Inhibition of GABA\textsubscript{A}-\textsubscript{ρ} receptors induces retina regeneration in zebrafish

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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\textsubscript{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\textsubscript{A} receptors (GABA\textsubscript{A}-\textsubscript{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\textsubscript{A}-\textsubscript{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

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\textsubscript{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\textsubscript{A} antagonist gabazine led to MG-derived proliferation in undamaged fish retinas (Rao et al., 2017). However, a second class of GABA receptors, GABA\textsubscript{A}-\textsubscript{ρ} (Blarre et al., 2014; Alexander et al., 2017)(originally referred to as GABA\textsubscript{C} receptors (Drew et al., 2017)) targets to induce mammalian retina regeneration. We previously used pharmacological and genetic methods to inhibit gamma aminobutyric acid A (GABA\textsubscript{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\textsubscript{A} receptors (GABA\textsubscript{A}-\textsubscript{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\textsubscript{A}-\textsubscript{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.

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The GABA<sub>A</sub> receptor antagonist bicuculline (Drew et al., 1984). Whether GABA<sub>A</sub>-<i>p</i> receptors might also be involved in regulating retina regeneration is unknown. The GABA<sub>A</sub>-<i>p</i> receptor was first discovered as a subtype of the GABA<sub>A</sub> receptor family that is insensitive to the GABA<sub>A</sub> receptor antagonist bicuculline (Drew et al., 1984). GABA<sub>A</sub>-<i>p</i> receptors have a similar structure to GABA<sub>A</sub> receptors in that they are both pentameric ionotropic ligand-gated ion channels (Connolly et al., 1996; Enz and Cutting, 1998; Ogurcu et al., 1999). However, the two types of receptors differ in the subunits that form the functional receptor. GABA<sub>A</sub> 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<sub>A</sub>-<i>p</i> receptors being insensitive to GABA<sub>A</sub> 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 p2a, with p1 and p3α showing significantly lower expression and even lower levels of p2b (Cocco et al., 2017). In contrast to the GABA<sub>A</sub> receptor, GABA<sub>A</sub>-<i>p</i> 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<sub>A</sub>-<i>p</i> 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)<sup>mi2001</sup> (Bernardos and Raymond, 2006) which marks mature MG and Tg(tuba1a:GFP) (Faussett and Goldman, 2006) which marks differentiated 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)<sup>mi2001</sup> 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) 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 incision was made in the sclera using a sapphire blade. A blunt-end 30-gauge needle was inserted into the vitreous incision was made in the sclera using a sapphire blade. A blunt-end 30-gauge needle was inserted into the vitreous, the amounts of which are indicated in the respective figure legends. Fiji image software 4.13 was used to process images (Schindelin et al., 2012).

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 image software 4.13 was used to process images (Schindelin et al., 2012).

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Tubular matrix-associated calcium-binding protein 1a (tuba1a), which marks mature MG and Tg(gfap:GFP)<sup>mi2001</sup> zebrafish.
polymerase chain reaction

Table 1

| Sequences (5’3’) | Product size (bp) |
|------------------|-------------------|
| 18S              | Forward: TTA CAG GCC CTC GAA AGA AGA 90 |
|                  | Reverse: AAA CGG CTA CCA CAT CCA AG 90 |
| asc1             | Forward: TGA GCC TCC GTA AAA GGA AAC T 139 |
|                  | Reverse: CGT GGT TTG CGG GTT TGT AT |
| insm1a           | Forward: CGG GGA CTT ACG AGA CAT AC 125 |
|                  | Reverse: GGG TGG GAA GCA CTG GTT TA 125 |
| sox2             | Forward: GAA AAA CAG CCC GGA CGG CAT GAC 252 |
|                  | Reverse: GTG TTG GTT TCT CGG GGT CTG TTG |
| dkk1b            | Forward: AAT GAC CCT GAC ATG ATT CAC G 213 |
|                  | Reverse: AGG CTT GCA GAT TTT GGA CC |

Inhibition of GABA_2 receptors induces proliferation in the undamaged retina

Our lab has previously shown that inhibition of GABA_2 receptor signaling induces spontaneous proliferation in an undamaged zebrafish retina (Rao et al., 2017). However, the p2a subunit of the GABA_2 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_2 receptors would also induce proliferation. For this, we injected the GABA_2 receptor antagonist TPMPA (Murata et al., 1996; Ragozzino et al., 1996) into undamaged eyes from Tg(1016tuba1a:gfp) transgenic fish and assessed proliferation using immunostaining against PCNA or by direct incorporation of EdU. The Tg(1016tuba1a:gfp) transgenic line specifically marks differentiated MG and MG-derived neural progenitors in actively regenerating retinas (Faussett and Goldman, 2006). TPMPA is a commercially available competitive antagonist of the GABA_2 receptor with only minimal effects on GABA_2 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_2 receptors compared to GABA_2 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_2 receptors. To complement the TPMPA experiments, we tested whether knocking down the p2a subunit (GABRR2a), the most abundant p 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
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morpholinos targeting the p2a subunit into undamaged retinas (Figure 2). Compared to control morpholin injections, injection of the two different morpholinos targeting the p2a 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<sub>ρ</sub>-p receptor induces a MG-based regenerative response.

