POTENTIAL CELLULAR AND IMMUNE MECHANISMS MEDIATING BILATERAL RETINAL GANGLION CELL RESPONSE TO UNILATERAL OPTIC NERVE INJURY

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Abstract

In vertebrates, retinal ganglion cells (RGCs) are the sole neurons that form the optic nerve and transmit visual information from the retina to the brain. Injury or degeneration of RGCs, as seen in diseases such as glaucoma, leads to irreversible vision loss in humans due to the limited regenerative capacity of central nervous system neurons. In contrast, zebrafish exhibit robust RGC survival and regeneration following injury. Understanding the mechanisms that support this regenerative capacity may inform therapeutic strategies for preserving vision in humans.

Previous single-cell transcriptomic analyses have shown that unilateral optic nerve transection (ONT) induces RGC dedifferentiation and activation of proliferative gene programs. Supporting these findings, fluorescence *in situ* hybridization revealed upregulation of regenerative gene markers not only in the ipsilateral (injured) retina but also in the contralateral (uninjured) eye across multiple timepoints, suggesting that optic nerve injury elicits a system-wide response.

This observation led to the hypothesis that early innate immune responses may mediate communication across the visual system and contribute to bilateral RGC activation. Because neutrophils are among the earliest immune cells recruited following injury and can influence inflammatory and regenerative signaling environments, they represent strong candidates for coordinating such responses. To investigate this, transgenic zebrafish with fluorescently labeled RGCs and neutrophils were used to characterize immune cell recruitment across the visual pathway (both eyes, optic nerves, and the midbrain containing the optic tectum) following ONT. Confocal microscopy was used to assess neutrophil distribution in the ipsilateral and contralateral retinae, as well as in the optic tectum, the primary target of RGC axons. Quantitative analysis revealed significant presence of neutrophils at the site of injury, with lower and more variable presence in contralateral and midbrain regions. While these findings indicate that immune recruitment is predominantly localized, the presence of neutrophils beyond the injury site and variability in their distribution suggest the presence of a broader systemic response.

Together, this work integrates transcriptional and cellular analyses to examine how localized injury may produce distributed effects across the visual system and explores the potential role of early immune dynamics in coordinating regenerative responses.

Background

Retinal ganglion cells and vision loss

Vision depends on a single population of neurons: retinal ganglion cells (RGCs). Located in the innermost layer of the retina, RGCs axons exit the eye to form the optic nerve, transmitting all visual information to the brain. Without RGCs, visual signals from photoreceptor inputs cannot reach central processing centers to create an understanding of spatial contrast, color, motion, and other visual stimuli [1]. Because of these functions, RGC survival is essential for maintaining visual function. In humans, degeneration of RGCs underlies several major diseases resulting in irreversible blindness. Glaucoma, the leading cause of irreversible blindness worldwide, is characterized by progressive axonal degeneration and gradual death of RGCs [2]. As RGCs die, the optic nerve deteriorates and visual field deficits form. Similar degeneration occurs in optic neuropathies and other retinal disorders involving axonal injury [3].

Central nervous system (CNS) neurons, including RGCs, have limited regenerative capacity in mammals. After injury that results in cell death, mammalian RGC axons fail to proliferate due to both intrinsic limitations in growth potential and extrinsic inhibitory environments such as inflammation that result in regeneration-preventing glial scarring [4]. As a result, vision loss caused by RGC death across a variety of degenerative diseases is typically permanent. However, this regenerative failure is not universal across vertebrates. The zebrafish (*Danio rerio*) is one vertebrate model that proves a powerful tool for studying neural injury and repair for development of future human therapies.

Zebrafish as a model for CNS regeneration

Zebrafish share conserved retinal structure and developmental pathways with humans, making them relevant for modeling human eye disease [5]. In contrast to humans, however, zebrafish possess a remarkable ability to regenerate CNS tissues. Following RGC injury models like optic nerve transection (ONT), approximately 90% of zebrafish RGCs can survive, regrow their axons, and re-establish functional connections with central targets in the optic tectum (OT), equivalent to the visual cortex in the human occipital lobe [6]. Importantly, zebrafish do not form the inhibitory glial scar that limits regeneration in mammals following CNS injury [7] Instead, injured neurons reactivate proliferative states that support axonal extension and reconnection. This contrast between regenerative success in zebrafish and failure in mammals raises a central question of what molecular mechanisms enable successful optic nerve regeneration.

System-wide response to injury in regeneration

Most studies of optic nerve regeneration focus on the local injury site. However, emerging observations suggest that responses may extend beyond the damaged tissue. Changes in retinal ganglion cell transcriptional profile to a more proliferative state have been observed in both eyes following unilateral optic nerve injury, indicating possible bilateral or system-wide signaling [8]. If immune responses are coordinated across both sides of the visual system, then focusing only on the injured nerve may overlook critical aspects of regeneration signaling and regulation. Determining whether immune cell migration and activation are restricted to the injured tissue or occur bilaterally is essential for fully characterizing the regenerative process. Mapping immune dynamics across both retinae, optic nerves, and targeted brain regions may therefore reveal previously unrecognized patterns of spatial and temporal coordination.

