Neurotoxic Reactive Astrocytes Drive Neuronal Death after Retinal Injury

Graphical Abstract

Highlights

- Astrogliosis occurs following acute optic nerve injury and in a mouse model of glaucoma
- Reducing astrogliosis stops neuron death after optic nerve crush or in a glaucoma model
- Neurons spared from death remain electrophysiologically functional
- Injury is required for neurons to become susceptible to astrocyte-mediated toxicity

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In Brief

Guttenplan et al. show that dampening the formation of reactive astrocytes prevents neuronal death following acute optic nerve crush or in a chronic model of glaucoma, with spared neurons remaining electrophysiologically functional. More generally, they show that injury is required to induce neurons to become susceptible to astrocyte-mediated toxicity.
Glaucoma is a neurodegenerative disease that features the death of retinal ganglion cells (RGCs) in the retina, often as a result of prolonged increases in intraocular pressure. We show that preventing the formation of neuroinflammatory reactive astrocytes prevents the death of RGCs normally seen in a mouse model of glaucoma. Furthermore, we show that these spared RGCs are electrophysiologically functional and thus still have potential value for the function and regeneration of the retina. Finally, we demonstrate that the death of RGCs depends on a combination of both an injury to the neurons and the presence of reactive astrocytes, suggesting a model that may explain why reactive astrocytes are toxic only in some circumstances. Altogether, these findings highlight reactive astrocytes as drivers of RGC death in a chronic neurodegenerative disease of the eye.

INTRODUCTION

Glaucoma is a neurodegenerative disease marked by axonal damage of retinal ganglion cells (RGCs) that results in RGC death and vision loss. Although we have long known the identity of the cells that degenerate in the disease, we still do not understand how or why these cells die. Considerable research into the mechanisms of glaucoma has been conducted in rodent models, with most leveraging an increase in fluid volume in the eye to increase outflow (John et al., 1998), and various experimental approaches that physically obstruct the fluid channels of the eye (Morrison et al., 2005). These models include inherited models, such as DBA/2 mice with mutations in Tyrp1 and Gpnmb that reduce aqueous outflow (John et al., 1998), and various experimental approaches that physically obstruct the fluid channels of the eye (Morrison et al., 1997; Ueda et al., 1998; Sappington et al., 2010; Chen et al., 2011; El-Danaf and Huberman, 2015). The resulting increase in IOP leads to RGC death and axonopathy, pathology that closely mimics the progression and cell-type specificity of human glaucoma. As in many neurodegenerative diseases, dysfunction in mouse models of glaucoma is coupled with a response of the surrounding glial cells in the retina and optic nerve known as gliosis, usually measured by increases in the protein and transcript of glial fibrillary acidic protein (GFAP) (Inman and Horner, 2007; Sun et al., 2017; Wang et al., 2017). Importantly, this response increases with disease progression (Bosco et al., 2016), suggesting a mechanistic link between gliosis and degenerative pathology.

We previously reported that microglia and astrocytes interact in response to acute injury to the optic nerve and in chronic neurodegenerative disease to produce a neurotoxic reactive astrocyte phenotype (Liddelow et al., 2017). This response by astrocytes is largely mediated by the microglial release of interleukin-1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α), and the classical complement component C1q. Altogether, these three factors are necessary and sufficient to induce neuroinflammatory reactive astrocytes, which can then release an as-yet-unidentified toxin to specifically kill neurons and mature oligodendrocytes. We have also shown that crushing the optic nerve is sufficient to induce both a pro-inflammatory microglial state and this neuroinflammatory reactive astrocyte phenotype. Blocking the activation of these astrocytes (while maintaining an otherwise normal microglia response) using either the neutralizing antibodies to IL-1α, TNF-α, and C1q or an Il1a−/−Tnf−/−C1qa−/− triple knockout (tKO) mouse line preserves RGC viability following axotomy. Thus, neurotoxic reactive astrocytes appear to contribute to neuronal death following acute injury in the optic system.

Here we investigate whether astrocytes drive neuronal death in chronic neurodegenerative diseases of the eye as they do in response to acute axotomy of RGCs. We show that the microbead-occlusion model of glaucoma induces a neurotoxic reactive astrocyte phenotype that is paired with loss of RGCs. We also show that blocking this reactive phenotype is sufficient...
to preserve RGC numbers. Given recent advances in encouraging the regrowth of axotomized RGCs (Park et al., 2008), we also ask whether neurons preserved from death in this model are still electrically active and thus of potential benefit to the regeneration of the retina. We perform electrophysiological and morphological analysis of RGCs protected from death following a glaucoma model and find that they are altered but still largely functional, with generally appropriate dendritic stratification and electrical response properties. Altogether, these findings suggest that reactive astrocytes are a potentially powerful target for therapeutic intervention in both acute and chronic injuries to the eye and that preventing the death of RGCs may provide a useful platform for the regeneration of the retina.

RESULTS

RGCs Die after Acute Axonal Injury
We first investigated the timing of RGC death following acute axotomy using the retro-orbital optic nerve crush (ONC) model (Frank and Wolburg, 1996; Tang et al., 2011). We previously reported that ONC induces reactive astrogliosis and that reducing astrogliosis prevents RGC death in this model (Liddelow et al., 2017). To better understand the mechanism of cell death following axotomy and to assess cell death at a time point comparable to mouse models of glaucoma, we performed a more detailed time course of RGC death following ONC in both mouse and rat. In both animal models, RGC death began at 7 days after injury and progressively worsened through 28 days after injury (Figure 1).

To confirm the role of reactive astrocytes in the death of RGCs after axotomy, we performed ONC in the absence of IL-1α, TNF-α, and C1q, three factors secreted from microglia that are necessary to induce neuroinflammatory astrocyte reactivity (Liddelow et al., 2017). In mouse, we performed ONCs in an Il1a−/−Tnf−/−C1qa−/− tKO generated in a previous study, because these mice fail to produce neurotoxic reactive astrocytes following activation of microglia (Figures 1A–1C) (Liddelow et al., 2017). In rat, we performed ONCs while simultaneously injecting neutralizing antibodies against IL-1α, TNF-α, and C1q or immunoglobulin G (IgG) control antibodies into the vitreous of the eye (Figures 1D–1F). In both animal models, RGC death began at 7 days after injury and progressively worsened through 28 days after injury (Figure 1).

