Research Article

Inhibition of $GABA_A$-ρ receptors induces retina regeneration in zebrafish

https://doi.org/10.4103/1673-5374.286972
Received: November 4, 2019
Peer review started: December 2, 2019
Accepted: February 20, 2020
Published online: August 24, 2020

Matthew R. Kent, Nergis Kara, James G. Patton*

Abstract

A potential treatment for retinal diseases is to induce an endogenous Müller glia (MG)-derived regenerative response to replace damaged neurons. In contrast to mammalian MG, zebrafish MG are capable of mediating spontaneous regeneration. We seek to define the mechanisms that enable retina regeneration in zebrafish in order to identify therapeutic targets to induce mammalian retina regeneration. We previously used pharmacological and genetic methods to inhibit gamma aminobutyric acid A ($GABA_A$) receptors in undamaged zebrafish retinas and showed that such inhibition could induce initiation of retina regeneration, as measured by the dedifferentiation of MG and the appearance of MG-derived proliferating progenitor cells. Here, we show that inhibition of a pharmacologically distinct subset of $GABA_A$ receptors ($GABA_A$-ρ) can also induce retina regeneration. Dual inhibition of both GABA receptor subtypes led to enhanced retina regeneration. Gene expression analyses indicate that inhibition of $GABA_A$-ρ receptors induces a canonical retinal regenerative response. Our results support a model in which decreased levels of GABA, such as would occur after retinal cell death or damage, induce dedifferentiation of MG and the generation of proliferating progenitor cells during zebrafish retina regeneration. Animal experiments were approved by the Vanderbilt's Institutional Animal Care and Use Committee (Protocol M1800200) on January 29, 2019.

Key Words: gamma aminobutyric acid; morpholino; Müller glia; neurotransmitter; regeneration; retina; stem cells; zebrafish

Chinese Library Classification No. R453; R364; R774

Introduction

The health and overall economic consequences of vision loss, whether due to injury or disease, is significant with 2014 estimates of annual costs by Prevent Blindness of $145 billion, a number that will certainly increase as life expectancy increases (Wittenborn and Rein, 2014). This has led to a concerted effort to identify therapies to restore loss of vision or reduce the effects of degenerative retinal disorders. One current treatment involves intravitreal injection of either stem cells or retinal precursor cells, but these treatments are not yet capable of fully restoring vision (Barber et al., 2013; Hanus et al., 2016; Gonzalez-Cordero et al., 2017; Stern et al., 2018). Recent gene therapy approaches have been used for delivery or overexpression of factors to induce retina regeneration or to restore expression of defective genes, but in certain cases this approach is limited to those diseases where the exact defective gene is known (Jorstad et al., 2017; Russell et al., 2017; Yao et al., 2018). An alternative to these approaches is to induce damaged or diseased retinas to undergo regeneration using resident adult stem cells. Zebrafish retinas undergo spontaneous retina regeneration in response to damage (Wan and Goldman, 2016). In contrast, the mammalian retina does not naturally regenerate, more often responding to damage via reactive gliosis (Bringmann et al., 2006). Intriguingly, the adult stem cell that is responsible for regeneration in zebrafish (Müller glia; MG)(Bernardos et al., 2007) is present in the mammalian retina, but for unknown reasons, MG-derived regeneration is blocked in mammals, possibly related to the hippo pathway (Rueda et al., 2019). In fish, MG respond to damage by dedifferentiation, asymmetric division, and the generation of proliferating neuronal progenitor cells which can then migrate and differentiate into any lost retinal cell type (Wan and Goldman, 2016).

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (Roberts and Kuriyama, 1968; Farrant and Nusser, 2005). Beyond its role in synaptic transmission, GABA has recently been shown to regulate neural stem cell activation in the mouse hippocampus (Catavero et al., 2018). We previously showed that inhibition of $GABA_A$ receptors can activate MG-derived stem cell proliferation during the initial stages of retina regeneration in zebrafish (Rao et al., 2017). We hypothesized that normal GABA levels maintain MG quiescence, but that disruption of the GABA levels, whether by damage, disease or pharmacological inhibition, activates MG leading to dedifferentiation, asymmetric division, production of proliferating progenitor cells, and overall induction of a regenerative response.

We previously showed that injection of the $GABA_A$ antagonist gabazine led to MG-derived proliferation in undamaged fish retinas (Rao et al., 2017). However, a second class of GABA receptors, $GABA_A$-ρ (Blarre et al., 2014; Alexander et al., 2017)(originally referred to as $GABA_C$ receptors (Drew et al.,

Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA
***Correspondence to:** James G. Patton, PhD, James.G.Patton@Vanderbilt.edu.
https://orcid.org/0000-0001-5749-0870 (James G. Patton)

***Funding:** This work was supported by grants from the NIH R01EY024354-S1 and UO1 EY027265 to JGP, and T32 EY021453, as well as additional support from the Stevenson family and Gisela Mosig endowments to Vanderbilt University.*
***How to cite this article:** Kent MR, Kara N, Patton JG (2021) Inhibition of $GABA_A$-ρ receptors induces retina regeneration in zebrafish. Neural Regen Res 16(2):367-374.*

1984)), is also expressed in the retina (Cutting et al., 1991, 1992; Boue-Grabot et al., 1998). Whether $GABA_A$-ρ receptors might also be involved in regulating retina regeneration is unknown. The $GABA_A$-ρ receptor was first discovered as a subtype of the $GABA_A$ receptor family that is insensitive to the $GABA_A$ receptor antagonist bicuculline (Drew et al., 1984). $GABA_A$-ρ receptors have a similar structure to $GABA_A$ receptors in that they are both pentameric ionotropic ligand-gated ion channels (Connolly et al., 1996; Enz and Cutting, 1998; Ogurusu et al., 1999). However, the two types of receptors differ in the subunits that form the functional receptor. $GABA_A$ receptors consist of two α subunits, two β subunits and a fifth subunit, most commonly a γ subunit. In contrast, the $GABA_A$-ρ receptors consist of five ρ subunits, of which there are three main types, typically homomeric (Connolly et al., 1996). This difference in subunit composition results in $GABA_A$-ρ receptors being insensitive to $GABA_A$ allosteric modulators such as benzodiazepines. While the ρ subunits were first discovered in mammals, they are also prevalent in zebrafish (Connaughton et al., 2008). The most abundant ρ subunit in the zebrafish retina is ρ2a, with ρ1 and ρ3a showing significantly lower expression and even lower levels of ρ2b (Cocco et al., 2017). In contrast to the $GABA_A$ receptor, $GABA_A$-ρ receptors display a more sustained response to activation (Bormann and Feigenspan, 1995) which could therefore better sustain retina regeneration. Here, we sought to test whether $GABA_A$-ρ receptors are involved in regulating initiation of retina regeneration in zebrafish.

Materials and Methods

Zebrafish lines and maintenance

Animal experiments were approved by the Vanderbilt's Institutional Animal Care and Use Committee (Protocol M1800200) on January 29, 2019. Zebrafish used in this study include Tg(*gfap*:GFP)^{mi2001} (Bernardos and Raymond, 2006) which marks mature MG and Tg(*tuba1a*:GFP) (Fausett and Goldman, 2006) which marks dedifferentiated MG. All fish were maintained at 28.5°C in a 14:10 hour light:dark cycle. All fish used were between 5 and 8 months old, and were a random mix of males and females. A total of 296 zebrafish were used in this study: 73 Tg(*gfap*:GFP)^{mi2001} zebrafish and 223 Tg(*tuba1a*:GFP) zebrafish.

Drug and morpholino injections

Pharmacological inhibitors and morpholinos were injected into the vitreous as described (Rao et al., 2017). Drugs included gabazine (S106, Sigma-Aldrich, St. Louis, MO, USA) and (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) (1040, Tocris, Minneapolis, MN, USA). Briefly, zebrafish were anesthetized in 0.016% tricaine and a small incision was made in the sclera using a sapphire blade. A blunt-end 30-gauge needle was inserted into the vitreous and 0.5 µL of drug (15, 20, 25, and 35 nmol) or morpholino (0.75 nmol) were injected into the vitreous, the amounts of which are indicated in the respective figure legends. Fish were then placed in recovery tanks for the times listed in each experiment. For 5-ethynyl-2′-deoxyuridine (EdU) injections, 20 µL of a 10 mM solution of EdU was administered via intraperitoneal injection as described by Kinkel et al. (2010).

Morpholino electroporation

Morpholinos (0.75 nmol) with a 3′-lissamine tag (Gene Tools, Philomath, OR, USA) were injected into the vitreous with or without drugs. 3 hours after injection, injected eyes were electroporated (75 V/pulse, two pulses, 1-second intervals between pulses). Fish were allowed to recover for the times indicated. Morpholinos used in this study were *Gabrr2a* MO1 (5′-AGT AGT GGC GCA GAT ATA ATG TCA T-3′), *Gabrr2a* MO2 (5′-TCG GCC TCA TAG TGA AGT CAT GAT C-3′), *ascl1a* MO1 (5′-ATC TTG GCG GTG ATG TCC ATT TCG C-3′) (Cau and Wilson, 2003), *ascl1a* MO2 (5′-AAG GAG TGA GTC AAA GCA CTA AAG T-3′) (Cau and Wilson, 2003), and a standard control morpholino (5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′).

Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling

Zebrafish were euthanized in 0.08% tricaine and treated eyes were removed and fixed in 4% paraformaldehyde overnight at 4°C. Eyes were then washed with 5% sucrose in PBS and then cryoprotected with 30% sucrose overnight at 4°C. Eyes were then transferred to a solution of two parts Tissue-Tek O.C.T. (25608-930, VWR, Radnor, PA, USA) and one part 30% sucrose for 3 hours before moving to 100% OCT for 30 minutes. Eyes were then embedded in OCT for cryosectioning. Slides were rehydrated in PBS, and then incubated in 10 mM sodium citrate at 95°C. Sections were then blocked in 3% donkey serum, 0.1% Triton X-100 in PBS. Antibodies used were mouse anti-proliferating cell nuclear antigen (PCNA) (1:500; ab29, Abcam, Cambridge, MA, USA) and rabbit anti-green fluorescent protein (GFP) (1:500; TP401, Torrey Pines Biolabs, Secaucus, NJ, USA) diluted in antibody solution (1% donkey serum, 0.05% Tween-20 in PBS) overnight at 4°C. Slides were washed with PBS, then secondary antibodies were applied: anti-mouse cy3 (1:100) and anti-rabbit Alexa fluor 488 (AF488) (Jackson Immuno Research, West Grove, PA, USA) with TO-PRO-3 (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) in antibody solution described above for 2 hours at room temperature. Slides were washed, dried, and coverslipped with Vectashield (Vector Laboratories, Inc, Burlingame, CA, USA). PCNA-positive cells were counted in the inner nuclear layer across the entirety of retinal sections. Two non-consecutive sections were counted and averaged for each eye. Images for immunofluorescence staining were taken using a META Zeiss LSM 510 Meta confocal microscope. Optical slice thickness is 0.44 µm. Images shown are stacks unless otherwise noted. The number of slices per stack are indicated in the respective figure legends. Fiji ImageJ software 4.13 was used to process images (Schindelin et al., 2012).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling was done using the *in situ* cell death detection kit, TMR Red (12156792910, Roche Life Science, Indianapolis, IN, USA) to detect apoptotic cells. EdU detection was done using the Click-iT EdU Alexa Fluor 555 Imaging Kit (C10338, Thermo Fisher Scientific) according to the manufacturer's instructions prior to immunohistochemistry. TUNEL-positive cells were counted across the entirety of retinal sections, in all layers of the retina. Each data point is from a single eye and is an average of counts from two-nonconsecutive sections, with each fish providing only one eye.

Fluorescent activated cell sorting

Fluorescent activated cell sorting (FACS) was used to isolate GFP+ cells from Tg(*gfap*:GFP)^{mi2001} zebrafish retinas, either undamaged or 24 hours after TPMPA injection, using BD FACSAria III (BD Biosciences, San Jose, CA, USA) in the VUMC Flow Cytometry Shared Resource. Retinas were dissociated according to Rajaram et al. (2014) with the following changes. Retinas were dissected and collected in Leibovitz L-15 media (21083-027, Thermo Fisher Scientific). Retinas were then treated with 1 mg/mL hyaluronidase (H3884, Sigma-Aldrich) rocking at room temperature for 15 minutes. Dead cells were detected via propidium iodide. A total of 24 retinas were collected and pooled from 12 adult fish per sorting.

Quantitative reverse transcription polymerase chain reaction

RNA was collected from sorted cells using TRIzol-LS (10296028, Thermo Fisher Scientific). Taqman small RNA assays (Thermo Fisher Scientific) were used to perform quantitative reverse transcription polymerase chain reaction (RT-qPCR) for let-7a. Briefly, 5 µL RNA was used per RT, which was then diluted

1:2. The diluted cDNA (1.33 µL) was used in a 10 µL qPCR reaction in technical triplicates. qPCR reactions were done in 384-well plates using the Bio-Rad CFX384 Real-time System (Bio-Rad Laboratories). The reactions were normalized to a U6 snRNA control. qPCR of mRNAs was done by first treating RNA with DNase (TURBO DNA free kit, AM1907, Thermo Fisher Scientific) and converted to cDNA using AccuScript High-Fidelity 1st Strand cDNA Synthesis Kit (Cat# 200820; Agilent Technologies, Stratagene, La Jolla, CA, USA). qPCR was performed using SYBR Green (Bio-Rad Laboratories). All qPCR primers spanned exon-exon junctions (Integrated DNA Technologies, Inc, Coralville, IO, USA). The reactions were normalized to 18S rRNA levels. qPCR reactions were done in 384-well plates using the Bio-Rad CFX384 Real-time System. Analysis was done using the ΔΔCt method (Livak and Schmittgen, 2001). The primers used for qRT-PCR are shown in **Table 1**.

In situ hydbridization

In situ hybridization of Gabrr2a and Gabrg2 was performed using RNAScope (Advanced Cell Diagnostics, Newark, CA, USA). Hybridizations were performed according to manufacturer's instructions for fixed frozen tissues on cryosections from Tg(*gfap*:GFP)^{mi2001} retinas, with the following changes. After creating a hydrophobic barrier around the tissue sections, RNAScope Protease III was applied for only 20 minutes rather than 30 minutes. After applying the HRP blocker solution, immunohistochemistry was performed according to ACDBio's instructions, using rabbit anti-GFP, and anti-rabbit AF488 and TOPRO-3 from above.

Statistical analysis

Two tailed Student's *t*-tests were used when comparing two sample means, a one-way analysis of variance was used to compare multiple means, and Fisher's LSD test was used for qRT-PCR analysis with ΔCt values. Each data point represents an average of two separate counts per eye. For each count, only the inner nuclear layer was counted. Eyes that were damaged were not used for analysis. Damage was indicated by disrupted morphology or high amounts of proliferation. For *in situ* hybridization analysis, a Costes' image randomization and evaluation of Pearson's coefficient was performed using the JACoP plugin for ImageJ across all optical slices per z-stack, each comprising between 68 and 70 optical slices (Costes et al., 2004; Bolte and Cordelieres, 2006).

Results

Inhibition of GABA$_A$-ρ receptors induces proliferation in the undamaged retina

Our lab has previously shown that inhibition of GABA$_A$ receptor signaling induces spontaneous proliferation in an undamaged zebrafish retina (Rao et al., 2017). However, the ρ2a subunit of the GABA$_A$-ρ receptor is also expressed in the whole retina (Cocco et al., 2017) and in purified MG by RNAseq (**Additional Table 1**). Thus, we tested whether pharmacological inhibition of GABA$_A$-ρ receptors would also induce proliferation. For this, we injected the GABA$_A$-ρ receptor antagonist TPMPA (Murata et al., 1996; Ragozzino et al., 1996) into undamaged eyes from Tg(*1016tuba1a:gf*p) transgenic fish and assessed proliferation using immunostaining against PCNA or by direct incorporation of EdU. The Tg(*1016tuba1a:gfp*) transgenic line specifically marks dedifferentiated MG and MG-derived neural progenitors in actively regenerating retinas (Fausett and Goldman, 2006). TPMPA is a commercially available competitive antagonist of the GABA$_A$-ρ receptor with only minimal effects on GABA$_A$ or GABAB receptors (Ragozzino et al., 1996). Intravitreal injection of TPMPA induced a significant increase in PCNA$^+$ cells compared to control PBS injections (**Figure 1**). Similar increases were detected using incorporation of EdU (**Additional Figure 1**). Induction of DNA replication was dose dependent up to 25 nmol, but we noticed a decrease in the number of PCNA positive cells at higher concentrations (**Additional Figure 2**). This is consistent with a specific effect of TPMPA, especially because it has been reported that TPMPA can act as an agonist at higher concentrations (Ragozzino et al., 1996). Importantly, all *tuba1a*-GFP$^+$ cells co-localized with PCNA$^+$ cells indicating that the proliferative cells were derived from MG (**Figure 1**). To ensure that the proliferation we observed was not an indirect consequence of cell death due to application of TPMPA, we utilized TUNEL staining which showed no difference in the number of apoptotic cells between the PBS-injected eyes and the TPMPA-injected eyes (**Additional Figure 3**). Combined with earlier work, the data indicate that impaired GABA signaling can activate MG and induce a regenerative response in undamaged zebrafish retinas.

While TPMPA is > 100-fold more potent against GABA$_A$-ρ receptors compared to GABA$_A$ receptors (Murata et al., 1996), it was possible that under the conditions of intravitreal injection, the effect of TPMPA could have been due to unexpected inhibition of GABA$_A$ receptors. To complement the TPMPA experiments, we tested whether knocking down the ρ2a subunit (GABRR2a), the most abundant ρ subunit in the undamaged retina (**Additional Table 1**), would also be sufficient to induce a proliferative response. For this, we independently electroporated two different antisense

Table 1 | **The primers used for quantitative reverse transcription polymerase chain reaction**

	Sequences (5'–3')	Product size (bp)
18S	Forward: TTA CAG GGC CTC GAA AGA GA	90
	Reverse: AAA CGG CTA CCA CAT CCA AG	
ascl1	Forward: TGA GCG TTC GTA AAA GGA AAC T	139
	Reverse: CGT GGT TTG CCG GTT TGT AT	
insm1a	Forward: CCG GGA CTT ACG AGA CAC AT	125
	Reverse: GGC TGG GAA GCA CTG GTT TA	
sox2	Forward: GAA AAA CAG CCC GGA CCG CAT GAG ACC	252
	Reverse: GTC TTG GTT TTC CTC CGG GGT CTG TAT TTG	
dkk1b	Forward: AAT GAC CCT GAC ATG ATT CAG C	213
	Reverse: AGG CTT GCA GAT TTT GGA CC	

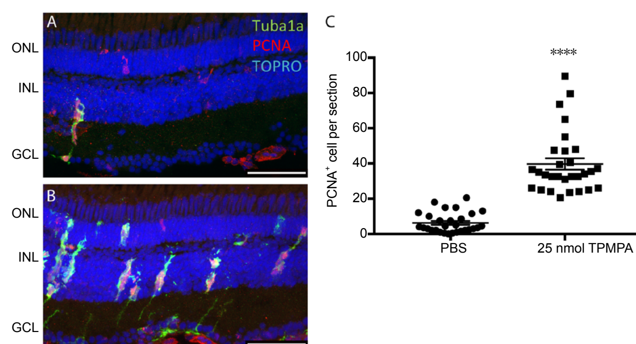


Figure 1 | **Inhibition of the GABA$_A$-ρ receptor in the absence of retina damage results in a Müller glia-derived proliferative response.**
Tg(*1016tuba1a:gfp*) zebrafish were intravitreally injected with either 1× PBS (A) or 25 nmol of the GABA$_A$-ρ receptor inhibitor (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA; B), and then allowed to recover for 48 hours before sectioning. Immunostaining was performed with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. Green: *tuba1a*:GFP; red: PCNA; blue: TO-PRO-3 in A and B. Scale bars: 50 µm. Number of optical slices: 53 (A) and 55 (B). GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. (C) Quantification of PCNA$^+$ cells. Each data point is from a separate eye and is an average of two sections, counting all PCNA$^+$ cells in the inner nuclear layer. Two-tailed Student's *t*-tests were used to test for significance. Error bars indicate the mean ± SEM. n = 29. ****$P = 1.12 \times 10^{-13}$, *vs*. PBS. GABA: Gamma aminobutyric acid; GFP: green fluorescent protein; PCNA: proliferating cell nuclear antigen.

morpholinos targeting the ρ2a subunit into undamaged retinas (**Figure 2**). Compared to control morpholino injections, injection of the two different morpholinos targeting the ρ2a subunit induced significantly higher proliferation (**Figure 2C** and **D**). Again, all *tuba1a*-GFP$^+$ cells co-localized with PCNA indicating that antisense inhibition of the GABA$_A$-ρ receptor induces a MG-based regenerative response.