The immune response following CNS injury

One major difference between regenerative and non-regenerative systems may lie in the immune response. Historically, inflammation in the CNS was viewed primarily as harmful. In mammals, prolonged immune activation contributes to scar formation and inhibits axon regrowth. However, recent evidence suggests that immune responses can also promote regeneration [9]. Following CNS injury, innate immune cells, including neutrophils and macrophages, rapidly migrate to the injury site. These cells clear debris and release signaling molecules to influence neuronal survival and axon regrowth. In zebrafish, immune recruitment appears to support rather than inhibit regeneration. Experimental stimulation of inflammatory pathways enhances optic nerve axon regrowth, and macrophage numbers increase during early stages of regeneration before resolving as repair progresses [10]. Rather than being purely destructive, early-responding immune cells may play a coordinated and time-dependent role in promoting neuronal survival and regeneration.

Overall aims and study rationale

The overall aims of the study are to determine how neutrophils respond spatially and temporally to optic nerve injury, and if this response is systemic across the visual system. Transgenic zebrafish expressing fluorescent markers in RGCs and neutrophils are used to visualize immune cell migration *in vivo* shortly following ONT injury. Confocal microscopy enables high-resolution imaging of fixed tissue sections. All experimental groups were analyzed for the resulting fluorescent signal of neutrophils in the ipsilateral and contralateral eye cups, optic nerves, and midbrain (containing OT regions).

The goal of this work is to improve understanding of spatial immune cell dynamics across the zebrafish visual system following optic nerve injury. By examining the visual system through ipsilateral, contralateral, and distal innervated tissues, this study aims to provide a small reference framework for understanding how early innate immune recruitment may contribute to RGC survival following injury.

Methods

Ethics statement

All animals were treated in accordance with provisions established by the University of Texas at Austin Institutional Animal Care and Use Committees (IACUC) under approved protocol AUP-2022-00179.

Zebrafish husbandry and transgenic lines

Two transgenic zebrafish lines were used for this study, Tg(-17.6isl2b:GFP)^zc7Tg [11] and Tg(lyz:TagRFP)^umw11Tg. The isl2b:GFP line was used for *in situ* hybridization, and a cross of isl2b:GFP;lyz:TagRFP were used for immune-related experiments. Zebrafish used in this study were adults between 3 and 9 months old, maintained on a 14:10 hour light:dark cycle at 28 degrees celsius. Previous work from our lab has shown that there was no difference in RGC survival based on sex of the fish [11], therefore both male and female fish were utilized for experiments in this study and no analysis was performed comparing the sexes.

Optic nerve transection

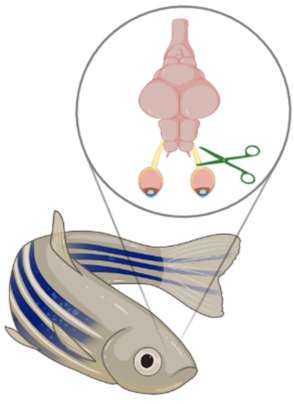

Figure 0: Unilateral optic nerve transection (ONT).
Adapted from Immunobites (2021).

Optic nerve transection (ONT) was performed as previously described in the lab [11]. Briefly, under a dissecting microscope, zebrafish were anesthetized with Tricaine methanesulfonate (MS-222, Syncaine). Tissue surrounding the eye cup was dissected away and the eye was pulled out slightly to allow the optic nerve to be transected completely using forceps. Sham surgeries were performed by clearing the surrounding tissue and pulling the eye out slightly without the subsequent transection. On the occasion the ophthalmic artery was cut resulting in bleeding, the animal was euthanized in an ice bath.

Tissue processing

All zebrafish were euthanized in ice, decapitated, and both corneas were punctured with a 20G needle. The heads were fixed in 4% paraformaldehyde in PBS overnight at 4 degrees celsius. For cryosectioning, whole-heads were processed through 25% sucrose in 1X PBS, 35% sucrose in 1X PBS, and OCT sequentially overnight at 4 degrees celsius. Tissues were then submerged in OCT in molds, flash-frozen on dry ice, and stored at -80 degrees celsius for later sectioning.

For retinal whole mounts, following overnight fixation, heads were washed in PBS and pinned to a silicon petri plate. Microscissors were used to make a radial cut through the cornea, then cut around the circumference to remove the top of the cornea and the lens. Two forceps were used to tear the sclera and remove the eye cup, allowing the retina to be loosened. The retinae were cleaned of any remaining RPE, choroid, sclera, and optic nerve tissue, and transferred to PBST. The harvested retinae were washed three times in PBST at 4 degrees celsius, washed twice in 100% MeOH at room temperature for dehydration, and stored in 100% MeOH at -20 degrees celsius.

Cryostat sectioning

OCT-embedded tissues were sectioned at a thickness of 16µm onto poly-L-lysine coated slides. Sections were collected sequentially in a series of 5 sections (80µm total) for each region. Central eye sections (used for ipsilateral and contralateral eye region analyses) were collected at the start of the visible optic nerve. Midbrain sections were collected at the start of the eye cup; this plane was chosen because it contains a central section of the optic tectum including the stratum opticum (SO) that contains all RGC axon endings [12].