Inhibition of GABA<sub>ρ</sub>-p signaling induces a regenerative response

To further test whether inhibition of GABA<sub>ρ</sub>-p 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 morpholin (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]<sub>maz01</sub> 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 (Vihelic 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<sub>ρ</sub>-p receptors by TPMPA induces expression of factors consistent with a canonical regenerative response in zebrafish.

Figure 2 | Inhibition of GABA<sub>ρ</sub>-p receptors via antisense morpholin 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 morpholin (Ctl-MO; A) or one of two independent antisense morpholinos targeting the p2a subunit of the GABA<sub>ρ</sub> 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: TOPRO-3 (A–D). 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 Ctrl-MO and n = 10 for gabrr2a-MO1 (C) or gabrr2a-MO2 (D). n = 6 for Ctrl-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. **** = 8.7 × 10⁻⁸ (C), *** = 0.0089 (D), vs. Ctrl-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<sub>α</sub>-p receptor) inhibitor TPMPA, 0.75 nmol of control morpholin (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-MO co-injection. (C) 25 nmol TPMPA/Ctl-MO co-injection. (D) 25 nmol TPMPA/ascl1-MO 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: TOPRO-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) **** = 5.8 × 10⁻¹⁰ (PBS+Ctl-MO vs TPMPA+Ctl-MO); **** = 1.2 × 10⁻¹³ (PBS+ascl1-MO1 vs TPMPA+Ctl-MO); **** = 3.7 × 10⁻¹¹ (TPMPA+Ctl-MO vs TPMPA+ascl1-MO1). (F) **** = 1.5 × 10⁻⁸ (PBS+Ctl-MO vs TPMPA+Ctl-MO); **** = 4 × 10⁻⁸ (PBS+ascl1-MO2 vs TPMPA+Ctl-MO); **** = 2.45 × 10⁻¹⁰ (TPMPA+Ctrl-MO vs TPMPA+ascl1-MO2). GFP: Green fluorescent protein; PCNA: proliferating cell nuclear antigen; TPMPA: [1,2,5,6-tetrahydroxypirin-4-y]methylphosphonic acid.

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GABA<sub>A,ρ</sub> receptors are localized to the inner and outer nuclear layers

If detection of reduced GABA<sub>A</sub> levels is mediated by MG, GABA<sub>A,ρ</sub> receptors should be expressed in MG. By RNAseq of undamaged retinas using the Tg(GFAP:GFP) mi2001 transgenic line, we found that GABA<sub>A</sub> and GABA<sub>A,ρ</sub> receptor subunits are indeed expressed in MG (Additional Table 1). Besides transcriptomic analysis, we also tested whether GABA<sub>A,ρ</sub> receptors co-localize with MG. We previously used immunostaining to show close association between GABA<sub>A,ρ</sub> receptors on MG processes flanking horizontal cell processes (Rao et al., 2017). Because antibodies against the zebrafish GABA<sub>A,ρ</sub> receptors are not available, we used in situ hybridization to localize RNAs encoding both the ρ2a and γ2 subunit (gabrr2a), 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<sub>A,ρ</sub> and GABA<sub>γ</sub> receptors

Individually, inhibition of GABA<sub>A,ρ</sub> (Figures 1 and 2) or GABA<sub>γ</sub> 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<sub>A</sub> receptor antagonist, and TPMPA. We observed a significant increase in the number of proliferating PCNA<sup>+</sup> cells compared to either treatment alone or the PBS control treatment (Figure 6).