Inhibition of GABA$_A$-ρ signaling induces a regenerative response

To further test whether inhibition of GABA$_A$-ρ receptors induces a bona fide regenerative response, we combined TPMPA injections with antisense inhibition of *ascl1a* (**Figure 3**). Ascl1a is a transcription factor that is required for MG-derived retina regeneration (Fausett et al., 2008; Ramachandran et al., 2010; Brzezinski et al., 2011; Ramachandran et al., 2011; Pollak et al., 2013; Ueki et al., 2015; Wohl and Reh, 2016). The prediction is that if TPMPA is inducing a bona fide regenerative response, loss of Ascl1a should reduce the proliferation observed after injection of TPMPA. As shown in **Figure 3**, after knockdown of *ascl1a*, we observed a significant decrease in the amount of proliferation compared to co-injection of TPMPA and a control morpholino (**Figure 3E** and **F**).

Lastly, to confirm that TPMPA-induced proliferation is indeed activating the canonical retina regeneration pathway, we used qRT-PCR on purified MG to determine if specific regeneration-associated genes are differentially expressed after treatment with TPMPA. Previous work has shown that retina regeneration in zebrafish results in an increase in the expression of *ascl1a*, *insm1a*, and *sox2*, and decreased expression of *dkk1b* and *let-7a*. Like Ascl1a, Insm1a and Sox2 are transcription factors that are required for retina regeneration (Ramachandran et al., 2012; Gorsuch et al., 2017). Downregulation of Dkk1b, a negative regulator of Wnt signaling, is also required for regeneration (Ramachandran et al., 2011) as is downregulation of *Let-7a*, a miRNA that represses several mRNAs encoding factors required for retina regeneration, including Ascl1a (Ramachandran et al., 2010). For these experiments, we used the Tg(*GFAP:GFP*)^{mi2001} zebrafish line in which MG expression of GFP is controlled by the GFAP (glial fibrillary acid protein) promoter (Bernardos and Raymond, 2006; Nagashima et al., 2013). After damage, expression of the GFP reporter and/or GFAP is increased in the zebrafish retina (Vihtelic et al., 2006; Bernardos et al., 2007; Lenkowski et al., 2013; Lenkowski and Raymond, 2014; Sifuentes et al., 2016). Thus, retinas from either undamaged fish or fish injected with TPMPA were dissociated and sorted to enrich for GFP$^+$ cells (**Figure 4**). RNA was isolated and qRT-PCR was performed to determine fold changes in expression between undamaged and TPMPA injected GFP$^+$ cells. We observed significantly increased levels of expression of *ascl1a* and *insm1a* and slightly upregulated expression of *sox2* (**Figure 4**). We also detected significantly decreased expression levels of *dkk1b* and *let-7a*. These results support the hypothesis that inhibition of GABA$_A$-ρ receptors by TPMPA induces expression of factors consistent with a canonical regenerative response in zebrafish.

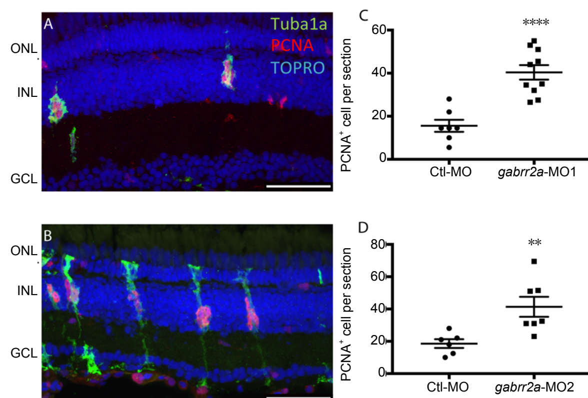


Figure 2 | Inhibition of GABA$_A$-ρ receptors via antisense morpholino injections in the absence of damage results in a proliferative response.
Tg(*1016tuba1a:gfp*) zebrafish were intravitreally injected with 0.75 nmol of either a control morpholino (Ctl-MO; A) or one of two independent antisense morpholinos targeting the ρ 2A subunit of the GABA ρ receptor (*gabrr2a*-MO1 (B, C) and *gabrr2a*-MO2 (D)) and then allowed to recover for 3 hours. Injected eyes were then electroporated and the fish were allowed to recover for 72 hours before sectioning. Immunostaining was performed with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of MG, respectively. Scale bars: 50 µm in A and B. Green: *tuba1a*:GFP; red: PCNA; blue: TO-PRO-3 in A and B. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Quantification of PCNA$^+$ cells compared to control morpholinos with either *gabrr2a*-MO1 (C) or *gabrr2a*-MO2 (D). *n* = 7 for Ctl-MO and *n* = 10 for *gabrr2a*-MO1 (C). *n* = 6 for Ctl-MO and *n* = 7 for *gabrr2a*-MO2 (D). Each data point is from a separate eye and is an average of two sections, counting all PCNA$^+$ cells in the inner nuclear layer. Two-tailed Student's *t*-tests were used to test for significance. Error bars indicate the mean ± SEM. ****$P = 8.7 \times 10^{-5}$ (C), **$P = 0.0089$ (D), *vs*. Ctl-MO. Number of optical slices: 52 (A) and 46 (B). GABA: Gamma aminobutyric acid; GFP: green fluorescent protein; PCNA: proliferating cell nuclear antigen.

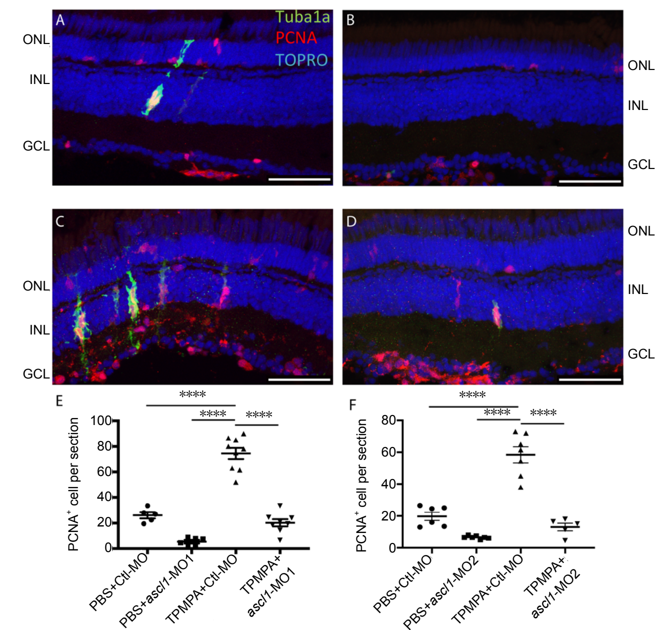


Figure 3 | Inhibition of ascl1a blocks TPMPA induced-proliferation.
Tg(*1016tuba1a:gfp*) zebrafish were intravitreally injected with either 1 × PBS, 25 nmol of the gamma aminobutyric acid A receptor (GABA$_A$-ρ receptor) inhibitor TPMPA, 0.75 nmol of control morpholino (Ctl-MO), 0.75 nmol ascl1a-MO targeting ascl1a, or combinations thereof. Fish were allowed to recover for 3 hours prior to electroporation, and an additional 45 hours before sectioning. (A) PBS/Ctl-MO co-injection. (B) PBS/*ascl1*-MO1 co-injection. (C) 25 nmol TPMPA/Ctl-MO co-injection. (D) 25 nmol TPMPA/*ascl1*-MO1 co-injection. Immunostaining was performed with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. Scale bars: 50 µm. Green: *tuba1a*:GFP; red: PCNA; blue: TO-PRO-3 (A–D). GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Number of optical slices: 41 (A), 46 (B), 46 (C), and 47 (D). (E, F) Quantification of PCNA$^+$ cells, with either *ascl1*-MO1 (E), or *ascl1*-MO2 (F). *n* = 5 for PBS+Ctl-MO, *n* = 6 for PBS+*ascl1*-MO1, *n* = 9 for TPMPA+Ctl-MO, and *n* = 8 for TPMPA+*ascl1*-MO1 (E). *n* = 7 for PBS+Ctl-MO, *n* = 10 for PBS+***ascl1***-MO2, *n* = 8 for TPMPA+Ctl-MO, and *n* = 8 for TPMPA+*ascl1*-MO2 (F). Each data point is from a separate eye and is an average of two sections, counting all PCNA$^+$ cells in the inner layer. One-way analysis of variance with Tukey's multiple comparison tests was used to test for significance. Error bars are the mean ± SEM. (E) ****$P = 5.8 \times 10^{-9}$ (PBS+Ctl-MO *vs*. TPMPA+Ctl-MO); ****$P = 1.2 \times 10^{-12}$ (PBS+*ascl1*-MO1 *vs*. TPMPA+Ctl-MO); ****$P = 3.7 \times 10^{-11}$ (TPMPA+Ctl-MO *vs*. TPMPA+*ascl1*-MO1). (F) ****$P = 1.5 \times 10^{-7}$ (PBS+Ctl-MO *vs*. TPMPA+Ctl-MO); ****$P = 4 \times 10^{-10}$ (PBS+*ascl1*-MO2 *vs*. TPMPA+Ctl-MO); ****$P = 2.45 \times 10^{-8}$ (TPMPA+Ctl-MO *vs*. TPMPA+*ascl1*-MO2). GFP: Green fluorescent protein; PCNA: proliferating cell nuclear antigen; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.