In situ hybridization

Whole retinae were rehydrated at room temperature in a series of MeOH washes 100%, 75%, 50%, 25%, and in PBST. *In situ* hybridization chain reaction v3.0 was performed following the previously described protocol [13]. Following HCR, retinae were cut radially and unrolled to lay flat on slides, then mounted with Vectashield DAPI and stored at 4 degrees celsius to be imaged the same or following day.

Confocal microscopy and image analysis

Images of retinal flat mounts were taken using an upright Nikon AXR laser scanning microscope. Retinal whole mounts were imaged following protocols previously described by the lab [7]. Images of sectioned tissue were acquired at 10X magnification using ND acquisition mode to stitch 3x3 plane images with 15% blending.

Fluorescence images of sectioned tissue were analyzed using ImageJ. Images were converted to 8-bit grayscale and thresholded to generate binary masks of neutrophil-positive signals. Threshold parameters were kept constant across all groups of images taken the same day (due to inconsistent outcomes of imaging between days; however, image groups are randomized by experimental group). Binary images were processed using watershed segmentation to separate any adjacent cells. Regions of interest (ROIs) were manually defined using the polygon selection tool to encompass the ipsilateral eye cups, contralateral eye cups, and midbrain, including a small margin of error surrounding each region to account for neutrophils on the edge of anatomical landmarks. For each section, neutrophils within the ROI were quantified using the Analyze Particles function with consistent size (10-60 pixels) and circularity

(0.30-1.00) thresholds to exclude debris. For each fish, neutrophil counts were averaged across five serial sections per region to generate a single value per region per animal for analysis.

Statistical analysis

All statistical analyses and data visualization were performed using GraphPad Prism. Data are presented as individual biological replicates (one point per fish) with mean ± SEM. Five biological replicates were analyzed for sham surgery conditions, and six biological replicates were analyzed for ONT conditions. To compare neutrophil counts across experimental groups, a two-way ANOVA was performed with factors of injury condition (ONT vs. sham) and anatomical region (ipsilateral eye, contralateral eye, and midbrain). A mixed-effects model (restricted maximum likelihood, REML) was used due to unequal sample sizes. Multiple comparisons were conducted using Šídák's correction to compare ONT and sham groups within each region.