Discussion

We previously showed that inhibition of GABA<sub>A</sub> receptors can induce retina regeneration in adult undamaged retinas (Rao et al., 2017). Here, we extend that work to show that inhibition of GABA<sub>A,ρ</sub> 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 (Faussett et al., 2008; Brzezinski et al., 2011; Jorstad et al., 2017) and knockdown of ascl1 blocks the effects of inhibition of GABA<sub>A,ρ</sub> receptors. Second, we observed the expected activation of ascl1 and insm1a (Ramachandran et al., 2012) following inhibition of GABA<sub>A,ρ</sub> 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<sub>A,ρ</sub> 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,
Gabazine/TPMPA). GABAA: Gamma aminobutyric acid A; GFAP: glial fibrillary acid protein; GFP: green fluorescent protein; | Tg(1016tuba1a:gfp) zebrafish were intravitreally injected with either 1× PBS (A), 6.25 nmol of the GABA antagonist gabazine (B), 12.5 nmol of the GABA-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 × 10−5 (PBS vs. Gabazine/TPMPA); **P = 0.0016 (Gabazine vs. Gabazine/TPMPA); ***P = 0.0006 (TPMPA vs. Gabazine/TPMPA). GABA-α: Gamma amino butyric acid α; GFAP: glial fibrillary acid protein; GFP: green fluorescent protein; PCNA: proliferating cell nuclear antigen; TPMPA: (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid.

**Figure 6** | Dual inhibition of GABAα and GABA-ρ receptors enhances proliferation.

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 GABAα and GABA-ρ receptors on bipolar cells (Connoughton 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 Ca2+ (Zhang et al., 2019) and subsequent proliferation (Pinto et al., 2015). The Ca2+ 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 Ca2+ 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-ρ receptors. While TPMPA is a highly selective antagonist of GABA-ρ receptors, it is also a weak antagonist of GABAα 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 GABAα receptors. However, the morpholino knockdown of GABA-ρ subunit ρ2a argues against this possibility and provides further support that the effect of TPMPA is through inhibition of GABA-ρ receptors. In addition, the dual inhibition of GABAα and GABA-ρ receptors provide further support that the effects are synergistic and not solely due to effects on GABAα 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, GABAα 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 GABAα receptors in regulating activation of MG in the zebrafish retina. Supporting our work in the retina, inactivation of GABAα 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. GABAα and GABA-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., 2017). 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 CoCl2 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<sub>ρ</sub> stimulate 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<sub>ρ</sub>-p receptors is sufficient to induce a regenerative response in the zebrafish retina in the absence of damage and that inhibiting both GABA<sub>ρ</sub>-p 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.

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**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.

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**Additional files:**

- **Additional file 1:** Open peer review reports 1 and 2.
- **Additional Figure 1:** Inhibition of the GABA<sub>ρ</sub>-p receptor in the absence of damage results in a proliferative response.
- **Additional Figure 2:** Dose dependent inhibition of the GABA<sub>ρ</sub>-p receptor.
- **Additional Figure 3:** Inhibition of the GABA<sub>ρ</sub>-p receptor does not result in increased apoptosis.
- **Additional Figure 4:** Localization of transcripts encoding the γ2 subunit of GABA<sub>ρ</sub> 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.

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Additional Figure 1 Inhibition of the GABA<sub>A</sub>-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<sub>A</sub>-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<sup>+</sup> cells. Each data point is a separate eye and is an average of two sections, counting all PCNA<sup>+</sup> 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 x 10<sup>-5</sup>, vs. PBS. EdU: 5-Ethynyl-2′-deoxyuridine; GABA<sub>A</sub>: 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_α-ρ receptor.

Tg(1016tuba1a:gfp) zebrafish were intravitreally injected with either 1× PBS (A) or 15 nmol of the GABA_α-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 × 10^{-9} (PBS vs. 25 nmol TPMPA); ****P = 1.8 × 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_α: 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\textsubscript{A}-\textrho receptor does not result in increased apoptosis.

Tg(10/6tuba1a:gfp) zebrafish were intravitreally injected with the indicated amounts of either PBS or the GABA\textsubscript{A}-\textrho 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\textsuperscript{+} 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\textsubscript{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\(_{\alpha\beta}\) receptors via the GABA\(_{\alpha\beta}\) receptor inhibitor TPMPA and/or GABA\(_{\alpha}\) 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\(_{\alpha}\) 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<sub>α</sub>-ρ subunit ρ1 | 9.665615 |
| Gabrr2a   | GABA<sub>α</sub>-ρ subunit ρ2a | 30.42483939 |
| Gabrr2b   | GABA<sub>α</sub>-ρ subunit ρ2b | 0.231149438 |
| Gabrr3a   | GABA<sub>α</sub>-ρ subunit ρ3a | 10.65283172 |
| Gabrr3b   | GABA<sub>α</sub>-ρ subunit ρ3b | 15.82386383 |
| Gabra1    | GABA<sub>α</sub> subunit α1 | 39.81043 |
| Gabrb2    | GABA<sub>α</sub> subunit β2 | 16.69505 |
| Gabrg2    | GABA<sub>α</sub> 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.