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The Microbead Occlusion Model of Glaucoma Induces Gliosis

We next asked whether astrocytes drive neuronal death following chronic central nervous system (CNS) injury as they do following acute injury. Sustained increase in IOP, the pathogenic mechanism in glaucoma, leads to the progressive death of RGCs in rodent models. In microbead occlusion models of glaucoma, ≤15 μM beads are injected into the anterior chamber of the eye, disrupting aqueous outflow and increasing IOP. We performed microbead occlusions in wild-type (WT) and tKO mice to compare the response of the retina with prolonged increases in IOP in the presence or absence of neuroinflammatory reactive astrocytes.

Two major responses of ocular pressure were observed in microbead occlusion retinas. Some animals showed sustained increase in IOP, with average daily pressures significantly higher than in the uninjected contralateral control eye at 7 days after injection (Figures 2A and 2B). Other animals showed a temporary increase in IOP that quickly resolved to baseline by 7 days after injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Other animals showed a temporary increase in IOP that quickly resolved to baseline by 7 days after injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Two major responses of ocular pressure were observed in microbead occlusion retinas. Some animals showed sustained increase in IOP, with average daily pressures significantly higher than in the uninjected contralateral control eye at 7 days after injection (Figures 2A and 2B). Other animals showed a temporary increase in IOP that quickly resolved to baseline by 7 days after injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D). Importantly, maximum increases in IOP were similar in both WT and injection (Figures 2C and 2D).

Although there was an ~25% decrease in RGCs 28 days after injection in WT mice with sustained increases in IOP compared with uninjected control eyes (as quantified by the RGC marker RNA binding protein with multiple splicing [RBPMs]), there was no change in RGC number in mice lacking the inducers of inflammatory astrogliosis (Figure 2G). SMI-32-positive RGCs were particularly vulnerable to increased IOP, with an ~50% decrease in cell number that was also prevented in the absence of IL-1α, TNF-α, and C1q (Figure 2H). Retinas in which IOP only increased transiently showed no change in RGC number in WT or tKO mice (Figure 2I), confirming that sustained increases in pressure are required to induce cell death.

These results suggest that reactive astrocytes are required for the death of RGCs following sustained increases in IOP. We next characterized the reactive response of astrocytes in the retina, optic nerve head, and optic nerve to the microbead occlusion model to better understand how astrocytes participate in degeneration. An inflammatory astrocyte response was observed in the retina, optic nerve head, and optic nerve of injected WT mice, with sustained increases in IOP relative to uninjected controls, and these responses were largely abated in tKO mice (Figures 2J–2N; Figures S2 and S3). Unexpectedly, although RGCs did not die in retinas with transient changes in IOP, WT retinas with transient changes in IOP still showed robust astrogliosis at the level of the retina (Figure 2J; Figures S2 and S3). These data suggest that both acute and chronic injuries to the eye are sufficient to drive formation of neurotoxic reactive astrocytes and that this activation is mediated by IL-1α, TNF-α, and C1q. Surprisingly, these data also suggest that the presence of neurotoxic astrocytes alone is not sufficient to induce death of RGCs.

Do Spared Neurons Have Altered Morphology?

Although RGCs can be spared from death in a microbead occlusion model of glaucoma, certain types of RGCs undergo dendritic remodeling following transient increases in IOP, potentially losing their normal circuit connectivity and function and thus their value to the regeneration of the retina (El-Danaf and Huberman, 2015). To determine whether spared neurons resemble uninjured neurons from the same mouse, we first tested whether OFF-S RGCs (RGCs with sustained rather than transient responses to the removal of a light stimulus and that undergo dendritic remodeling after transient IOP increases) retain their stereotyped dendritic morphology. We dye filled randomly selected OFF-S RGCs from WT mice 30–35 days after increasing IOP and performed confocal imaging of their dendritic arbors through the depth of the inner plexiform layer (IPL). We chose this time because several studies show changes in RGC numbers from 2 to 6 weeks using this model (Cone et al., 2010; Sappington et al., 2010; El-Danaf and Huberman, 2015; Sabharwal et al., 2017), and we wanted to study chronic effects of IOP increase on RGC maintenance. Counterstaining for choline acetyltransferase (ChAT) allowed the position of the RGC dendrites within the IPL to be measured and compared between animals (Figures 4A and 4B). To quantify dendritic localization within the IPL, the normalized fluorescence intensity of the lps (LPS) injection, in which neuroinflammatory astrogliosis occurs throughout the brain without the widespread death of neurons (Castro et al., 1998; Zamanian et al., 2012; Liddelow et al., 2017). We hypothesized that the observed discrepancy could be explained by the requirement of a second signal, such as an injury, to induce the target neurons to become vulnerable to the toxicity of reactive astrocytes. To test this hypothesis, we directly injected astrocyte-conditioned media (ACM) from quiescent or reactive immunopanned astrocytes (Liddelow et al., 2017) into the retinas of WT mice. The toxicity of the reactive ACM was already tested in in vitro assays using both primary neurons and oligodendrocytes (data not shown), and only ACM that was validated as toxic was used in further experiments. Injecting equal protein concentrations of either control or reactive ACM into the vitreous of an uninjured WT eye failed to induce cell death (Figure 3). Thus, toxic factors secreted from reactive astrocytes alone are not sufficient to induce the death of uninjured RGCs. As described previously (Figure 1), tKO mice lack inflammatory reactive astrocytes and do not suffer the normal death of RGCs after axotomy by ONC, presumably because the neurons are injured by the nerve crush, but the absence of reactive astrocytes leads to a lack of production of the astrocyte-derived toxic factors (Figure 2; Figures S2 and S3). However, if we injected reactive ACM into the retinas of tKO mice following axotomy and thus restore the presence of astrocyte-derived toxic factors to the injured retina, the death of RGCs normally seen after ONC is restored to WT levels (Figure 3). Altogether, these experiments suggest that neurons of the retina are normally resistant to astrocyte-mediated toxicity but become vulnerable after axotomy. A similar mechanism may explain the lack of cell death seen in the retinas of mice with transient IOP, in which reactive astrogliosis occurs but in the absence of the prolonged changes in ocular pressure necessary to induce neurons into a vulnerable state.