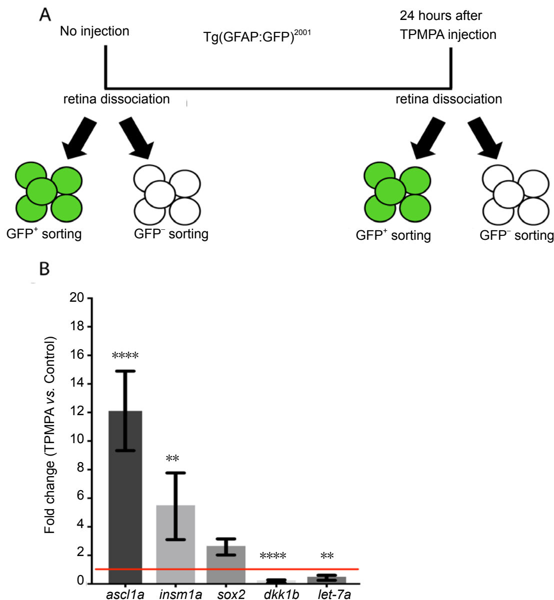


Figure 4 | Inhibition of the GABA$_A$-ρ receptor induces gene expression changes consistent with canonical retina regeneration.
(A) Retinas from *Tg(GFAP:GFP)^{mi2001}* zebrafish expressing GFP in Müller glia were dissected, dissociated, and fluorescence sorted to obtain GFP$^-$ and GFP$^+$ cells without injection of the gamma aminobutyric acid A receptor (GABA$_A$-ρ receptor) inhibitor TPMPA or 24 hours after 25 nmol TPMPA injection. (B) Quantitative reverse transcription PCR was performed on RNA from GFP$^+$ and GFP$^-$ pools and fold changes in expression were determined for the indicated mRNAs. Fold changes are displayed as $2^{-\Delta\Delta Ct}$. The red line indicates no change in expression. Fisher's least significant difference tests were used to analyze fold change expression of the indicated RNAs in TPMPA-injected retinas compared to the uninjected retinas. Error bars are shown as the mean ± SEM. n = 3 biological replicates, each with 3 technical triplicates. ****$P = 3.3 \times 10^{-6}$ (*ascl1a*); **P = 0.0037 (*insm1a*); ****$P = 5.4 \times 10^{-6}$ (*dkk1b*); **P = 0.0056 (*let-7a*). GFAP: Glial fibrillary acid protein; GFP: green fluorescent protein; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.

GABA$_A$-ρ receptors are localized to the inner and outer nuclear layers

If detection of reduced GABA levels is mediated by MG, GABA receptors should be expressed in MG. By RNAseq of undamaged retinas using the Tg(*GFAP:GFP*)^{mi2001} transgenic line, we found that GABA$_A$ and GABA$_A$-ρ receptor subunits are indeed expressed in MG (**Additional Table 1**). Besides transcriptomic analysis, we also tested whether GABA$_A$-ρ receptors co-localize with MG. We previously used immunostaining to show close association between GABA$_A$ receptors on MG processes flanking horizontal cell processes (Rao et al. 2017). Because antibodies against the zebrafish GABA$_A$-ρ receptors are not available, we used in situ hybridization to localize RNAs encoding both the ρ2a and γ2 subunits of the GABA$_A$-ρ and GABA$_A$ receptors, respectively. RNA transcripts encoding ρ2a subunits (gabrr2a) were detected in the outer plexiform layer, the inner nuclear layer, and cell bodies of the outer nuclear layer (**Figure 5**). GABA$_A$-ρ receptors are known to be expressed in both horizontal and bipolar cells, (Qian and Dowling, 1993; Fletcher et al., 1998; Lopez-Chavez et al., 2005), but we also detected ρ2a transcripts associated with MG processes (**Figure 5**). To better determine whether ρ2a subunits co-localize with MG processes, we used the ImageJ plug-in JACoP (Bolte and Cordelieres, 2006) to evaluate the extent of co-localization and also applied Costes' image randomization and evaluation of Pearson's coefficient on three sets of optical slices (single slice shown in **Figure 5B**) from one of which the Z stack in **Figure 5** was generated. This analysis resulted in an average Pearson's coefficient of 0.133667. For these analyses, Pearson's coefficients can range from 1 (perfect correlation) to –1 (no correlation). Thus, there is a positive correlation for co-localization between RNA transcripts encoding ρ2a subunits

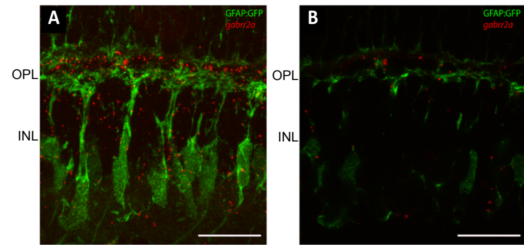


Figure 5 | Localization of GABA$_A$-ρ transcripts via *in situ* hybridization.
Retinas from *Tg*(*GFAP*:GFP)^{mi2001} zebrafish expressing GFP in Müller glia were immunostained with antibodies against GFP to mark Müller glia. In situ hybridization was performed on the same sections using probes against the ρ2a subunit of the GABA$_A$-ρ receptor (*gabrr2a*). (A) Z-stack formed from 69 optical slices. Optical slice thickness is 0.439 µm. (B) Representative optical slice from the z-stack in A. Scale bars: 20 µm. INL: Inner nuclear layer; OPL: outer plexiform layer. Green: *GFAP*:GFP; red: gabrr2a (A, B). GABA$_A$: Gamma aminobutyric acid A; GFAP: glial fibrillary acid protein; GFP: green fluorescent protein; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.

with MG processes. The correlation is weak, but the resulting *P*-value is 1.0, meaning high confidence (> 95%) that the colocalization is not due to random chance.

Additionally, we used in situ hybridization to localize the γ2 subunit (gabrg2), which showed that RNA transcripts encoding the γ2 subunit are broadly expressed across the retina (**Additional Figure 4**). Closer examination of the merged image revealed that puncta corresponding to γ2 subunits are detectable in retinal layers containing both MG cell bodies and processes, consistent with previous immunostaining. Combined, immunostaining, RNA localization, and RNAseq support the hypothesis that MG processes are in position to sense GABA levels in the retina.

Synergistic activation of regeneration by simultaneous inhibition of GABA$_A$-ρ and GABA$_A$ receptors

Individually, inhibition of GABA$_A$-ρ (**Figures 1** and **2**) or GABA$_A$ receptors (Rao et al., 2017) is sufficient to induce proliferation as part of a canonical retina regenerative response. However, since both receptors are associated with MG processes, overall detection of GABA levels by MG could be mediated by both receptors. If true, combined inhibition of both receptors should synergize to activate MG during regeneration. To test this, we co-injected gabazine, a GABA$_A$ receptor antagonist, and TPMPA. We observed a significant increase in the number of proliferating PCNA$^+$ cells compared to either treatment alone or the PBS control treatment (**Figure 6**).

Discussion

We previously showed that inhibition of GABA$_A$ receptors can induce retina regeneration in adult undamaged retinas (Rao et al., 2017). Here, we extend that work to show that inhibition of GABA$_A$-ρ receptors can induce a similar regenerative response. Several lines of evidence support that the induction of proliferation that we observe is mediated by MG in a canonical regenerative pathway. First, the transcription factor Ascl1 must be activated during retina regeneration in both fish and mice (Fausett et al., 2008; Brzezinski et al., 2011; Jorstad et al., 2017) and knockdown of ascl1 blocks the effects of inhibition of GABA$_A$-ρ receptors. Second, we observed the expected activation of ascl1 and insm1a (Ramachandran et al., 2012) following inhibition of GABA$_A$-ρ receptors, and we also observed reduced levels of dkk1b and let-7a (Ramachandran et al., 2010, 2011). We did not observe significant activation of sox2, but this seems to be more related to timing. Sox2 is normally activated during regeneration, reaching its peak by 31 hours of light damage (Gorsuch et al., 2017) whereas we examined sox2 levels only 24 hours after inhibition of GABA$_A$-ρ receptors. Given the significant differential expression of the other factors, it is likely that sox2 would show higher expression if tested at a later timepoint. Altogether,

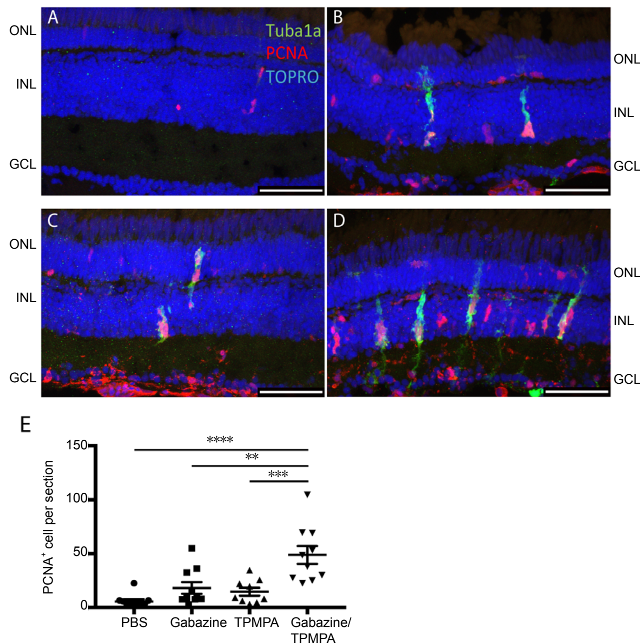


Figure 6 | Dual inhibition of GABA$_A$ and GABA$_A$-ρ receptors enhances proliferation.