Results

Bilateral upregulation of regenerative gene expression in RGCs

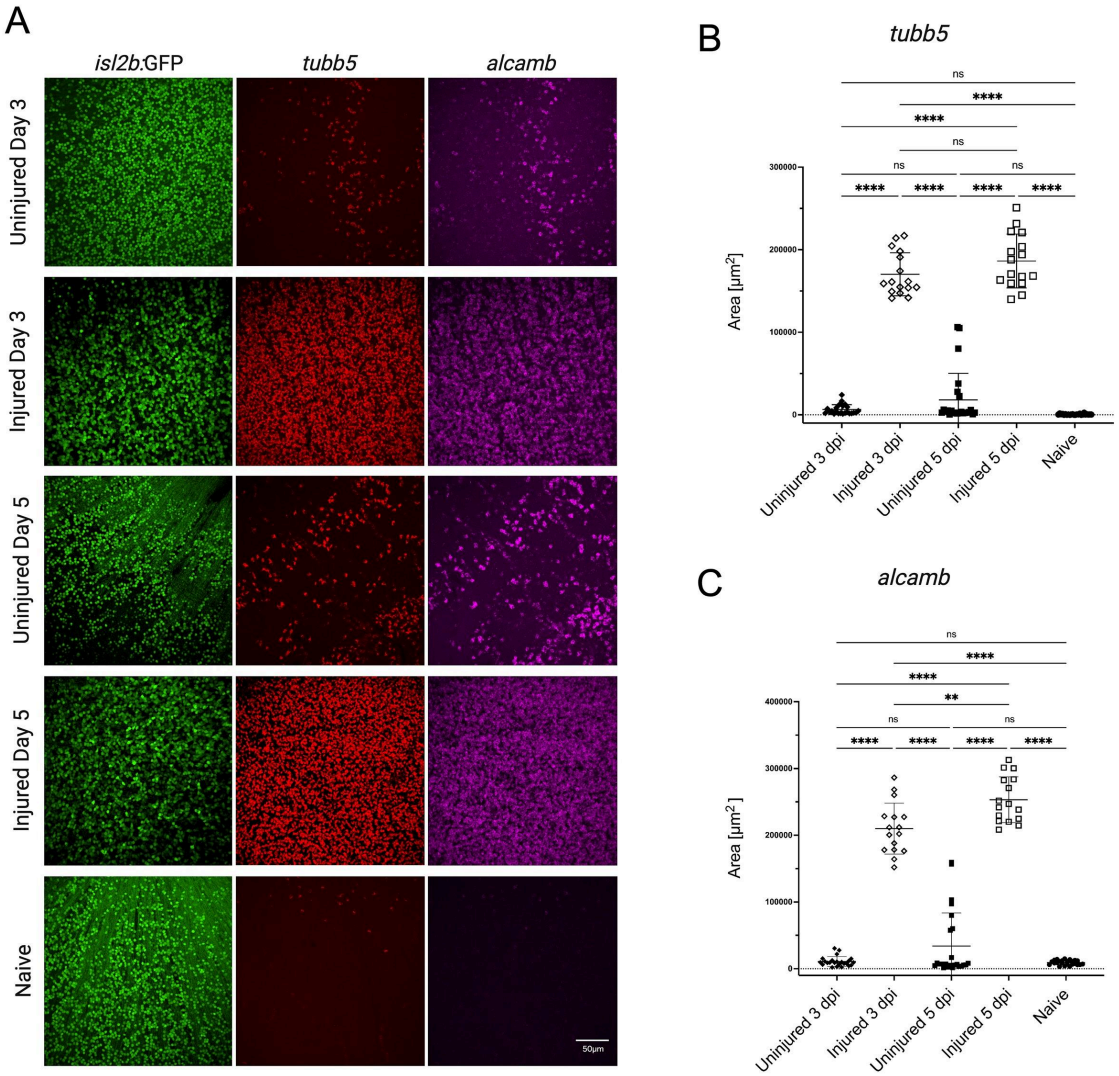

Figure 1: Expression of proliferative and regenerative gene markers in retinal ganglion cells following ONT. *A) tubb5 and alcamb expression in RGCs is robust throughout the retina at both 3 and 5 days post injury B) tubb5 expression is significant as early as 3 dpi compared to uninjured and naive controls ($P < 0.0001$) and increases significantly at 5 dpi. C) Expression of alcamb is significant as early as 3 dpi compared to uninjured and naive controls ($p < 0.0001$) and increases significantly at 5 dpi. From Ali et al. (2025).*

To validate previous findings that optic nerve injury elicits responses beyond the site of damage [8], fluorescence *in situ* hybridization (FISH) was performed on retinal whole mounts following unilateral optic nerve transection (ONT). Expression of regenerative RGC subtype 3 gene markers *alcamb* and *tubb5* was assessed in both the ipsilateral (injured) and contralateral (uninjured) retinae across early post-injury timepoints of 3 and 5 days post-injury (Fig. 1A), in comparison to naive samples.

