OPEN PEER REVIEW REPORT 1

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-19-00657
Title: Inhibition of GABAA-ρ receptors induces retina regeneration in zebrafish
Reviewer's Name: Salvatore L. Stella
Reviewer's country: USA
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COMMENTS TO AUTHORS

The authors present a manuscript focused on the neuroregenerative role of changing GABA levels in the retina, resulting in the induction of the Müller cell-derived regenerative response in zebrafish retina. The goal of this study was to determine whether activation of GABAc receptors contributes to the mechanisms that enable retinal regeneration in zebrafish. The authors in a previous study used both pharmacological and genetic methods to inhibit GABAA receptors or alter glutamate response secondarily to the GABA effect in undamaged zebrafish retinas. Initiation of retinal regeneration was measured by the dedifferentiation of Muller cells and the appearance of Muller cell derived proliferating progenitor cells. In this study, the authors attempt to demonstrate that inhibition of a pharmacologically distinct subset of GABA receptors (GABAA-ρ or GABAc receptors) can also induce retina regeneration.

The authors found that inhibiting both GABAA and GABAC receptor subtypes led to enhanced retinal regeneration in undamaged retina. Gene expression analyses indicate that inhibition of GABAA-ρ receptors induces a canonical retinal regenerative response. These findings are important in understanding how zebrafish retinal neurons regenerate and how decreased levels of GABA, like those that could be present during retinal cell death or damage, could induce dedifferentiation of Muller cels and the generation of proliferating retinal neurons. The authors took advantage of transgenic zebrafish that selectively label Muller cells and utilized in vivo studies with pharmacological agents and morpholinos targeted to both GABA receptors and regeneration driven transcription factors involved in proliferation. This is an intriguing study with a tantalizing hypothesis and uses complementary approaches to address a very compelling finding regarding the role of GABA in retinal regeneration and repair in lower vertebrates with the hopes that these clues can be someday translated to mammals and humans once the mechanism has been worked out.

For the most part the manuscript is well written. This reviewer's major concerns deal primarily with the GABA pharmacology, some technical aspects of the interpretation of the authors data, and the discussion. In addition, there are some minor points dealing with much needed clarifications within the text.

Major:

1. The pharmacology of the drugs used are at the lower ends of the IC50s for most of the drugs tested in this study, for example generally TPMPA, a classic GABAc antagonist has an IC50 in the low micromolar range (1-2 µM), but for these studies only nM concentrations were used. The authors state that they injected 0.5 µl into the eye, however was the final concentration 12.5 nM or was that the amount injected in the 0.5 ul which means that the actual amount hitting the retina is much lower. For example, did the authors inject 12.5 nM in the eyes, this would result in a much lower final concentration seen by the retina. If this is the case, the authors should provide a reasonable estimate of the final concentration seen by the retina. Thus, if the authors injected 12.5 nM, the actual final

concentration based upon the eye volume would be at least 1 log unit lower, meaning that the final concentration that the retina would receive is about 1/8-1/10th of the 12.5 nM, placing it in the low single digit nM range. Would you expect the GABAc antagonist, TPMPA to effectively inhibit GABAc receptors at this concentration? Most GABAc responses are not effectively blocked at this concentration. How do you reconcile this issue with your findings?

2. Figure 5, the authors' state on page 13 lines 3-5 (9-13) that "GABAA-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 (Fig. 5)." However, the in situ signal seems to be absent from the Muller cells or there is very little signal if any in the Muller cells which is unlike the gamma subunit which is clearly expressed in the GFP positive Muller cell bodies in Fig. S4. How do the authors reconcile this issue. My suggestion is to try the in situ on isolated Muller cells, or attempt to use one of the commercially available GABAc antibodies that were intended for other species but work in zebrafish. Also Ralph Enz has devloped a GABAc antibody that works in zebrafish (Koulen et al, J Comp Neurol. 1997 Apr 21;380(4):520-32). Remember, Yazulla and Studholme also published a zebrafish atlas (Journal of Neurocytology 30, 551-592, 2001) using most antibodies that were originally generated for mammalian epitopes in zebrafish that worked rather well. If you look at that paper the GABAc/GABAa antibodies work well in zebrafish.

3. The authors need to elaborate on the functional significance of their findings at the end of the discussion. Presently, the discussion abruptly ends with the influence of GABA and pancreatic beta-cell regulation in mouse. The focus of the study is on how activation of GABAc receptors in addition to GABAa receptors can prevent spontaneous activation of Muller progenitor cells in the zebrafish retina.

4. For the discussion, where is the GABA derived form inner or outer retina. Horizontal cells or amacrine cells, or both elaborate?

Minor issues:

1. P10, when the authors introduce the Tg(tuba1a-GFP) zebrafish they need to explain to the reader the rationale or utility of using the Tg(tuba1a-GFP) zebrafish in the text, for example, "for these experiments we used the Tg(tuba1a-GFP) zebrafish which selectively labels dedifferentiated Muller cells.."
2. Fig. 6. Strong PCNA signal is presnt in the GCL in panel C and D. Why would you expect more PCNA positive RGCs or proliferative cells in the GCL when treating with TPMPA or GABAzine? This is also apparent in Figure 2 and 3 when using the morpholinos, you see a great effect on the inner retina and the RGCs.
3. What was the total number of zebrafish used to obtain the TUNEL and PCNA data, from the test in the methods it appears that "wo non-consecutive sections were counted and averaged for each eye." From one fish? or multiple, fish? and how many? Add this information to the Materials and Methods.
4. Are the images stacks, single 2D images, maximum projection etc., and if so how many? What is the optical thickness? If it is different for each image, add that to the figure legend, if it's the same for most/all sections include that under imaging in the Materials and methods.