Only Damaged Neurons Are Susceptible to Reactive Astrocyte Toxin-Induced Death

In the case of transient increases in IOP, astrogliosis occurs robustly at the level of the retina but does not induce RGC death. A similar phenomenon occurs in systemic lipopolysaccharide (LPS) injection, in which neuroinflammatory astrogliosis occurs throughout the brain without the widespread death of neurons (Castro et al., 1998; Zamanian et al., 2012; Liddelow et al., 2017). We hypothesized that the observed discrepancy could be explained by the requirement of a second signal, such as an injury, to induce the target neurons to become vulnerable to the toxicity of reactive astrocytes. To test this hypothesis, we directly injected astrocyte-conditioned media (ACM) from quiescent or reactive immunopanned astrocytes (Liddelow et al., 2017) into the retinas of WT mice. The toxicity of the reactive ACM was already tested in in vitro assays using both primary neurons and oligodendrocytes (data not shown), and only ACM that was validated as toxic was used in further experiments. Injecting equal protein concentrations of either control or reactive ACM into the vitreous of an uninjured WT eye failed to induce cell death (Figure 3). Thus, toxic factors secreted from reactive astrocytes alone are not sufficient to induce the death of uninjured RGCs. As described previously (Figure 1), tKO mice lack inflammatory reactive astrocytes and do not suffer the normal death of RGCs after axotomy by ONC, presumably because the neurons are injured by the nerve crush, but the absence of reactive astrocytes leads to a lack of production of the astrocyte-derived toxic factors (Figure 2; Figures S2 and S3). However, if we injected reactive ACM into the retinas of tKO mice following axotomy and thus restore the presence of astrocyte-derived toxic factors to the injured retina, the death of RGCs normally seen after ONC is restored to WT levels (Figure 3). Altogether, these experiments suggest that neurons of the retina are normally resistant to astrocyte-mediated toxicity but become vulnerable after axotomy. A similar mechanism may explain the lack of cell death seen in the retinas of mice with transient IOP, in which reactive astrogliosis occurs but in the absence of the prolonged changes in ocular pressure necessary to induce neurons into a vulnerable state.
Figure 2. Microbead Occlusion Model of Glaucoma Increases IOP and Causes Astrocyte-Dependent RGC Loss

(A–D) Bead injection produced increases in IOP in WT mice (A) and tKO mice (B). In some WT (C) and tKO (D) animals, this increase in IOP was only transient and returned to baseline within 48 h. Data in (C) and (D) are normalized around the day of peak IOP increase. Refer to Figure S1 for individual animal plots.

(E) IOP maximum increase was approximately 20%–30% in both WT and tKO mice. Similar maximal levels were reported in mice that had sustained (S) increase (A and B) or transient (T) increase (C and D) in IOP.

(F) Representative RBPMS+ staining of whole-mount retinas from WT and tKO animals following sustained and transient IOP increase. Quantified in (G) and (H).

(G and H) IOP increase paired with death of RBPMS+ RGCs in WT mice, but not tKO mice, following bead occlusion (compared with the contralateral eye) (G). There was a particularly large drop in SMI-32+ RGCs (H).

(I) Mice with transient increase in IOP (C and D) had no loss of RGCs.

(J) Heatmap of Z scores from microfluidic qPCR analysis highlights enrichment of reactive astrocyte transcripts in retina, optic nerve head, and optic nerve of bead-injected eyes, but not PBS-injected eyes from tKO mice.

(K–N) Average fold induction of all reactive astrocyte transcripts (taken from J). Astrogliosis is similar in the retinas of mice with both sustained (K) and transient (L) increases in IOP, as well as in the optic nerve head (M) and optic nerve (N) of mice with sustained increase in IOP. In all instances, astrogliosis is reduced in tKO mice (see also Figures S2 and S3).

*p < 0.05, one-way ANOVA followed by Tukey’s multiple comparison test. Individual data points representative of individual animals are plotted, and error bars represent mean ± SEM. The scale bar is 20 μm for all micrographs in (F).
The altered kinetics of spared OFF-S RGCs suggests that these cells might be integrating input from presynaptic partners differently. To test whether the integration of spatial information might be altered in OFF-S RGCs, we next probed the size tuning of cells by recording responses to spots of negative contrast of increasing size. Although the spike rates of spared OFF-S RGCs increased with spot diameter, they were significantly lower than control cells for smaller spots before reaching equivalent spike rates at the largest spot diameters tested (Figure 5D). These results suggest that the ability of spared RGCs to integrate spatial information was altered. To better quantify this change, normalized size tuning functions were calculated for each cell (Figure 5E; STAR Methods) and used to determine the spot size that produced a half-maximal response in spared OFF-S RGCs (Figure 5E). These findings indicate an impairment in the ability of spared OFF-S RGCs to integrate spatial information and are consistent with changes in the presynaptic input they receive.

The changes in light response kinetics and spatial integration of spared OFF-S RGCs could arise if these cells received input from novel presynaptic partners. We next tested whether this was the case by probing the spatial summation properties of dendritic signal was plotted relative to that of the ChAT signal in a small region of the IPL (Figure 4C). This normalized measure was used to quantify the depth of the dendritic stratification within the retina, as well as the total width of the dendritic arborization (Figure 4D). Although the depth at which the dendritic arbors stratified in the IPL did not change, the width of those arbors significantly increased (Figures 4D and 4E). Thus, although the dendrites of spared RGCs are likely receiving presynaptic input from neurons in the correct region of the IPL (i.e., OFF versus ON), it is possible for them to receive input from a wider range of presynaptic neurons.

In addition to stratifying at stereotypical depths within the IPL to ensure proper connectivity with presynaptic partners, the dendrites of RGCs also extend radially in conjunction with their spatial summation properties. We performed 3D reconstruction of dye-filled OFF-S RGCs to analyze their radial dendritic arborization (Figures 4F–4H). Example images of reconstructed dendritic arbors in control or elevated IOP Il1a−/− Tnf−/− C1qa−/− retinas show that spared RGCs retain high levels of dendritic branching (Figure 4G), and side views of the reconstructed dendrites recapitulate the increase in width of stratification within their target layer found by confocal microscopy (Figure 4H). Indeed, Sholl analysis suggests that the average radius of an RGC from the elevated IOP mouse is significantly smaller than those from uninjured retina from the same mouse, but the peak level of branching is largely similar in both cases (Figures 4I and 4J). Although there was no difference in branching complexity, spared RGCs had slightly smaller dendritic arbors, with a decrease in the total cell radius (maximum dendrite extension) in spared RGCs (Figure 4K). We also measured the total neurite length of RGCs from control and bead-injected eyes and found no change in the total dendritic arborization (Figure 4L).

Altogether, these data suggest that OFF-S RGCs spared from death following sustained increases in IOP undergo dendritic remodeling but that the overall targeting and branching characteristics of the dendritic arbor are largely maintained when compared with undamaged tKO RGCs.