Tg(*1016tuba1a:gfp*) zebrafish were intravitreally injected with either 1× PBS (A), 6.25 nmol of the GABA$_A$ antagonist gabazine (B), 12.5 nmol of the GABA$_A$-ρ receptor inhibitor TPMPA (C), or both (D). Fish were allowed to recover for 48 hours before sectioning and immunostaining with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars: 50 µm. Green: *tuba1a*:GFP; red: PCNA; blue: TO-PRO-3 (A–D). Number of optical slices: 37 (A), 49 (B), 48 (C), and 61 (D). (E) Quantification of PCNA$^+$ cells. Each data point is a separate eye and is an average of two sections, counting all PCNA$^+$ cells in the inner nuclear layer. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are the mean ± SEM. n = 10 PBS, n = 10 Gabazine, n = 9 TPMPA, and n = 10 TPMPA/Gabazine. ****$P = 1.2 \times 10^{-5}$ (PBS *vs*. Gabazine/TPMPA), **P = 0.0016 (Gabazine *vs*. Gabazine/TPMPA); ***P = 0.0006 (TPMPA *vs*. Gabazine/TPMPA). GABA$_A$: Gamma aminobutyric acid A; GFAP: glial fibrillary acid protein; GFP: green fluorescent protein; PCNA: proliferating cell nuclear antigen; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.

immunostaining (Rao et al., 2017), *in situ* hybridization, and RNAseq support the idea that MG are positioned to respond to reduced levels of GABA and induce activation of MG (**Additional Figure 5**). Normally, this cascade would be induced after retinal injury, but our experiments show that simply mimicking the loss of GABA in an undamaged retina can induce regeneration.

Direct *vs*. indirect effects of GABA inhibition

Because multiple cell types express GABA receptors in the retina, it remains possible that the effects we observe are not directly due to sensing of decreased GABA levels by MG. This could explain the increase in PCNA$^+$ cells in the ganglion cell layer, as GABA receptors have been found in retinal ganglion cells (Popova, 2015). Inhibition of GABA$_A$ and GABA$_A$-ρ receptors on bipolar cells (Connaughton et al., 2008) could result in excess glutamate leading to excitotoxic damage (Olney, 1982) and MG-derived proliferation. Excess glutamate could also activate AMPA receptors on MG, leading to an influx of Ca^{2+} (Zhang et al., 2019) and subsequent proliferation (Pinto et al., 2015). The Ca^{2+} activated protein CAPN5 is upregulated in MG after damage (Coomer and Morris, 2018); excess glutamate activating AMPA receptors on MG could be responsible for upregulation of CAPN5 and other Ca^{2+} activated proteins during regeneration. Lastly, it is also possible that damage induced by injection could be causing cell death leading to induction of a regenerative response, as opposed to regeneration as a consequence of inhibition of GABA signaling. We did not observe increased levels of apoptosis as measured by TUNEL assays, but it remains formally possible that damage leading to necrosis or autophagy could also result in initiation of regeneration. Definitive testing of the model that GABA levels are directly sensed by MG awaits generation of transgenic zebrafish lines with inducible, MG-specific knockouts of these receptors.

There also remains the possibility that the effects of TPMPA are not entirely due to inhibition of GABA$_A$-ρ receptors. While TPMPA is a highly selective antagonist of GABA$_A$-ρ receptors, it is also a weak antagonist of GABA$_A$ receptors and an even weaker agonist of GABAB receptors (Ragozzino et al., 1996). It is therefore formally possible that the effects of TPMPA we observe could be mostly due to inhibition of GABA$_A$ receptors. However, the morpholino knockdown of GABA$_A$-ρ subunit ρ2a argues against this possibility and provides further support that the effect of TPMPA is through inhibition of GABA$_A$-ρ receptors. In addition, the dual inhibition of GABA$_A$ and GABA$_A$-ρ receptors provide further support that the effects are synergistic and not solely due to effects on GABA$_A$ receptors. This synergistic effect is likely due to more GABA receptors being inhibited. As shown, both here and in our previous work (Rao et al., 2017), higher doses of the inhibitors increase the resulting number of PCNA$^+$ cells.

GABA receptors and adult neurogenesis

Neural stem cell activity in the mouse hippocampus has been proposed to be regulated by sensing of non-synaptic GABA levels (Chell and Frisen, 2012; Song et al., 2012; Catavero et al., 2018). Our proposed activation of MG (**Additional Figure 5**) is very similar to that proposed by Song et al. (2012) although we do not have evidence of long range GABAergic inputs (Bao et al., 2017) which would not seem to be necessary in the retina. Interestingly, GABA$_B$ receptors have been proposed to play a role in adult neurogenesis in the mouse hippocampus (Giachino et al., 2014). We have no evidence thus far for an involvement in G-protein coupled GABA$_B$ receptors in regulating activation of MG in the zebrafish retina. Supporting our work in the retina, inactivation of GABA$_A$ receptors was shown to inhibit proliferation of cultured progenitor cells from adult mouse retina (Wang et al., 2019). Thus, despite some differences, there appears to be a an evolutionarily conserved mechanism involving stem cell activity and the sensing of GABA levels. A major question, then, is how loss of GABA signaling mechanistically induces regeneration in the zebrafish retina. GABA$_A$ and GABA$_A$-ρ are both ionotropic receptors that selectively transport Cl^- ions either into or out of the cell depending on membrane potential. Recently, reduced levels of intracellular Cl^- were found to induce tumor necrosis factor α in endothelial cells (Yang et al., 2012). Tumor necrosis factor α has been shown to be involved in the early stages of retina regeneration (Nelson et al., 2013). It is therefore possible that loss of GABA signaling after retina damage results in reduced intracellular Cl^-, which then leads to an upregulation of tumor necrosis factor α.

As above, excess glutamate in the retina can be excitotoxic (Olney, 1982) which could be due to TPMPA acting on bipolar cells. While our previous work showed that inhibiting glutamate receptors in the retina leads to MG-derived proliferation (Rao et al., 2017), inhibiting AMPA receptors in an injury model involving injection of $CoCl_2$ led to reduced proliferation, an apparent neuroprotective effect (Medrano et al., 2018). While other explanations are possible, these seemingly contradictory results could simply be due to a CoCl2-mediated injury response, whereas we blocked AMPA receptors in undamaged retinas.

GABA and β-cell regeneration

Beyond the mouse hippocampus and the zebrafish retina, GABA

levels can drive pancreatic α cells to a β cell fate (Ben-Othman et al., 2017; Li et al., 2017). Intriguingly, the role of GABA in this case, both in adult mice and cultured cells, is the opposite of what we observe in the retina in that increased GABA levels or administration of indirect agonists of the $GABA_A$ stimulated increased numbers of α cells derived from glucagon secreting β cells. Even though the mechanism of action of GABA is the opposite, the common finding is that altered GABA signaling can activate regeneration.

Conclusion

We have shown that inhibiting $GABA_A$-ρ receptors is sufficient to induce a regenerative response in the zebrafish retina in the absence of damage and that inhibiting both $GABA_A$ $GABA_A$-ρ receptors simultaneously produces a synergistic effect. It will be important to determine if this effect is directly mediated through MG, but, together, our results suggest a novel approach to induce a regenerative response in the mammalian retina.

Acknowledgments: *We would like to thank members of the Patton lab for constructive discussions. Zebrafish were maintained by the Stevenson Zebrafish facility with help from Qiang Guan. Zebrafish lines were shared by Pamela Raymond (Tg(gfap:GFP)mi2001) and Daniel Goldman (Tg(tuba1a:GFP).*

Author contributions: *MRK designed the study, collected and analyzed data, and drafted and edited the manuscript. NK collected and analyzed data and helped with drafting the manuscript. JGP helped design the study, helped with analyzing data, drafting and editing the manuscript. All authors approved the final version of the paper.*

Conflicts of interest: *The authors have no conflict of interest.*

Financial support: *This work was supported by grants from the NIH R01EY024354-S1 and UO1 EY027265 to JGP, and T32 EY021453 to Milam Brantley, as well as additional support from the Stevenson family and Gisela Mosig endowments to Vanderbilt University.*

Institutional review board statement: *Animal experiments were approved by the Vanderbilt's Institutional Animal Care and Use Committee (Protocol M1800200) on January 29, 2019.*

Copyright license agreement: *The Copyright License Agreement has been signed by all authors before publication.*

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*

Plagiarism check: *Checked twice by iThenticate.*

Peer review: *Externally peer reviewed.*

Open access statement: *This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.*

Open peer reviewers: *Salvatore L. Stella, Penn State College of Medicine, USA; JiaJie Teoh, Columbia University Irving Medical Center, USA.*

Additional files:

Additional file 1: *Open peer review reports 1 and 2.*

Additional Figure 1: *Inhibition of the GABAA-ρ receptor in the absence of damage results in a proliferative response.*

Additional Figure 2: *Dose dependent inhibition of the GABAA-ρ receptor.*

Additional Figure 3: *Inhibition of the GABAA-ρ receptor does not result in increased apoptosis.*

Additional Figure 4: *Localization of transcripts encoding the γ2 subunit of GABAA receptors.*

Additional Figure 5: *Model of GABA receptor inhibition-induced proliferation.*

Additional Table 1: *Average RPKM values of GABA subunits from sequencing of sorted Müller glia.*

References

Alexander SP, Peters JA, Kelly E, Marrion NV, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA; CGTP Collaborators (2017) THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: Ligand-gated ion channels. Br J Pharmacol 174 Suppl 1:S130-159.

Bao H, Asrican B, Li W, Gu B, Wen Z, Lim SA, Haniff I, Ramakrishnan C, Deisseroth K, Philpot B, Song J (2017) Long-range GABAergic inputs regulate neural stem cell quiescence and control adult hippocampal neurogenesis. Cell Stem Cell 21:604-617.