Neutrophils are recruited to the injury site following ONT

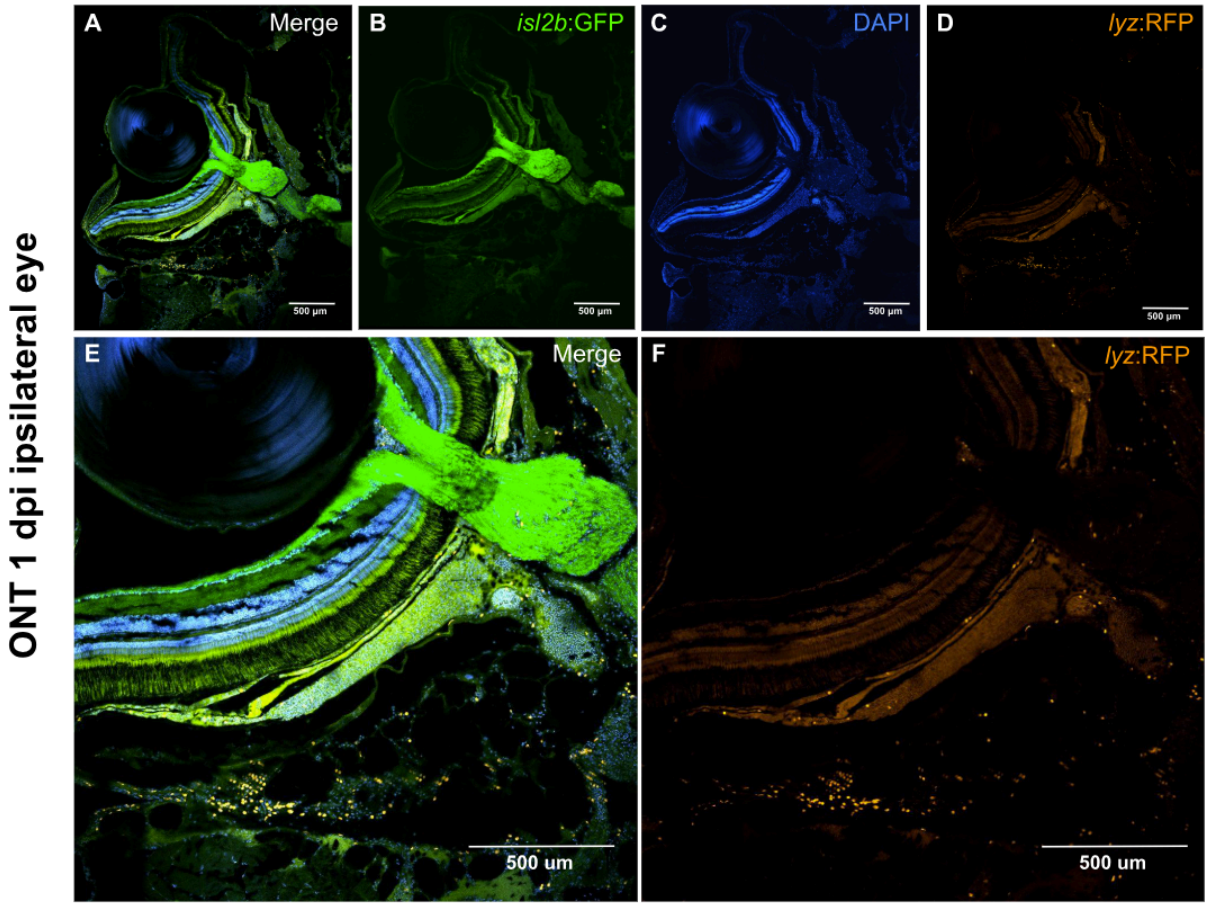

Figure 2: Robust recruitment of neutrophils to the ipsilateral eye region following ONT.
A) Merged image demonstrating the region of interest; eye cup, retinal layers, and optic nerve. B) GFP fluorescence marks RGC cell bodies and axons. C) DAPI staining for all nuclei. D) RFP fluorescence shows all neutrophils. E–F) Accumulation of neutrophils in tissues surrounding the eye cup, and in small numbers within the optic nerve fibers and optic nerve head of the ipsilateral eye. Unpublished data (2026).

To investigate whether early immune responses may contribute to this system-wide effect, neutrophil dynamics were examined using transgenic zebrafish expressing fluorescently labeled RGCs and neutrophils. Confocal imaging of cryosectioned tissue revealed clear accumulation of neutrophils surrounding the site of optic nerve injury.

Compared to sham injury controls, ONT animals exhibited a marked increase in neutrophil presence surrounding the injured optic nerve. Neutrophils were frequently observed clustered at the optic nerve head and the occasional neutrophil within the optic nerve axons (Fig. 2F). Very few neutrophils were found in retinal layers across technical and biological replicates.

Neutrophils are recruited to the contralateral eye following ONT

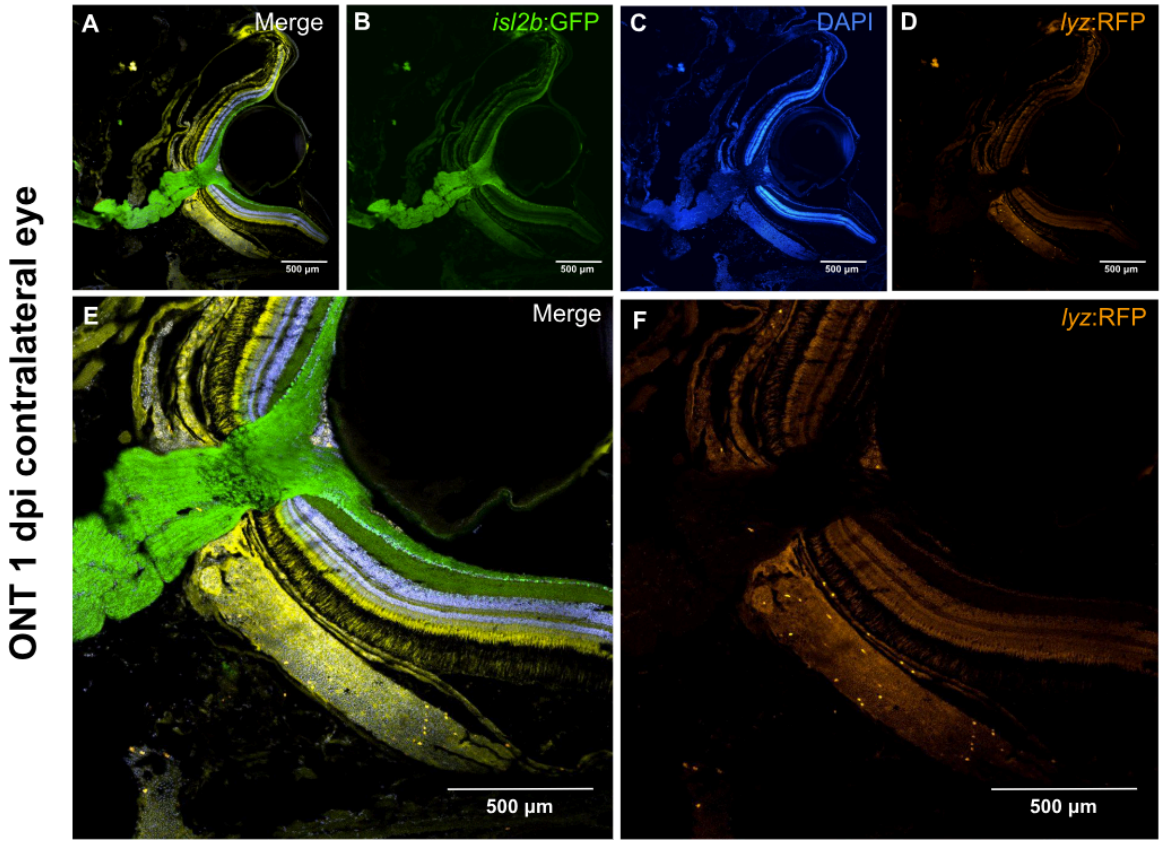

Figure 3: Neutrophil recruitment to the contralateral eye region following ONT.
A) Merged image demonstrating the region of interest; eye cup, retinal layers, and optic nerve. B) GFP fluorescence marks RGC cell bodies and axons. C) DAPI staining for all nuclei. D) RFP fluorescence shows all neutrophils. E–F) Accumulation of neutrophils in tissues surrounding the eye cup, and in small numbers within the optic nerve fibers and optic nerve head reflecting the patterns seen in the ipsilateral eye following ONT. Unpublished data (2026).