Are Spared Neurons Still Functional?
The light response properties of RGCs arise partly from the patterns of input they receive from their presynaptic partners. This presynaptic partner choice is dictated primarily by the level of the IPL at which they stratify their dendrites. The changes in morphology and stratification of spared OFF-S RGCs following sustained increases in IOP in tKO mouse retina raised the possibility that the light response properties of RGCs might be altered. To address this possibility, we investigated whether spared RGCs were functionally similar to uninjured tKO RGCs. We analyzed the activity of OFF-S RGCs by recording whole-cell current-clamp responses to visual stimuli in tKO mice following 30–35 days of elevated IOP. OFF-S RGCs have been well characterized and can be targeted reliably based on cell soma size and light response properties (Krieger et al., 2017). All spared OFF-S RGCs from which we recorded remained light responsive, although their light responses were qualitatively different compared with control cells (Figure 5A). To quantify these changes, light response kinetics were compared. Spared OFF-S RGCs had a significantly decreased peak spike rate, but latency and decay of the light response, as well as the final/peak ratio, were unchanged (Figures 5B and 5C). These data indicate that neurons spared from astrocyte-targeted death following increased IOP are still active and light responsive.

The altered kinetics of spared OFF-S RGCs suggests that these cells might be integrating input from presynaptic partners differently. To test whether the integration of spatial information might be altered in OFF-S RGCs, we next probed the size tuning of cells by recording responses to spots of negative contrast of increasing size. Although the spike rates of spared OFF-S RGCs increased with spot diameter, they were significantly lower than control cells for smaller spots before reaching equivalent spike rates at the largest spot diameters tested (Figure 5D). These results suggest that the ability of spared RGCs to integrate spatial information was altered. To better quantify this change, normalized size tuning functions were calculated for each cell (Figure 5E; STAR Methods) and used to determine the spot size that produced a half-maximal response in spared OFF-S RGCs (Figure 5E). These findings indicate an impairment in the ability of spared OFF-S RGCs to integrate spatial information and are consistent with changes in the presynaptic input they receive.

The changes in light response kinetics and spatial integration of spared OFF-S RGCs could arise if these cells received input from novel presynaptic partners. We next tested whether this was the case by probing the spatial summation properties of
spared OFF-S RGCs. Spatial summation properties, including the nonlinear subunit structure within the receptive field, are known to be generated by inputs from specific populations of bipolar cells (Borghuis et al., 2013; Demb et al., 1999). To probe spatial summation, RGCs were presented with visual stimuli consisting of contrast reversing gratings at a range of spatial frequencies and temporal phases. This stimulus produced robust spiking responses in spared OFF-S RGCs that modulated mainly at the temporal frequency of the stimulus (F1 component) (Figure 6A). The F1 component of the response was also highly phase dependent, with null responses at two spatial phases separated by 180°, indicating that spared cells could encode specific features of the stimuli used (Figure 6B). To more completely quantify the spatial summation response properties, we calculated the F1 and F2 Fourier harmonics as a function of spatial frequency (Petrusca et al., 2007). Both spared and control
OFF-S RGCs showed similar subunit structures that were dominated by the F1 harmonic at all spatial frequencies tested (Figure 6C). There was also an F2 component, but it was not strongly spatial frequency dependent, suggesting that both populations of cells integrated spatial information linearly. We also quantified the level of nonlinearity of spatial summation (F2/F1) and found that there were no significant differences between spared and control OFF-S RGCs (Figure 6D).

Collectively, these data indicate that although spared OFF-S RGCs stratify their dendrites over a broader region of the IPL and integrate spatial information differently compared with the uninjured RGCs of the contralateral eye, they likely do not connect to novel presynaptic partners, because the nonlinear subunit structure of their receptive fields does not change.

**DISCUSSION**

Here we demonstrate the contribution of reactive astrocytes to the death of RGCs in both acute and chronic injuries of the retina. By inhibiting or knocking out IL-1α, TNF-α, and C1q, the factors secreted from microglia that induce neurotoxic reactive astrocytes, we can largely prevent the death of RGCs induced following ONC or following prolonged increases in IOP. The neurotoxicity of reactive astrocytes in glaucoma, combined with studies from other groups highlighting the contribution of this astrocytic response to models of Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease (Shi et al., 2017; Yun et al., 2018; Diaz-Castro et al., 2019; Joshi et al., 2019) suggests that neurotoxic reactive astrocytes may be a common feature in the complex and heterogeneous milieu of the CNS’s response to prolonged injury. This response stands in stark contrast to the growing literature that other forms of astrocyte reactivity, such as those governed by the transcription factor STAT3, can be beneficial to CNS recovery after injury, including in the case of glaucoma (Qu and Jakobs, 2013; Anderson et al., 2016; Sun et al., 2017). Furthermore, eliminating neurotoxic reactive astrocytes in a model of prion infection actually worsened outcomes (Hartmann et al., 2019), suggesting that these phenotypes have highly contextual functions. The divergent impact of different reactivity states on cell health and regeneration highlights the need to carefully manipulate and consider astroglia as a potentially heterogeneous response, and it is still unclear what mechanisms link the increase in IOP to the activation of glial cells or how homogeneous/heterogeneous the glial response is in space and time. This will no doubt be the focus of future studies across many disease paradigms, especially with the proliferation of techniques to analyze cell responses at the single-cell level (Menon et al., 2019; Tran et al., 2019).

In addition to showing that RGCs can be spared from death following acute axotomy and prolonged IOP, we found that spared RGCs are still functional, with many electrophysiological
and morphological features similar to those of the uninjured retina of the contralateral eye. There are many ways to prevent cell death by eliminating final executioners of the cell death process. Unfortunately, surviving cells are sometimes irreversibly changed by the intracellular signaling processes that would normally lead to cell death or, in the case of neurons, may feature dramatically altered electrophysiological processes that make the surviving cell more detrimental to the normal function of a circuit than if the cell had simply died. Thus, our observation that the electrophysiological and morphological features of RGCs spared from cell death were similar to those of the uninjured retina suggests that eliminating the factors that induce reactive astrocytes prevents many pathological changes that often occur before eventual cell death.

There were noticeable changes in certain properties in spared compared with uninjured RGCs, such as the decrease in peak firing rate or the increase in width of dendritic arborization. However, these cells retained largely normal light response properties, which are governed by the cells’ presynaptic inputs, suggesting that they retained many correct presynaptic inputs and that damaged or diseased neurons, if encouraged to fully regenerate their pre- and postsynaptic connections, might be able to reconnect into normal circuitry.