Barber AC, Hippert C, Duran Y, West EL, Bainbridge JW, Warre-Cornish K, Luhmann UF, Lakowski J, Sowden JC, Ali RR, Pearson RA (2013) Repair of the degenerate retina by photoreceptor transplantation. Proc Natl Acad Sci U S A 110:354-359.

Ben-Othman N, Vieira A, Courtney M, Record F, Gjernes E, Avolio F, Hadzic B, Druelle N, Napolitano T, Navarro-Sanz S, Silvano S, Al-Hasani K, Pfeifer A, Lacas-Gervais S, Leuckx G, Marroquí L, Thévenet J, Madsen OD, Eizirik DL, Heimberg H, et al. (2017) Long-term GABA administration induces alpha cell-mediated beta-like cell neogenesis. Cell 168:73-85. e11.

Bernardos RL, Raymond PA (2006) GFAP transgenic zebrafish. Gene Expr Patterns 6:1007-1013.

Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci 27:7028-7040.

Blarre T, Bertrand HO, Acher FC, Kehoe J (2014) Molecular determinants of agonist selectivity in glutamate-gated chloride channels which likely explain the agonist selectivity of the vertebrate glycine and GABAA-rho receptors. PLoS One 9:e108458.

Bolte S, Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224:213-232.

Bormann J, Feigenspan A (1995) GABAC receptors. Trends Neurosci 18:515-519.

Boue-Grabot E, Roudbaraki M, Bascles L, Tramu G, Bloch B, Garret M (1998) Expression of GABA receptor rho subunits in rat brain. J Neurochem 70:899-907.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A (2006) Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25:397-424.

Brzezinski JAt, Kim EJ, Johnson JE, Reh TA (2011) Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. Development 138:3519-3531.

Catavero C, Bao H, Song J (2018) Neural mechanisms underlying GABAergic regulation of adult hippocampal neurogenesis. Cell Tissue Res 371:33-46.

Cau E, Wilson SW (2003) Ash1a and Neurogenin1 function downstream of Floating head to regulate epiphysial neurogenesis. Development 130:2455-2466.

Chell JM, Frisen J (2012) Noisy neurons keep neural stem cells quiet. Cell Stem Cell 11:282-284.

Cocco A, Ronnberg AM, Jin Z, Andre GI, Vossen LE, Bhandage AK, Thornqvist PO, Birnir B, Winberg S (2017) Characterization of the gamma-aminobutyric acid signaling system in the zebrafish (Danio rerio Hamilton) central nervous system by reverse transcription-quantitative polymerase chain reaction. Neuroscience 343:300-321.

Connaughton VP, Nelson R, Bender AM (2008) Electrophysiological evidence of GABAA and GABAC receptors on zebrafish retinal bipolar cells. Vis Neurosci 25:139-153.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ (1996) Assembly and cell surface expression of heteromeric and homomeric gamma-aminobutyric acid type A receptors. J Biol Chem 271:89-96.

Coomer CE, Morris AC (2018) Capn5 expression in the healthy and regenerating zebrafish retina. Invest Ophthalmol Vis Sci 59:3643-3654.

Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys J 86:3993-4003.

Cutting GR, Curristin S, Zoghbi H, O'Hara B, Seldin MF, Uhl GR (1992) Identification of a putative gamma-aminobutyric acid (GABA) receptor subunit rho2 cDNA and colocalization of the genes encoding rho2 (GABRR2) and rho1 (GABRR1) to human chromosome 6q14-q21 and mouse chromosome 4. Genomics 12:801-806.

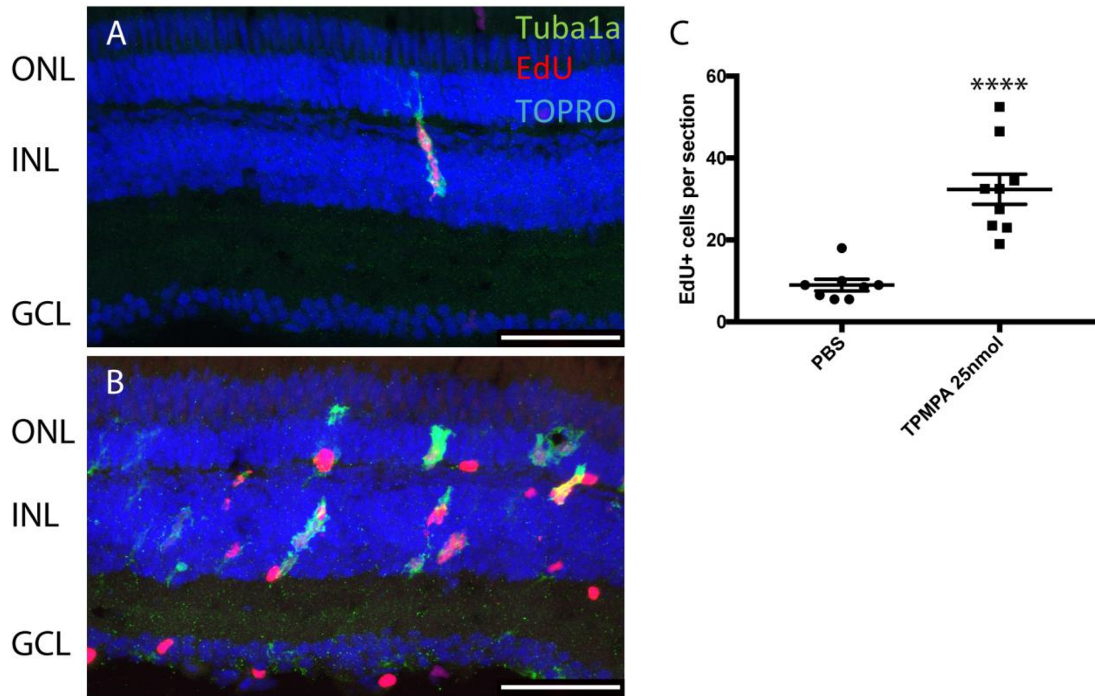
Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, et al. (1991) Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. Proc Natl Acad Sci U S A 88:2673-2677.

Drew CA, Johnston GA, Weatherby RP (1984) Bicuculline-insensitive GABA receptors: studies on the binding of (-)-baclofen to rat cerebellar membranes. Neurosci Lett 52:317-321.

Enz R, Cutting GR (1998) Molecular composition of GABAC receptors. Vision Res 38:1431-1441.