Similar distribution of neutrophils were observed in the contralateral eye region. While fewer in number than the corresponding ipsilateral eye in each animal (Fig. 5B), the presence of neutrophils in the optic nerve head and optic nerve axons reflects the overall trends seen in the ipsilateral eye (Fig. 3F).

Neutrophil presence in midbrain following ONT

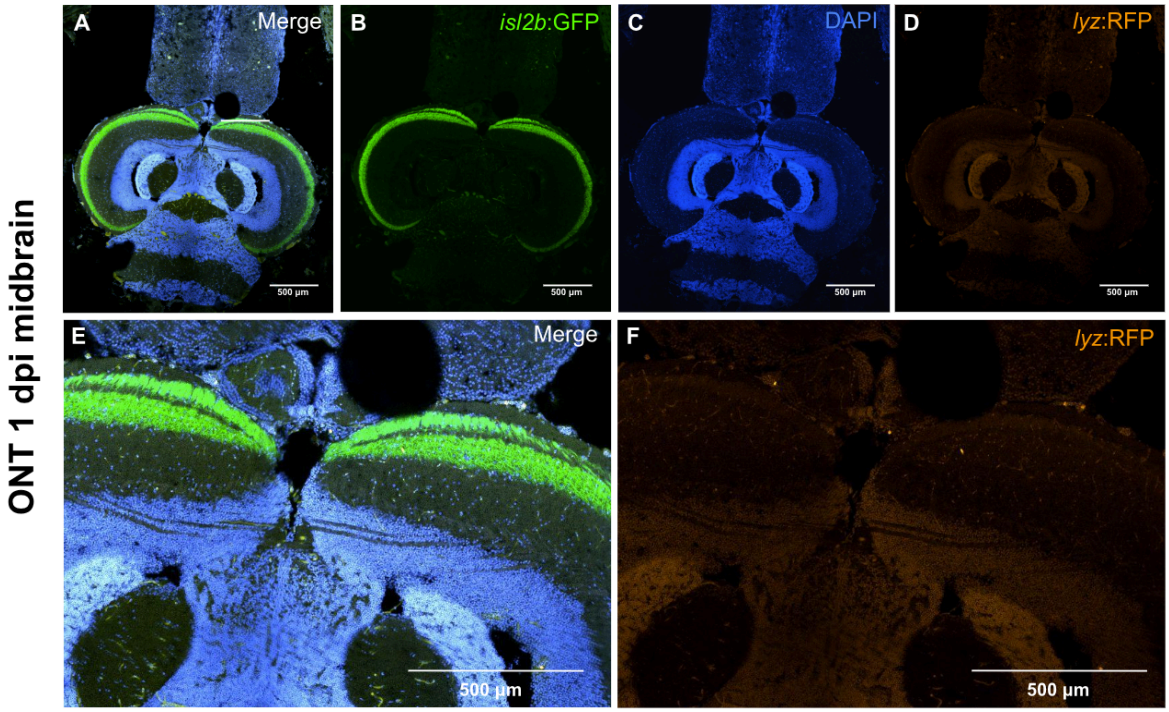

Figure 4: Neutrophil presence in the optic tectum following optic nerve transection (ONT).
A) Merged image demonstrating the region of interest; the optic tectum are the 2 outermost layers of the midbrain. B) GFP fluorescence marks RGC axon innervations into the optic tectum stratum opticum (SO) layer. C) DAPI staining for all nuclei. D) RFP fluorescence shows all neutrophils. E–F) Small numbers of neutrophils seen in the SO and stratum marginale, the outermost OT layer. Unpublished data (2026).

Neutrophils were also observed in small numbers in the optic tectum (Fig. 4F). Although neutrophil numbers in this region were lower than those observed at the injury site, their presence was consistently detected across multiple samples. This suggests sham surgeries may cause sufficient damage to the optic tissues to initiate systemic neutrophil response, however further investigation and comparison to baseline neutrophil levels in uninjured tissues is necessary.