The exact mechanism of the dendritic morphology changes that occur is unclear at this point. Although previous studies have highlighted C1q and TNF-α directly in synaptocytic remodeling (Stellwagen and Malenka, 2006; Stevens et al., 2007), we believe that the observed changes are not directly linked to the lack of these proteins, because such dendritic changes occur in mouse models of glaucoma even when these factors are still present (El-Danaf and Huberman, 2015), and our previous studies have implicated reactive astrocytes more directly in synaptic remodeling separate from the direct influence of these signaling molecules (Liddelow et al., 2017). Future experiments should use alternative methods for increasing IOP that allow analysis of visual acuity to determine whether tKO mice also regain the components of vision normally lost following RGC death, as well as to determine whether other compensatory changes in the spared neurons or their pre/postsynaptic partners would help reestablish normal vision after changes to spike rate or dendritic patterning. Alternatively, investigations into similar microglia-astrocyte-derived neurotoxicity models in other brain regions may highlight other important components of this complex cascade.

Finally, observations from mice with transient rather than prolonged increases in IOP, as well as experiments combining...
ONC with the injection of toxic or control ACM from primary rodent astrocyte culture, have suggested that the presence of astrocyte-secreted toxic factors or the injury of RGCs alone is not sufficient to induce RGC death. Rather, both toxin and injury are required to induce cell death, which has led us to a model in which cellular injury makes the target cell susceptible to the eventual cell death signal provided by the astrocytes. Another potential interpretation of the lack of in vivo neuron death seen when injecting toxin into the retina without ONC is that IL-1α, TNF-α, and CT1q induce changes to other cells in the visual system that are required for the neurons to die, rather than a cell-intrinsic change in RGCs. However, we have previously demonstrated that RGCs are vulnerable to the toxic factor when cultured in the absence of other cell types and in the presence of full trophic support (Liddelow et al., 2017). Furthermore, we greatly reduce the abundance of these cytokines by the size-dependent concentration of media before injection, which renders the proteins undetectable by mass spectrometry and makes the media incapable of further activating astrocytes in vitro (results not shown). Altogether, these results suggest that the changes that make a neuron susceptible are cell intrinsic, but experiments should be performed to further test this hypothesis. In this model, we predict that cultured neurons are injured during their removal from the brain, an event that necessarily causes axotomy, and this damage induces the neurons to become susceptible to the toxin. This model also aligns with observations from systemic injection of LPS, in which widespread astrogliosis occurs throughout the CNS in the absence of the cataclysmic death of all neurons and mature oligodendrocytes. In these large systemic insults, reactive astrocytes may be present and secreting factors toxic to neurons, but neurons are not susceptible because of a lack of conditioning injury. Future experiments should take advantage of techniques such as ATAC-seq to try and identify the cellular changes that make cells susceptible to astrocyte-mediated death, because these changes would be powerful therapeutic targets in a range of injuries and diseases. Regardless of whether therapies to stop this process are found, this model adds a satisfying logic to the otherwise precarious presence of toxic astrocytes in the CNS, as well as cautions a thoughtful approach to stopping neuronal cell death in some contexts, because injured neurons may be more detrimental to the system alive than dead in certain diseases.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Mice
  - Rats
- **METHOD DETAILS**
  - Retro-orbital optic nerve crush
  - Microbead injection and intraocular pressure measurements
  - Microfluidic quantitative PCR
  - Electrophysiology
  - Immunohistochemistry
  - Sholl analysis

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107776.

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AUTHOR CONTRIBUTIONS

S.A.L., R.N.E.-D., B.K.S., and A.D.H. developed the study, K.A.G., B.K.S., R.N.E.-D., D.I.A., A.E.M., M.K.W., and S.A.L. performed experiments. B.K.S., K.A.G., D.I.A., and S.A.L. performed analyses. K.A.G., B.K.S., R.N.E.-D., and S.A.L. wrote the manuscript. All authors discussed the results and provided feedback on the manuscript.

DECLARATION OF INTERESTS

S.A.L. is an academic founder of AstronauTx Ltd.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| Anti-TNF-α neutralizing antibody | Cell Signaling Technology | Cat# 7321S; RRID:AB_10925386 |
| Anti-C1q neutralizing antibody | Quidel | Cat# A301; RRID:AB_452502 |
| Anti-IL-1α neutralizing antibody | abcam | Cat# ab9614; RRID:AB_308732 |
| Bandeiraea Simplicifolia Lectin I | Vector Labs | Cat# B-1105; RRID:AB_2336489 |
| Donkey anti-Goat Alexa Fluor 647 secondary antibody | Thermo Fisher | Cat# A21447; RRID:AB_2535864 |
| Donkey anti-Rabbit Alexa Fluor 488 secondary antibody | Thermo Fisher | Cat# A21206; RRID:AB_2535792 |
| Goat anti-mouse IgM μ-chain | Jackson ImmunoResearch | Cat# 115-005-020; RRID:AB_2338450 |
| Goat anti-mouse IgG+IgM (H+L) | Jackson ImmunoResearch | Cat# 115-005-044; RRID:AB_2338451 |
| Goat anti-rat IgG (H+L) | Jackson ImmunoResearch | Cat# 112-005-167; RRID:AB_2338101 |
| Goat polyclonal antibody against ChAT | Sigma | Cat# AB144P; RRID:AB_2079751 |
| Mouse anti-human Integrin beta 5 | eBioscience | Cat# 14-0497-82; RRID:AB_467288 |
| O4 hybridoma supernatant | Sommer and Schachner, 1981 | N/A |
| Rabbit polyclonal antibody against Lucifer Yellow | Invitrogen | Cat# A-5750; RRID:AB_2536190 |
| Rabbit polyclonal antibody against RBPMS | PhosphoSolutions | Cat# 1830-RBPMS; RRID:AB_2492225 |
| Rat anti-mouse CD45 | BD PharMingen | Cat# 550539; RRID:AB_2174426 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Ames’ medium | Sigma | Cat# A1420 |
| Apo-transferrin | Sigma | Cat# T1147 |
| ATP-magnesium salt | Sigma | Cat# A9187 |
| Bovine serum albumin (BSA) | Sigma | Cat# A4161 |
| DMEM (high glucose) | GIBCO | Cat# 11960-044 |
| DNase I | Worthington | Cat# DPRFS |
| Donkey serum | EMD Millipore | Cat# S30-100ML |
| Earle’s balanced salt solution | Sigma | Cat# E7510 |
| EDTA-free Protease Inhibitor Cocktail | Sigma | Cat# 04693159001 |
| EGTA | Sigma | Cat# E3889 |
| Fetal calf serum (FCS) | GIBCO | Cat# 10437-028 |
| FluoSpheres Polystyrene Microspheres, 10 μm, red fluorescent (580/605) | Invitrogen | Cat# F8834 |
| Goat serum | MP Biomedicals | Cat# 0219135680 |
| GTP-sodium salt | Sigma | Cat# G8877 |
| HEPES | Sigma | Cat# H3375 |
| L-cysteine hydrochloride monochloride | Sigma | Cat# C7880 |
| L-glutamine | GIBCO | Cat# 25030-081 |
| N-acetyl cysteine | Sigma | Cat# A9165 |
| Native hC1q | Mybiosource | Cat# MBS143105 |
| Neurobasal | GIBCO | Cat# 21103-049 |
| Ovomucoid trypsin inhibitor | Worthington | Cat# LS003086 |
| Papain | Worthington | Cat# LS003126 |
| Paraformaldehyde Aqueous Solution | Electron Microscopy Sciences | Cat# 15710 |
| Penicillin/ Streptomycin | GIBCO | Cat# 15140-122 |
| Poly-D-lysine hydrobromide | Sigma | Cat# P6407 |