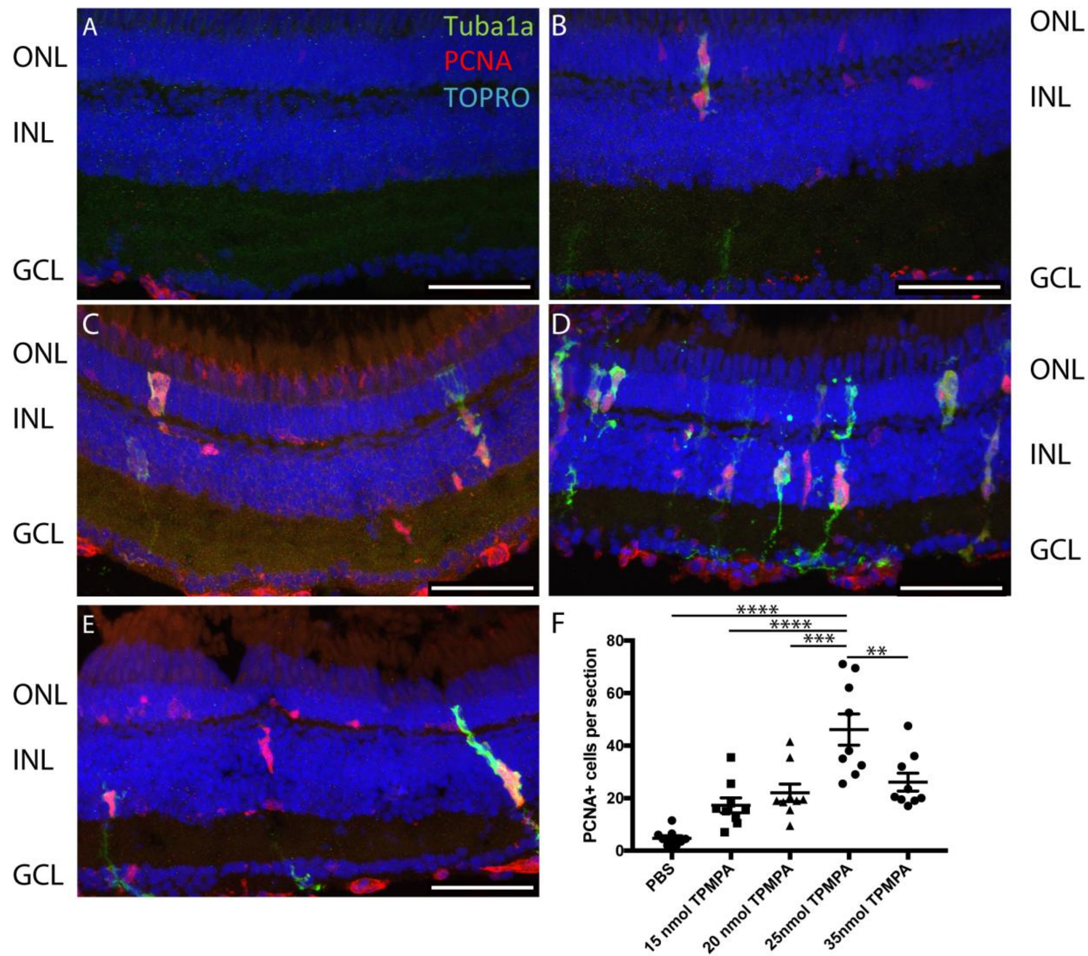
Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6:215-229.
Fausett BV, Goldman D (2006) A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci 26:6303-6313.
Fausett BV, Gumerson JD, Goldman D (2008) The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci 28:1109-1117.
Fletcher EL, Koulen P, Wassle H (1998) GABAA and GABAC receptors on mammalian rod bipolar cells. J Comp Neurol 396:351-365.
Giachino C, Barz M, Tchorz JS, Tome M, Gassmann M, Bischofberger J, Bettler B, Taylor V (2014) GABA suppresses neurogenesis in the adult hippocampus through GABAB receptors. Development 141:83-90.
Gonzalez-Cordero A, Kruczek K, Naeem A, Fernando M, Kloc M, Ribeiro J, Goh D, Duran Y, Blackford SJI, Abelleira-Hervas L, Sampson RD, Shum IO, Branch MJ, Gardner PJ, Sowden JC, Bainbridge JWB, Smith AJ, West EL, Pearson RA, Ali RR (2017) Recapitulation of human retinal development from human pluripotent stem cells generates transplantable populations of cone photoreceptors. Stem Cell Reports 9:820-837.
Gorsuch RA, Lahne M, Yarka CE, Petravick ME, Li J, Hyde DR (2017) Sox2 regulates Muller glia reprogramming and proliferation in the regenerating zebrafish retina via Lin28 and Ascl1a. Exp Eye Res 161:174-192.
Hanus J, Zhao F, Wang S (2016) Current therapeutic developments in atrophic age-related macular degeneration. Br J Ophthalmol 100:122-127.
Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, Wong RO, Rieke F, Reh TA (2017) Stimulation of functional neuronal regeneration from Muller glia in adult mice. Nature 548:103-107.
Kinkel MD, Eames SC, Philipson LH, Prince VE (2010) Intraperitoneal injection into adult zebrafish. J Vis Exp doi: 10.3791/2126.
Lenkowski JR, Raymond PA (2014) Muller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. Prog Retin Eye Res 40:94-123.
Lenkowski JR, Qin Z, Sifuentes CJ, Thummel R, Soto CM, Moens CB, Raymond PA (2013) Retinal regeneration in adult zebrafish requires regulation of TGFbeta signaling. Glia 61:1687-1697.
Li J, Casteels T, Frogne T, Ingvorsen C, Honoré C, Courtney M, Huber KVM, Schmitner N, Kimmel RA, Romanov RA, Sturtzel C, Lardeau CH, Klughammer J, Farlik M, Sdelci S, Vieira A, Avolio F, Briand F, Baburin I, Májek P, et al. (2017) Artemisinins Target GABAA Receptor Signaling and Impair alpha Cell Identity. Cell 168(1-2):86-100.e15.
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
Lopez-Chavez A, Miledi R, Martinez-Torres A (2005) Cloning and functional expression of the bovine GABA(C) rho2 subunit. Molecular evidence of a widespread distribution in the CNS. Neurosci Res 53:421-427.
Medrano MP, Pisera Fuster A, Sanchis PA, Paez N, Bernabeu RO, Faillace MP (2018) Characterization of proliferative, glial and angiogenic responses after a CoCl2-induced injury of photoreceptor cells in the adult zebrafish retina. Eur J Neurosci 48:3019-3042.
Murata Y, Woodward RM, Miledi R, Overman LE (1996) The first selective antagonist for a GABA(C) receptor. Bioorg Med Chem Lett 6:2073-2076.
Nagashima M, Barthel LK, Raymond PA (2013) A self-renewing division of zebrafish Muller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. Development 140:4510-4521.
Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR (2013) Tumor necrosis factor-alpha is produced by dying retinal neurons and is required for Muller glia proliferation during zebrafish retinal regeneration. J Neurosci 33:6524-6539.
Ogurusu T, Yanagi K, Watanabe M, Fukaya M, Shingai R (1999) Localization of GABA receptor rho 2 and rho 3 subunits in rat brain and functional expression of homooligomeric rho 3 receptors and heterooligomeric rho 2 rho 3 receptors. Receptors Channels 6:463-475.
Olney JW (1982) The toxic effects of glutamate and related compounds in the retina and the brain. Retina 2:341-359.
Pinto MC, Kihara AH, Goulart VA, Tonelli FM, Gomes KN, Ulrich H, Resende RR (2015) Calcium signaling and cell proliferation. Cell Signal 27:2139-2149.
Pollak J, Wilken MS, Ueki Y, Cox KE, Sullivan JM, Taylor RJ, Levine EM, Reh TA (2013) ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. Development 140:2619-2631.
Popova E (2015) GABAergic neurotransmission and retinal ganglion cell function. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 201:261-283.
Qian H, Dowling JE (1993) Novel GABA responses from rod-driven retinal horizontal cells. Nature 361:162-164.
Ragozzino D, Woodward RM, Murata Y, Eusebi F, Overman LE, Miledi R (1996) Design and in vitro pharmacology of a selective gamma-aminobutyric acidC receptor antagonist. Mol Pharmacol 50:1024-1030.
Rajaram K, Harding RL, Hyde DR, Patton JG (2014) miR-203 regulates progenitor cell proliferation during adult zebrafish retina regeneration. Dev Biol 392:393-403.
Ramachandran R, Fausett BV, Goldman D (2010) Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol 12:1101-1107.
Ramachandran R, Zhao XF, Goldman D (2011) Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. Proc Natl Acad Sci U S A 108:15858-15863.
Ramachandran R, Zhao XF, Goldman D (2012) Insm1a-mediated gene repression is essential for the formation and differentiation of Muller glia-derived progenitors in the injured retina. Nat Cell Biol 14:1013-1023.
Rao MB, Didiano D, Patton JG (2017) Neurotransmitter-regulated regeneration in the zebrafish retina. Stem Cell Reports 8:831-842.
Roberts E, Kuriyama K (1968) Biochemical-physiological correlations in studies of the gamma-aminobutyric acid system. Brain Res 8:1-35.
Rueda EM, Hall BM, Hill MC, Swinton PG, Tong X, Martin JF, Poche RA (2019) The hippo pathway blocks mammalian retinal muller glial cell reprogramming. Cell Rep 27:1637-1649.
Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, Tillman A, Wittes J, Pappas J, Elci O, McCague S, Cross D, Marshall KA, Walshire J, Kehoe TL, Reichert H, Davis M, Raffini L, George LA, Hudson FP, Dingfield L, et al. (2017) Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet 390:849-860.
Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676-682.
Sifuentes CJ, Kim JW, Swaroop A, Raymond PA (2016) Rapid, dynamic activation of muller glial stem cell responses in zebrafish. Invest Ophthalmol Vis Sci 57:5148-5160.
Song J, Zhong C, Bonaguidi MA, Sun GJ, Hsu D, Gu Y, Meletis K, Huang ZJ, Ge S, Enikolopov G, Deisseroth K, Luscher B, Christian KM, Ming GL, Song H (2012) Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. Nature 489:150-154.
Stern JH, Tian Y, Funderburgh J, Pellegrini G, Zhang K, Goldberg JL, Ali RR, Young M, Xie Y, Temple S (2018) Regenerating eye tissues to preserve and restore vision. Cell Stem Cell 23:453.
Ueki Y, Wilken MS, Cox KE, Chipman L, Jorstad N, Sternhagen K, Simic M, Ullom K, Nakafuku M, Reh TA (2015) Transgenic expression of the proneural transcription factor Ascl1 in Muller glia stimulates retinal regeneration in young mice. Proc Natl Acad Sci U S A 112:13717-13722.
Vihtelic TS, Soverly JE, Kassen SC, Hyde DR (2006) Retinal regional differences in photoreceptor cell death and regeneration in light-lesioned albino zebrafish. Exp Eye Res 82:558-575.
Wan J, Goldman D (2016) Retina regeneration in zebrafish. Curr Opin Genet Dev 40:41-47.
Wang S, Du L, Peng G, Li W (2019) GABA inhibits proliferation and self-renewal of mouse retinal progenitor cell. Cell Death Discov 5:80.
Wittenborn JS, Rein DB (2014) The future of vision: forecasting the prevalence and cost of vision problems. Chicago, IL: University of Chicago.
Wohl SG, Reh TA (2016) miR-124-9-9* potentiates Ascl1-induced reprogramming of cultured Muller glia. Glia 64:743-762.
Yang H, Huang LY, Zeng DY, Huang EW, Liang SJ, Tang YB, Su YX, Tao J, Shang F, Wu QQ, Xiong LX, Lv XF, Liu J, Guan YY, Zhou JG (2012) Decrease of intracellular chloride concentration promotes endothelial cell inflammation by activating nuclear factor-kappaB pathway. Hypertension 60:1287-1293.
Yao K, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, Liu X, Chang B, Zenisek D, Crair MC, Demb JB, Chen B (2018) Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. Nature 560:484-488.
Zhang RW, Du WJ, Prober DA, Du JL (2019) Muller glial cells participate in retinal waves via glutamate transporters and AMPA receptors. Cell Rep 27:2871-2880. e2.

C-Editors: Zhao M, Li CH; T-Editor: Jia Y

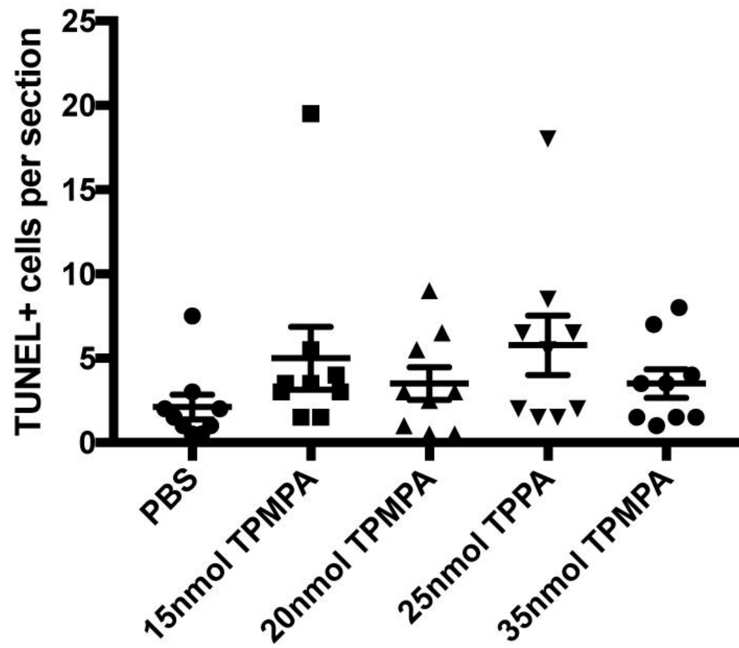


Additional Figure 1 Inhibition of the GABA$_A$-ρ receptor in the absence of damage results in a proliferative response.