OPEN PEER REVIEW REPORT 2

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-19-00657
Title: Inhibition of GABAA-ρ receptors induces retina regeneration in zebrafish
Reviewer's Name: JiaJie Teoh
Reviewer's country: USA
Date sent for review: 2019-12-2

COMMENTS TO AUTHORS

I applaud the idea to examine the effect of a relatively understudied GABA receptor type (in this case, the GABAA-rho receptor), in retinal regeneration involving Muller glial (MG) cells. The authors used convincing zebrafish models that could help to distinguish mature and nascent/proliferative form of MG in response to the inhibition experiments. The authors show that specific inhibition of GABAA-rho receptor with TPMPA increases Muller glial proliferation, evidence of regeneration, in undamaged zebrafish retina. This finding is reinforced with specific blocking of receptor rho2a subunit using two different morpholinos. The authors also show the expression of GABAA-rho receptor mRNA in OPL, where the processes of Muller glial are highly concentrated. This supports the idea that Muller glia is sensitive to GABAA-rho receptor inhibition. I like the experiment showing specific inhibition of GABAA-rho receptor (using TPMPA), together with other GABAA receptor types (using gabazine, which is insensitive to GABAA-rho receptor) gives an additive effect on Muller glia proliferation. The scope of the study is clearly defined and the flow of the experimental planning is easy to understand. However, some of the references cited bring questions, especially when the focus is "regeneration in undamaged retina", and the cited references are mostly about the regeneration in injured retina.

Major comments.

1. Although the authors did not mention in text, the Gabrr2a MO1 seems to complement the base 376-400 with reference to zebrafish Gabrr2a mRNA (Genbank NM_001045376). Then, Gabrr2a MO2 complements base 333-357. However, in MO2, the first two nucleotides from 5' are mismatched. Is this a mismatch strategy or a typo? Does a 2 nucleotide 5' mismatch still allow the MO2 to block its target properly?

2. In the first section of Results, the authors performed the inhibition with TPMPA 25nmol and two morpholinos, specifically in undamaged retina. The response is encouraging where the PCNA+ MG increases after treatment. There is no induction of injury mentioned throughout the manuscript, so I presumed the authors focus only on undamaged retinal regeneration. However, in the second result section, the authors used Ascl1a, Insm1a, Let-7a, dkk1b expression levels to measure regeneration response. These proteins increase only after retina injury, as shown in multiple references cited by the authors. For example, Ramachandran et al.(2011) reported Ascl1a induction is injury-dependent. In uninjured retina, GSK-3Beta was sufficient to induce MG dedifferentiation. Ramachandran et.al. (2012) also reported Isnm1a expression after retina injury.

-Why did the authors decided to use Ascl1a instead of GSK-3Beta? Can the authors provide references relating Ascl1a, Insm1a, Let-7a, dkk1b expression level to uninjured retina? Elsaeidi et al. (2018) might help.

This brings up another question. Do the authors consider TPMPA injection or morpholino injection as

a mode of injury induction? In Fig.1C, PBS injection doesn't seem to induce injury regeneration response. The use of references quoting results from damaged retina further complicated this matter (i.e. the sentences before Fig.4). Do the authors have results from uninjected retina to tell whether if the PBS injection indeed shows the baseline PCNA+ cell number? If the PCNA+ cell number is even lower in uninjected retina, incision with a sapphire blade and injection could be a mode of injury. Then, using injury dependent markers sounds more reasonable.

-I noticed the authors also tested Sox2 with reference to the works of Gorsuch et al. (2017). This is the only reference that shows result in uninjured retina (and it is in zebrafish). Gorsuch et al. (2017; Fig.6) showed PCNA+ cells increases following Sox2 overexpression in uninjured retina. What appears to contradict the current manuscript is that Gorsuch's wt control retina with basal Sox2 level doesn't have much PCNA+ cells. The authors showed in Fig.4b of current manuscript, Sox2 level in TPMPA-treated retina is similar to the basal level in untreated control. However, these TPMPA-treated retinas showed more PCNA+ cells in Fig.1. Can the authors discuss this matter further in Discussion with relevant references? i.e. elaborate on why the authors think it is related to timing and why result from different time points is not included in this study?

3. In Fig.5A, the authors did a great job showing the localization of GABAA-Rho receptor in retina using in situ hybridization. It is the protein that does the job. Because the target of detection is RNA, two different mRNAs localize at a same spot is not a definitive way to correlate whether their protein transcripts will stay together. Can the authors provide supporting reference about this method?

Minor comments.

Introduction,

1. The authors mentioned "…for unknown reasons, MG-derived regeneration is blocked in mammals". Ross Poche lab recently published a paper [Rueda EM et al. (2019)], showing evidence that it might be related to hippo pathway.
2. Most of the references cited are not in zebrafish and are decades old. They are a range of molusk, nematode, mammalian studies.
3. Cocco et al. 2017 studied p3a not p3.
4. The scope of this work did not cover sustainability of regeneration.