(Continued on next page)
### continued

| REAGENT or RESOURCE SOURCE IDENTIFIER |
|--------------------------------------|
| Potassium methanesulfonate Sigma Cat# 83000 |
| Progesterone Sigma Cat# P-8783 |
| Putrescine Sigma Cat# P-5780 |
| Recombinant HB-EGF PeproTech Cat# 100-47 |
| Recombinant hTNFa Cell Signaling Technology Cat# 8902 |
| Recombinant IL-1a Sigma Cat# I3901 |
| Sodium chloride Sigma Cat# S9888 |
| Sodium pyruvate Sigma Cat# P5280 |
| Sodium selenite Sigma Cat# 8902 |
| SYBR Green PCR Master Mix Thermo Fisher Cat# 4385612 |
| TEA-Cl Sigma Cat# T2265 |
| Triton X-100 Sigma Cat# T8787 |
| Trypsin Sigma Cat# T9935 |

### Critical Commercial Assays

| Assay |
|-------|
| 96.96 Dynamic Array IFC for Gene Expression Fluidigm Cat# 101-0349 |
| Bradford Assay Bio-Rad Cat# 500-0006 |
| High-Capacity RNA-to-cDNA Kit Thermo Fisher Scientific Cat# 4387406 |
| RNeasy Micro Kit Qiagen Cat# 74004 |

### Experimental Models: Organisms/Strains

- **Mouse:** C57BL/6J Jackson Laboratory Strain Code 000664
- **Mouse:** Il1a−/− Tnf−/− C1qa−/− Liddelow et al., 2017 N/A
- **Rat:** Sprague Dawley Charles River Strain Code 400

### Software and Algorithms

- Fiji/ImageJ NIH N/A
- MATLAB MathWorks N/A
- Psychophysics Toolbox https://github.com/kleinerm/Psychtoolbox-3
- Fluidigm Melting Curve Analysis Software 1.1.0 build 20100514.1234 Fluidigm N/A
- BioMark Data Collection Software 2.1.1 build 20090519.0926 Fluidigm N/A
- Real-time PCR Analysis Software 2.1.1 build 20090521.1135 Fluidigm N/A
- pClamp 11.0 Molecular Devices N/A

### Other

- 1.0 Neutral Density Filter Chroma Cat# ND 1.0
- 30 kDa MWCO centrifugal filter unit Sartorius Cat# VS2022
- 5 μl Hamilton Syringe Hamilton Company Cat# 87919
- DLP® LightCrafter E4500 MKII Fiber Couple EKB Technologies Cat# E4500MKII
- icare® TONOLOAB rebound tonometer icare Cat# TV02
- IR (830 nm) LED Spotlight Edmunds Optics Cat# 66-860
- Picospritzer III - Intracellular Microinjection Dispense Systems Parker Precision Fluidics Cat# 051-0500-900
- Poly-D-Lysine coated coverslips (12 mm) Neuvitro Cat# NC0565504
- Polystyrene Microspheres, 10 μm Invitrogen Cat# F-8834
- UV DLP Projector EKB Technologies Cat# DPM-E4500UVGOAMKII
RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Shane Liddelow (shane.liddelow@nyulangone.org).

Materials Availability
This study did not generate new unique reagents. However, there are restrictions to the availability of the Il1a<sup>−/−</sup> Tnf<sup>−/−</sup> C1qa<sup>−/−</sup> mouse line due to MTAs. Please contact Shane Liddelow (shane.liddelow@nyulangone.org) for more information.

Data and Code Availability
This study did not generate or utilize any dataset or code that requires distribution. The data that support the findings of this study are available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
All mouse procedures were conducted in accordance with guidelines from the National Institute of Health and Stanford University’s Administrative Panel on Laboratory Animal Care. Mice (postnatal day, P 14-42) were housed with food and water available ad libitum in a 12-h light/dark environment. Wild-type (WT) C57BL/6J mice were obtained from Jackson Laboratories. Triple knockout (Il1a<sup>−/−</sup> Tnf<sup>−/−</sup> C1qa<sup>−/−</sup>) neuroinflammatory reactive astrocyte-deficient mice (tKO mice) were from a previous study in our laboratory (Liddelow et al., 2017). All lines were maintained by breeding with C57BL/6J mice. Animals were randomly assigned numbers and thereafter evaluated blind (to both experimental condition and genotype). Only female mice were used for glaucoma studies.

Rats
All rat procedures (P 5-7, and P 14-42) were conducted in accordance with guidelines from the National Institute of Health and Stanford University’s Administrative Panel on Laboratory Animal Care. All rats were housed with food and water available ad libitum in a 12-h light/dark environment. Sprague Dawley rats were from obtained from Charles River. Mixed genders were used for both optic nerve crush studies as well as primary cell culture studies.