Quantitative analysis reveals trends consistent with distributed immune response

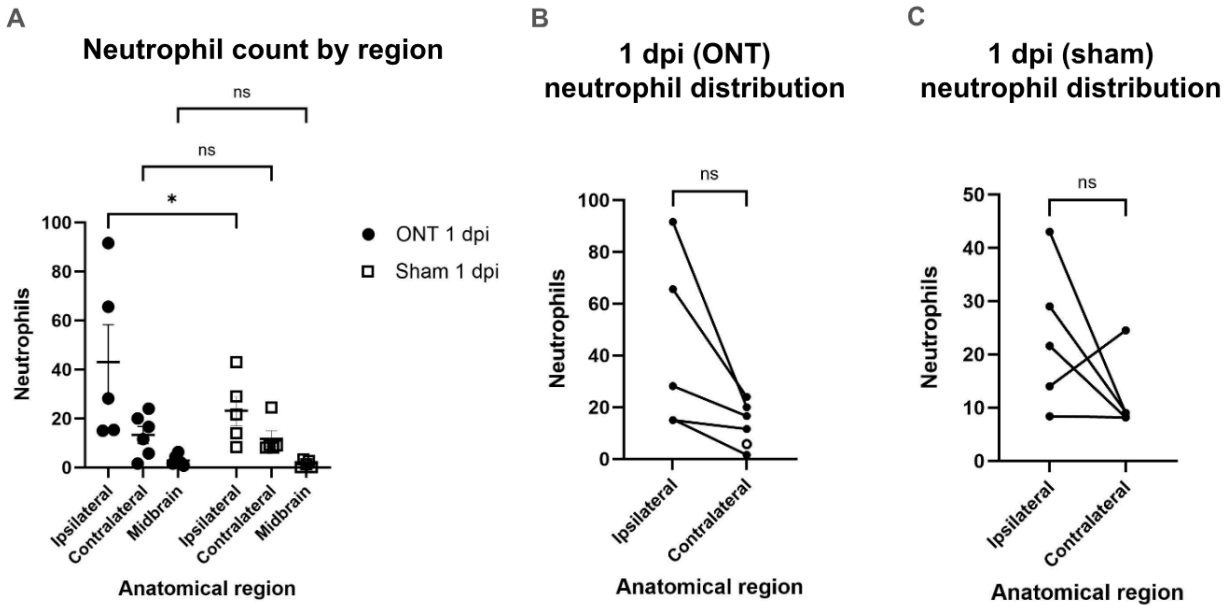

Figure 5: Quantification of neutrophil distribution across anatomical regions following ONT.
A) Neutrophil counts were quantified in the ipsilateral eye, contralateral eye, and midbrain for both ONT and sham-operated animals. B–C) Paired comparisons by individual fish ID illustrate within-animal differences between ipsilateral and contralateral regions. A significant increase in neutrophil abundance was observed in the ipsilateral eye of ONT animals compared to sham controls, while no significant differences were detected in the contralateral eye or midbrain. Data are presented as individual biological replicates with mean ± SEM. Unpublished data (2026).

Localized enrichment of neutrophils at the site of injury

To quantitatively assess regional differences, a mixed-effects model was used to compare neutrophil counts across anatomical regions and experimental conditions. Within the ipsilateral eye region, ONT animals exhibited a significant increase in neutrophil counts compared to sham controls (mean difference = 21.43, $p = 0.0276$). No significant differences between ONT and sham animals were detected in the contralateral retina or midbrain (Fig. 5A).

Within ONT animals, neutrophil counts were significantly higher in the ipsilateral retina compared to both the contralateral retina ($p = 0.0092$) and midbrain ($p = 0.0005$), while no difference was observed between contralateral retina and midbrain. In contrast, sham animals showed no significant regional differences (Fig. 5A). These results indicate that neutrophil recruitment is strongly enriched at the injury site, with lower levels in distal regions.

Paired comparisons suggest a trend toward ipsilateral enrichment following injury

To assess within-animal differences, paired comparisons of ipsilateral and contralateral neutrophil counts were performed. ONT animals exhibited a trend toward higher neutrophil counts in the ipsilateral retina compared to the contralateral side (mean difference = -28.40, $p = 0.0868$). This did not reach statistical significance (Fig. 5B), further demonstrating the contralateral enrichment of neutrophils.

Discussion

This study investigated whether optic nerve injury in zebrafish induces a spatially coordinated immune response that extends beyond localized damage, and whether these immune dynamics may relate to previously observed bilateral transcriptional changes in RGCs. Using transgenic imaging and quantitative histological analysis, we show that ONT leads to robust neutrophil recruitment at the injury site, accompanied by lower but detectable immune cell presence in contralateral retinal tissue and distal midbrain regions. While the strongest immune response remains localized to the injured optic nerve, the presence of neutrophils outside the primary injury site (Figs. 3 & 4) suggests that optic nerve injury may induce a broader and more distributed inflammatory response than previously appreciated.

Early bilateral upregulation of proliferative gene markers

Consistent with prior single-cell transcriptomic analyses, regenerative gene expression was significantly upregulated in the ipsilateral retina following injury (Fig. 1B). Notably, increased expression of these markers was also observed in the contralateral retina, despite the absence of direct localized injury to these RGCs (Fig. 1A). This bilateral response was seen across multiple timepoints and reproduced in all samples, confirming that ONT must induce a system-wide transcriptional response in RGCs soon after injury. These findings establish that RGC activation following injury is not restricted to only the damaged tissue, and suggest the presence of signaling mechanisms that coordinate bilateral responses.

