluded that fluorescence persisted after RGC death and was phagocytosed by microglia.\textsuperscript{18} Wang et al.\textsuperscript{18} used Iba-1 (an astrocyte marker) immunoreactivity and morphologic analysis to determine the presence of CFP and FluoroGold fluorescence in glial cells. The authors described the appearance of phagocytosed fluorescent markers as “spindle-shaped” or “bright punctate bodies.”\textsuperscript{18} In the present study, while we did not stain for Iba-1 immunoreactivity, we did not observe the presence of GFP-positive spindle-shaped or condensed cells, characteristic of microglia uptake observed by Wang et al.\textsuperscript{18} Furthermore, we did not observe any additional GFP immunofluorescence colocalized with amacrine cell markers in retinas of the ONT groups. Taken together, these results show that GCaMP3 expressed under the control of the Thy1 promotor is specific for RGCs and remains as such after ONT, suggesting that this transgenic line can be used as a reporter to selectively study RGC function in experimental models of retinal disease.

GCaMP3 and its successors (GCaMP6, along with others) have been used extensively to measure changes in intracellular calcium in neurons.\textsuperscript{27–32} However, the present study is the first, to our knowledge, to examine GCaMP3 responses in the retinas of Thy1-GCaMP3 transgenic mice in both control and ONT conditions. Results showed that GCaMP3 exhibited concentration-dependent increases in fluorescence in response to KA in Thy1-GCaMP3 retinas, suggesting that this transgenic line can be used as a sensitive calcium reporter molecule in RGCs. In control group retinas, GCaMP3 transients were similar to those found in previous studies from our group with the traditional small-molecule calcium indicator dye, fura-2.\textsuperscript{10} Fura-2 has been used extensively by our group to study calcium dynamics in RGCs.\textsuperscript{10,53} and while fura-2 has excellent properties for measuring changes in intracellular calcium, uptake of the dye is not selective to RGCs. Furthermore, it is not clear whether cells will take up small-molecule dyes as readily after an injury such as ONT. Thus, a GECI specifically expressed in RGCs prior to any other intervention allows the examination of cellular changes caused by ONT.

Previous studies of other genetically expressed fluorescent proteins have shown quenching of fluorescence after cellular injury.\textsuperscript{54} which makes it impossible to study changes in functional responses after an insult. In the present study, baseline GCaMP3 fluorescence levels were not different between retinas in control and ONT groups, while calcium transient amplitudes were reduced in RGCs from retinas in the ONT group. In fact, many GCaMP3-positive cells in retinas of the ONT groups had negligible calcium transient amplitudes. It is possible that the expression levels of GCaMP3 were reduced in retinas of the ONT groups, which may result in smaller calcium transients reported by the indicator. Indeed, previous studies have shown downregulation of Thy1 in RGCs after injury\textsuperscript{35}; however, in the present study, baseline levels of fluorescence were not different between experimental groups, which we interpret as relatively stable levels of intracellular GCaMP3. We would expect that if levels of GCaMP within cells were reduced by downregulation of the Thy1 promoter, or otherwise, then the baseline fluorescence intensity reported by GCaMP3 would also be reduced. One alternate possibility for reduced calcium transient amplitudes in retinas of the ONT group is compartmentalization and sequestration of a portion of GCaMP3 within RGCs, which would have persistent resting fluorescence levels\textsuperscript{56} but fail to fully report changes in intracellular calcium levels upon application of KA. Wang et al.\textsuperscript{18} observed bright, punctate cells expressing CFP after ONT and concluded that these cells were in the process of dying, undergoing shrinkage, and being removed from the retina by microglia. Thus, it is possible that in the present study, GCaMP3-positive cells in retinas of the ONT groups had begun cell death processes and GCaMP3 is being sequestered, making it functionally unavailable. Furthermore, alterations in the intracellular environment may influence the ability of GCaMP3 to reliably report changes in calcium levels. Studies have shown pH-dependent changes in the fluorescence of GFP,\textsuperscript{57} which may be extrapolated to GCaMP owing to some similarities in the protein structure. Also, the dynamic conformational changes in the GCaMP protein in the calcium bound and unbound states, which influence emitted fluorescence, may be altered in a stressed cellular environment, and this could impact reporter activity. However, to our knowledge, this has not been studied. Results of the present study demonstrated a concentration-dependent increase in the proportion of RGCs responding to KA, reaching near 100% response rate at 50 μM KA in retinas of the control group. However, in cells from retinas of the ONT groups, there was a shift in the sensitivity to KA. At all time points post ONT (3, 5, and 7 days), 100 μM KA was required to elicit calcium transients in most RGCs. Despite the possibility that GCaMP may be altered in some way by the post ONT cellular environment, the present study showed responsiveness, albeit reduced, of nearly 100% of remaining cells using 100 μM KA in the ONT groups, suggesting a decreased sensitivity and capacity of RGCs to respond to depolarizing stimuli after ONT. Furthermore, the finding that there was a concentration-dependent effect of KA on GCaMP3-reported calcium transient amplitudes in retinas of the ONT groups suggests that GCaMP3 is sensitive enough to measure calcium dynamics during periods of cellular distress; however, further studies examining the properties of GCaMPs in varied cellular environments are warranted.