Cell culture and conditioned media collection
Astrocytes were purified by immunopanning from P5 rat forebrains and cultured as previously described (Foo et al., 2011). Briefly, cortices were dissected and enzymatically digested by papain at 37°C and 10% CO₂. Tissue was then mechanically triturated using a 5ml serological pipette at room temperature and the suspension filtered through a 70μm nitex filter to generate a single-cell suspension. The suspension was negatively panned for microglia (CD45), endothelial cells (BSLI), and oligodendrocyte-lineage cells (O4) followed by positive panning for astrocytes (Itgb5). Astrocytes were removed from the final positive selection plate by brief digestion with 0.025% trypsin and plated on poly-D-lysine coated 10cm tissue culture plates. Astrocytes were cultured in defined, serum-free medium containing 50% neurobasal, 50% DMEM, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 292 μg/mL L-glutamine, 1 μM SATO (neurobasal media supplemented with 100 μg/mL transferrin, 100 μg/mL BSA, 16 μg/mL putrescine, 60 ng/mL progesterone, 40 ng/mL sodium selenite), 5 μg/mL of N-acetyl-L-cysteine, and 5ng/mL HBEGF (Peprotech, 100-47).

After 6 days in culture, astrocytes were treated with IL-1α (3 ng ml<sup>−1</sup>, Sigma, I3901), TNFα (30 ng ml<sup>−1</sup>, Cell Signaling Technology, 8902SF) and C1q (400 ng ml<sup>−1</sup>, MyBioSource, MBS143105) with 0.025% trypsin and plated on poly-D-lysine coated 10cm tissue culture plates. Astrocytes were cultured in defined, serum-free medium containing 50% neurobasal, 50% DMEM, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 292 μg/mL L-glutamine, 1 μM SATO (neurobasal media supplemented with 100 μg/mL transferrin, 100 μg/mL BSA, 16 μg/mL putrescine, 60 ng/mL progesterone, 40 ng/mL sodium selenite), 5 μg/mL of N-acetyl-L-cysteine, and 5ng/mL HBEGF (Peprotech, 100-47). After 6 days in culture, astrocytes were treated with IL-1α (3 ng ml<sup>−1</sup>, Sigma, I3901), TNFα (30 ng ml<sup>−1</sup>, Cell Signaling Technology, 8902SF) and C1q (400 ng ml<sup>−1</sup>, MyBioSource, MBS143105) for 24hrs to generate neuroinflammatory reactive astrocytes or treated with an identical volume of PBS (control). Conditioned media was removed after 24hrs, supplemented with complete protease inhibitor cocktail (Roche), and concentrated with 30-kDa size exclusion Vivaspin ultrafiltration units (Sartorius). Control and reactive conditioned media were concentrated to the same final volume, and equivalent final protein concentration confirmed by Bradford protein concentration analysis (BioRad, 500-0006). In indicated experiments, 1 μl of either control or reactive ACM of equivalent protein concentration was injected into the vitreous of the eye using a Hamilton syringe (Hamilton Company, 87919) in either unmanipulated rats or rats that received an optic nerve crush (see below) immediately prior to the injection.

METHOD DETAILS

Retro-orbital optic nerve crush
Postnatal day 21 wild-type (WT, C57BL/6J) and tKO mice or postnatal day Sprague Dawley rats (Charles River) were anaesthetized with 2.5% inhaled isoflurane in 2.0 L O₂ per min. Without incision to the orbital rim, the supero-external orbital contents were blunt-dissected, the superior and lateral rectus muscles teased apart, and the left optic nerve exposed. The nerve was crushed for 3–5 s approximately 2 mm distal to the lamina cribrosa. After surgery, the eye fundi were checked to ensure retinal blood flow was intact. 1, 3, 7, 14, and 28 days following crush retinas were collected for immunofluorescence to determine viability of retinal ganglion cells. In a separate set of experiments, WT and tKO mice had optic nerves crushed, retinas removed, and microfluidic qPCR analyses conducted. These mice had expected levels of astrocyte reactivity as reported previously (Liddelow et al., 2017), and this was similar to...
activation seen in experimental glaucoma via bead injection at 28 days (data not shown). In rat optic nerve crush experiments, some rats received a 2 µl intravitreal injection of neutralizing antibodies to II-1α (150 µg µl−1, Abcam, ab9614), TNF (150 µg µl−1, Cell Signaling Technology, 7321), and C1q (Quidel, A301), or rabbit IgG control (150 µg µl−1, Abcam, ab27472) at day 0 (the time of optic nerve crush) and every 7 days until retinas were collected.

**Microbead injection and intraocular pressure measurements**

The microbead occlusion model was used to achieve intraocular pressure (IOP) elevation applying previously described protocols (Sappington et al., 2010; Chen et al., 2011; El-Danaf and Huberman, 2015). Mice were anesthetized with a mixture of ketamine/xylo-
lazine and a small hole was made in the cornea using a glass micropipette attached to a picospritzer. A 1–2 µL volume of polystyrene microbeads (10 µm diameter; Invitrogen F-8834) was injected into the anterior chamber of one eye. The other eye served as an internal control, in which a hole was made and no beads were delivered (El-Danaf and Huberman, 2015). In some experiments, control animals received an equal volume (1–2 µl) of sterile PBS. IOPs were measured daily, around the same time every day (±60 min), using a rebound tonometer (TonoLab, Colonial Medical Supply). The reported IOP for each day consisted of an average reading of 10 consecutive IOP measurements. Baseline IOP values were obtained by monitoring the pressure for 2–3 days prior to bead injections. Pressures were then measured for 4–6 days and at 28 days following the bead injections to detect any elevations in IOP. Mice showing no IOP elevation resulting from bead leakage were omitted from the study.

**Microfluidic quantitative PCR**

Total RNA was extracted from whole retinas using the RNeasy Plus kit (QIAGEN) and cDNA synthesis performed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to supplier protocols. Microfluidic qPCR was performed using a 96.96 Dynamic Array chip (Fluidigm Corporation) run on a BioMark Real-Time PCR System (Fluidigm) using a cycling program of 10 min at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, as described previously (Zhang et al., 2014; Liddelow et al., 2017; Clarke et al., 2018). Data were collected using BioMark Data Collection Software 2.1.1 build 20090519.0926 (Fluidigm). Three reference genes *Aldh111*, *Gapdh* and *Rplp0* were used to normalize transcript expression depending on cell type. Data pre-
processing and analysis was completed using Fluidigm Melting Curve Analysis Software 1.1.0 build 20100514.1234 (Fluidigm) and Real-time PCR Analysis Software 2.1.1 build 20090521.1135 (Fluidigm) to determine valid PCR reactions. Reactive astrocyte primers were used as described previously (Liddelow et al., 2017).