Localized immune activation dominates the early injury response

The most robust and statistically significant finding from this study is the enrichment of neutrophils in the ipsilateral retina and optic nerve 1dpi following ONT. This is consistent with established models of CNS injury in zebrafish, where innate immune cells are rapidly recruited to sites of axonal damage within hours of injury and contribute to debris clearance and regenerative signaling [14]. In zebrafish optic nerve regeneration specifically, neutrophils have been shown to indirectly support axonal regrowth through a pro-regenerative inflammatory environment rather than inhibitory glial scars, as seen in mammals [15].

The absence of significant neutrophil infiltration into retinal layers themselves, despite strong accumulation at the optic nerve head, is also consistent with previous observations that immune cells preferentially localize to axonal injury sites rather than neuronal somata during early phases of regeneration [16]. This spatial restriction likely reflects regulated chemotactic signaling from damaged axons and surrounding glia, rather than diffused inflammatory activation across the retinal layers.

Evidence for distributed immune signaling across the visual system

Although immune activation was strongest at the injury site, neutrophils were consistently detected in contralateral retinal tissue and the optic tectum. While these signals were weaker and more variable than ipsilateral responses, their reproducibility across samples suggests that ONT may trigger systemic immune signaling across the visual pathway.

This finding aligns with emerging evidence that CNS injury is not strictly a local event but can induce bilateral or brain-wide immune modulation. In zebrafish, unilateral brain injury has been shown to induce contralateral proliferative and transcriptional responses, mediated in part by cytokine diffusion and systemic immune activation [17]. Similarly, optic nerve injury has been associated with changes in gene

expression in both eyes, suggesting inter-eye communication mechanisms that may involve immune or glial signaling pathways [7, 1].

The presence of neutrophils in the optic tectum is particularly notable, as this distal region is not directly injured but receives synaptic input from RGC axons. Immune presence in this region may reflect axon-associated signaling or retrograde transport of injury cues from local to distal tissues. Comparable long-range immune effects have been described in mammalian CNS injury models, where peripheral immune activation and cytokine signaling can influence distal brain regions [18].

Relationship between immune dynamics and bilateral RGC transcriptional changes

A key motivation for this study was the observation that regenerative gene expression in RGCs occurs not only in the injured retina but also in the contralateral, uninjured eye. The immune distribution patterns observed here provide a potential mechanistic link to that phenomenon.

Neutrophils, while traditionally viewed as short-lived first responders, are increasingly recognized as active regulators of tissue regeneration. They can secrete cytokines, reactive oxygen species, and proteases that modulate stem cell behavior and neuronal plasticity [19, 20]. In zebrafish, early inflammatory signaling has been shown to be necessary for successful regeneration of multiple tissues, including the retina and spinal cord [10, 20]. It is therefore plausible that early neutrophil recruitment contributes indirectly to the induction of regenerative transcriptional programs in RGCs.

However, the weak and variable nature of contralateral neutrophil presence in this dataset suggests that immune cells alone are unlikely to fully explain bilateral gene expression changes. Instead, immune signaling may act in concert with neuronal activity dependent signaling pathways, such as circulating inflammatory mediators that prime neural tissue following injury.

Limitations of the study

Several limitations should be considered when interpreting these results. First, analysis was restricted to fixed tissue snapshots, preventing assessment of dynamic immune cell migration over time. As neutrophil recruitment is highly transient, peak contralateral or midbrain responses may have been missed. Second, reliance on threshold-based image segmentation may underestimate low-intensity or partially labeled immune cells, especially due to unexpected variability in image outcomes using confocal microscopy. Additionally, sample size limitations reduce power to detect subtle bilateral differences. While neutrophils were the focus of this study, other immune populations such as macrophages and microglia likely play equally or more important roles in coordinating regenerative signaling. Previous work has shown that macrophage-derived signals are essential for axon regeneration in zebrafish optic nerve injury [14], suggesting that neutrophils represent only one component of a broader immune network.

Future directions

Future work should focus on resolving the temporal sequence of immune activation across the entire visual system using various timepoints or live imaging. Sequential time points or time-lapse imaging of neutrophil and macrophage dynamics in *vivo* would allow determination of whether contralateral immune activation precedes, follows, or occurs independently of ipsilateral responses.

Additionally, perturbation experiments targeting early inflammatory signaling pathways or neutrophil recruitment pathways would help establish causality between immune activation and bilateral RGC transcriptional changes. Single-cell RNA sequencing of both retinal and immune populations following ONT could further clarify whether systemic injury induces shared transcriptional programs across immune and neuronal compartments.

Conclusion

Overall, this study supports a model in which optic nerve injury induces a predominantly localized neutrophil response at the site of axonal damage, but also generates weaker, variable immune signals in distal regions of the visual system. These findings, together with prior evidence of bilateral RGC proliferative gene activation, suggest that optic nerve injury in zebrafish elicits a partially distributed immune and transcriptional response across the visual pathway. While neutrophils likely contribute to early neuroprotective signaling following injury, they are unlikely to be the sole mediators of these system-wide effects. Instead, RGC survival may arise from coordinated interactions between localized inflammation, systemic signaling, and intrinsic neuronal plasticity.

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