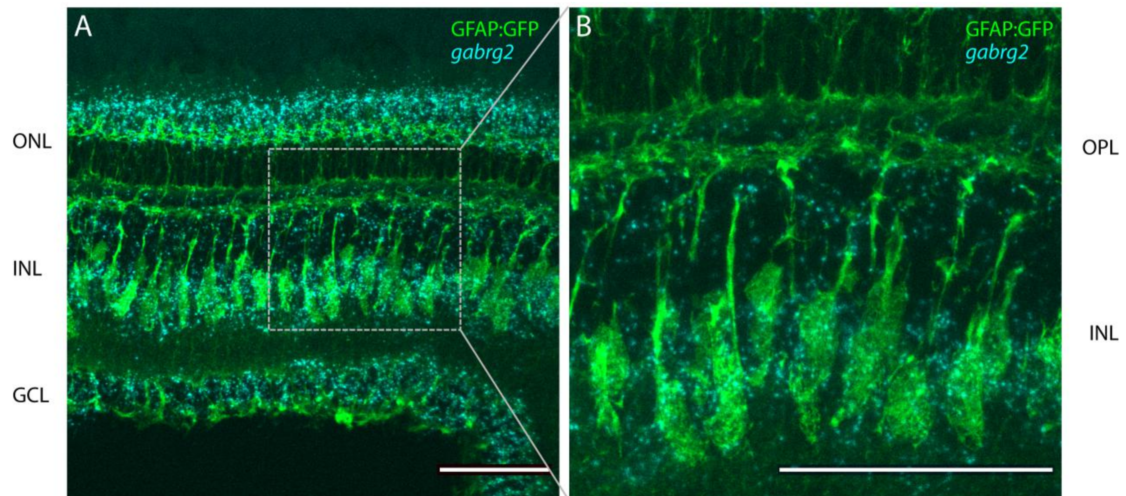
Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with either PBS (A) or 25 nmol of the GABA$_A$-receptor inhibitor TPMPA (B). After 3 hours, fish were given an intraperitoneal injection of 20 µL of 10 mM EdU with a second intraperitoneal injection of EdU after 24 hours. Eyes were collected 48 hours after the TPMPA injection and sectioned, followed by immunostaining with antibodies against GFP to monitor dedifferentiation of MG and detection of EdU incorporation using the Click-iT EdU labeling kit to monitor DNA replication. Scale bar: 50 µm. Green: *tuba1a*:GFP; red: EdU; blue–TO-PRO-3 (A, B). ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Number of optical slices: 47 (A) and 45 (B). (C) Quantification of EdU$^+$ cells. Each data point is a separate eye and is an average of two sections, counting all PCNA$^+$ cells in the inner nuclear layer. Two-tailed Student's *t*-tests were used to test for significance. Error bars are the mean ± SEM. n = 9. ****P = 4.7 × 10^{-5}, *vs.* PBS. EdU: 5-Ethynyl-2′-deoxyuridine; GABA$_A$: Gamma aminobutyric acid A; GFP: green fluorescent protein; PCNA: proliferating cell nuclear antigen; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.



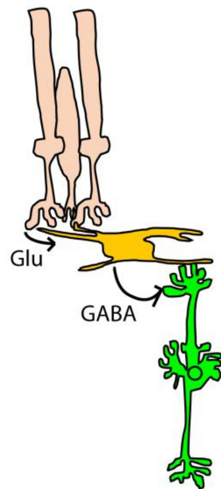
Additional Figure 2 Dose dependent inhibition of the $GABA_A$-ρ receptor.
Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with either 1× PBS (A) or 15 nmol of the $GABA_A$-receptor inhibitor TPMPA (B), 20 nmol TPMPA (C), 25 nmol TPMPA (D), or 35 nmol TPMPA (E). Fish were allowed to recover for 48 hours before sectioning and immunostaining with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Scale bar: 50 μm. Green: *tuba1a*:GFP; red: PCNA; blue: TO-PRO-3 (A-E). Number of optical slices: 49 (A), 45 (B), 49 (C), 44 (D), and 53 (E). (F) Quantification of $PCNA^+$ cells. Each data point is from a separate eye and is an average of two sections, counting all $PCNA^+$ cells in the inner nuclear layer. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are the mean ± SEM. n = 9. ****$P = 8.4 \times 10^{-9}$ (PBS *vs*. 25 nmol TPMPA); ****$P = 1.8 \times 10^{-5}$ (15 nmol TPMPA *vs*. 25 nmol TPMPA); ***$P = 0.0003$ (20 nmol TPMPA *vs*. 25 nmol TPMPA); ****$P = 0.0025$ (35 nmol TPMPA *vs*. 25 nmol TPMPA). $GABA_A$: Gamma aminobutyric acid A; GFP: green fluorescent protein; PCNA: proliferating cell nuclear antigen; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.

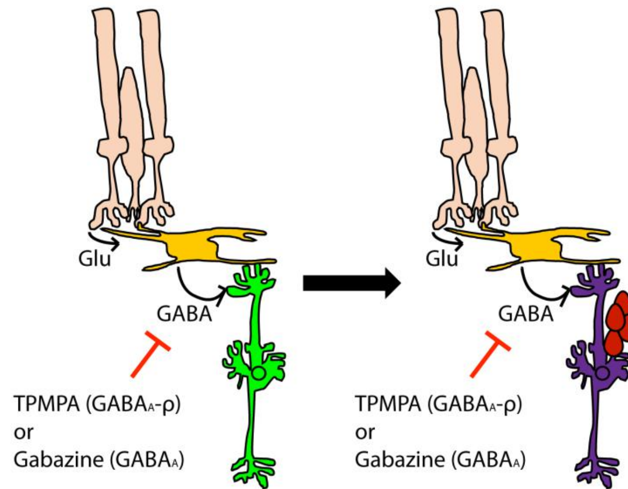


Additional Figure 3 Inhibition of the GABA$_A$-ρ receptor does not result in increased apoptosis. Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with the indicated amounts of either PBS or the GABA$_A$-ρ receptor inhibitor TPMPA. After injection, fish were allowed to recover for 48 hours followed by analysis of apoptotic cells using TUNEL staining. Each data point is a separate eye and is an average of two sections, counting all TUNEL$^+$ cells in the retina section. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are shown as the mean ± SEM. n = 10 for PBS, n = 9 for 15 nmol TPMPA, n = 9 for 20 nmol TPMPA, and n = 8 for 25 nmol TPMPA. GABA$_A$: Gamma aminobutyric acid A; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.



Additional Figure 4 Localization of transcripts encoding the γ2 subunit of GABA$_A$ receptors.
Representative image of a retina from the Tg*(GFAP:GFP)*^{mi2001} transgenic line immunostained with GFP antibody to mark Müller glia (green) and probed for GABA receptor γ2 subunit (*gabrg2*) (cyan) expression by *in situ* hybridization. (A) High magnification image taken with a 40× objective. Outlined area is magnified and shown in B. Scale bars: 50 μm. GABA$_A$: Gamma aminobutyric acid A; GCL: Ganglion cell layer; GFAP: glial fibrillary acid protein; INL: inner nuclear layer; ONL: outer nuclear layer; OPL: outer plexiform layer; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.





Additional Figure 5 Model of GABA receptor inhibition-induced proliferation.
(A) Model of neuronal circuit maintaining Müller glia quiescence. Photoreceptors (taupe) normally release glutamate which is sensed by Horizontal cells (yellow) which release GABA that is then sensed by Müller glia (green) to remain quiescent. (B) Inhibiting $GABA_A$-ρ receptors via the $GABA_A$-ρ receptor inhibitor TPMPA and/or $GABA_A$ receptors via gabazine mimics disruption of the normal circuit as would occur after damage or disease. In this model, Müller glia sense decreased levels of GABA, dedifferentiate (purple) and generate proliferating progenitor cells (red) as part of a regenerative response. Pharmacological blocking of GABA receptors induces a regenerative response in the absence of damage. GABA: Gamma aminobutyric acid; gabazine: $GABA_A$ antagonist.

Additional Table 1 Average RPKM values of GABA subunits from sequencing of sorted Müller glia

Gene name	Subunit name	Average RPKM values
Gabrr1	$GABA_A$-ρ subunit ρ1	9.665615
Gabrr2a	$GABA_A$-ρ subunit ρ2a	30.42483939
Gabrr2b	$GABA_A$-ρ subunit ρ2b	0.231149438
Gabrr3a	$GABA_A$-ρ subunit ρ3a	10.65283172
Gabrr3b	$GABA_A$-ρ subunit ρ3b	15.82386383
Gabra1	$GABA_A$ subunit α1	39.81043
Gabrb2	$GABA_A$ subunit β2	16.69505
Gabrg2	$GABA_A$ subunit γ2	36.93309

Retinas were collected from undamaged Tg(*gfap:gfp*) adult fish. Tg(*gfap:gfp*) transgenic zebrafish express GFP in Müller glia driven by the glial fibrillary acidic protein promoter (Bernardos and Raymond, 2006). Fluorescence activated cell sorting was used to enrich for populations of Müller glia from undamaged Müller glia. RNA was isolated from the cell pools and RNA-seq was performed. Read data from undamaged retinas are shown as reads per kilobase of transcript per million mapped reads (RPKM). GABA: Gamma aminobutyric acid.