**Electrophysiology**

Electrophysiological procedures were similar to those described previously (Stafford et al., 2014; Osterhout et al., 2015). Briefly, retinas were harvested and dissected in gassed (95% O2 and 5% CO2) Ames medium (Sigma) under infrared illumination. A piece of ventral retina was placed in a chamber on an upright microscope and superfused (±5 ml/min) with gassed Ames medium heated to 33–35°C. RGCs with large somas were targeted under IR illumination to bias recordings toward OFF-S cells for recording. A glass electrode (tip resistance, 4–6 MΩ) was filled with Ames’ medium for loose-patch recording and the type of RGC was determined by measuring light responses. Once a presumed OFF-S cell was located, a second glass electrode was filled with intracellular solution and the same cell was targeted for whole-cell recording. The intracellular solution contained (in mM): 120 K-methanesulphonate 10 HEPES, 5 NaCl, 0.1 EGTA, 2 ATP-Mg2+, and 0.3 GTP-Na, titrated to pH 7.3. Chemicals were purchased from Sigma-Aldrich or Tocris. Custom programs in MATLAB were used for spike thresholding and analysis of spiking parameters. For analysis of normalized spiking responses as a function of spot size, the average normalized response from at least three trials was fit with a Nakao-Rushton equation to determine the spot size that generated a half maximal response.

Patterned light stimuli were generated by custom software developed in Psychophysics Toolbox and MATLAB. Stimuli were pro-
jected onto the retina using a custom DLP projector (EKB Technologies; E4500MKII) custom fitted with a UV LED (final emission, 385 nm), attenuated by a neutral density 1.0 filter, and focused using a 10X objective to the level of rod and cone outer segments. The mean luminance generated 2.6 × 10^3 R²/S-cone/sec. The receptive field center was mapped by recording responses to square-wave modulations of a 300 µm diameter spot presented at eight positions. In subsequent experiments, stimuli were pre-
sent at a contrast pulse (100% Weber contrast), 100–800 µm in diameter or as a patch of contrast reversing gratings (800 µm patch diameter) modulated at 2 Hz at 5 spatial frequencies and 12 spatial phases. Responses to drifting square-wave gratings (500 µm/ cycle; 100% Michelson contrast) modulated at 1 Hz against a background mean luminance were also recorded from a subset of cells.

Following recordings, pieces of retina were fixed in 4% PFA for one hour at room temperature, transferred to a blocking solution containing 1x PBS, 0.5% Triton X-100, and 10% donkey serum for two hours at room temperature, then incubated with antibodies against Lucifer yellow (1:1000; Invitrogen A-5750) and cholera acetyltransferas (ChAT, 1:100, Millipore AB-144P) diluted in blocking solution while rocking for three days at 4°C. Following washing and staining with appropriate fluorescently conjugated secondary antibodies, retinas were mounted on coverslips for confocal imaging. Fluorescent signals from the filled RGC and ChAT staining were imaged through the entire depth of the retina from a region that covered the full dendritic field of the filled cell. Morphological analyses of electrophysiologically recorded cells was conducted by determining the position of the dendrites relative to the ChAT bands as described previously (Manookin et al., 2008). Briefly, confocal z stacks were analyzed in small regions that adequately re-
lected the stratification of the RGC dendrites and where the dendritic signal and ChAT signals displayed minimal tissue warping/ compression. A maximum projection of each fluorescent signal in these regions was generated by averaging the stacks across
the z-dimension (Figures 4A and 4B). The fluorescent signals were analyzed as a function of distance across the IPL, the boundaries of which were defined by the ChAT cell bodies in the INL and GCL (Figure 4B, yellow box). Third-order polynomials were fitted around six to eight points (3-4 μm) at the fluorescent peaks associated with the ChAT signal to determine the percentage depth of the two ChAT bands in the IPL. The dendritic signal was analyzed and the width of the dendritic signal was determined as follows: the positions corresponding to the first time the dendritic signal rose above, and the last time it dropped below, 0.5 were identified (Figure 4C). For all cells, these positions were manually checked by eye to confirm they captured the extent of the dendritic signal. These positions were then normalized relative to the two fitted peaks of the ChAT signal in the same tissue where the On ChAT band = 0, and the Off ChAT band = 1. The stratification width of the dendrites was considered the difference between these two positions. This normalization procedure allows the stratification position and width of dendrites to be compared from cells in tissue from different animals.

**Immunohistochemistry**
Mice were euthanized by inhaled CO₂ and cervical dislocation. Whole eyes were dissected and placed in ice-cold 4% paraformaldehyde for 10 min, and then washed in DPBS before dissecting the retina away from the rest of the eye and post-fixing in 4% paraformaldehyde overnight at 4°C. Retinas were blocked with 10% goat serum (MP Biomedicals, 0219135680) and stained with rabbit anti-RBPMS (1:500, PhosphoSolutions 1830-RBPMS) diluted in phosphate buffered saline and visualized with appropriate secondary antibodies conjugated with Alexa fluorophores (Invitrogen). Immunofluorescent images were collected using a Keyence BZ-X700 Fluorescence Microscope (Keyence) and final multi-fluorescent images prepared using FIJI (Schindelin et al., 2012). Z stacked dye-filled RGCs were reconstituted in FIJI (Schindelin et al., 2012)

**Sholl analysis**
Z stacked images of dye-field RGCs from tKO mice injected with either microbeads or control preparations were uploaded to FIJI (Schindelin et al., 2012) for Sholl analysis (Ferreira et al., 2014). Each RGC neurite was traced by an evaluator blind to experimental conditions using the FIJI Simple Neurite Tracer (SNT) plugin (Longair et al., 2011). Sholl analysis was performed on each traced RGC using a 10 μm radius step size. The radius from the center of the soma to the terminal trace of the longest neurite was used as a marker of total RGC radius. Traces were skeletonized using the SNT render/skeletonize function then oriented and collapsed along the +YZ plane using the FIJI 3D viewer (Schmid et al., 2010).

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Statistical analyses were completed using GraphPad Prism 7.00 software. Most data were analyzed by one-way ANOVA followed by Dunnett’s multiple post hoc test for comparing more than three samples, and two-sample unpaired t tests for comparing two samples with 95% confidence. Power calculations were performed using G* Power Software V 3.1.9.2 (Faul et al., 2007). Group sizes were calculated to provide at least 80% calculable power with the following parameters: probability of type I error (0.05), conservative effect size (0.25). Four to ten individual animals per treatment group with multiple measurements were obtained per replicate. Further details on the exact tests and data presentation for each experiment can be found in its accompanying figure legend.