Functional deficits in mouse RGCs following optic nerve trauma have been suggested by studies examining the effect of ONT on the ERG waveform. Specifically, both the negative and positive scotopic threshold response components of the full-
field mouse ERG have been linked to RGC function and decrease in amplitude as early as 3 days following ONT.\textsuperscript{37,38,39} Studies examining single cell changes after axonal injury have shown dendritic retraction and RGC soma shrinkage leading up to cell death,\textsuperscript{40,41} which would result in the loss of connectivity of RGCs with bipolar and amacrine cells and lead to dysfunction within the visual pathway. It is possible that the head decreased synaptic input to RGCs, from interneurons as RGC dendrites retract and connections are lost, contributed to the reduction in calcium transients in retinas of the ONT groups reported here. The changes in RGC intracellular calcium dynamics in response to extracellular stimulation reported in the present study are likely indicative of multiple levels of cellular dysfunction occurring in the retina after ONT and may also contribute to widespread defects in cellular processes mediated by improper calcium handling, including action potential properties, neurotransmitter release onto target structures, and gene expression.\textsuperscript{2-6} At a system level, these defects may manifest as reduction in or even loss of visual function occurring prior to the actual death of the cell, as demonstrated by full-field ERG.\textsuperscript{37}

With increasing time after ONT, the sensitivity of remaining GCaMP3+ RGCs to KA was diminished to the point that many remaining cells did not respond to 50 \( \mu \)M KA, which generated a maximal response in most normal RGCs. These results show that cells labeled with a structural marker (GCaMP3, in this case) are not necessarily functioning normally. The current results suggest that neuronal dysfunction at a cellular level, manifested as the loss of ability to respond to external stimuli, is widespread and precedes the structural loss of RGCs assayed commonly with immunohistochemistry in animal models of optic nerve and retinal disease. Much research relies entirely on structural assessment of RGCs to predict viability and evaluate putative treatments.\textsuperscript{15,16,42,43} Though these methods give important insights into gross changes in the structure of the retina, overall function is likely overestimated by labeling methods alone. The results of the present study show that GCaMP3, expressed under the Thy1 promoter, can be used to study functional changes in RGCs after ONT. While the present study used ONT as a model of rapid and severe RGC damage, we expect that GCaMP3 would also be applicable in studies of chronic RGC injury, such as elevated IOP.

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References

10. Daniels BA, Baldridge WH. \( \delta \)-Serine enhancement of NMDA receptor-mediated calcium increases in rat retinal ganglion cells. \textit{J Neurochem}. 2010;112:1180–1189.