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Glycine inhibition on-off directionally selective retinal ganglion cells.

Madeline C Mitchell
madeline.mitchell@louisville.edu

Maureen McCall Ph.D
maureen.mccall@louisville.edu

GLYCINE INHIBITION OF ON-OFF DIRECTIONALLY SELECTIVE RETINAL GANGLION CELLS

Madeline Mitchell and Maureen McCall Ph.D

Department of Anatomical Sciences and Neurobiology, University of Louisville

Abstract

Retinal ganglion cells (RGCs) are a group of over 40 different types that use both GABAergic and Glycenergic inhibition. There are four different types of Glycine receptors (GlyRs) known as GlyRα1, GlyRα2, GlyRα3, and GlyRα4 (Sanes & Masland, 2015; C. Zhang & McCall, 2012). Electrophysiological data has supported the theory that the RGC cell type On-Off directionally selective (ooDS) cells express both GlyRα4 and GlyRα2. If ooDS cells do express only GlyRα4 and GlyRα2, an immunohistochemical analysis of these cells should support this theory. By comparing the expression of GlyRα4 and GlyRα2 in Glra4$^{-/-}$, Glra2$^{-/-}$ Glra4$^{-/-}$, and Glra2$^{-/-}$ the expression we can support or weaken the findings made by previous electrophysiological data. Comparing cell images of colocalized dendrites and GlyRα4 or GlyRα2 in an original and randomized orientation can determine if an immunohistochemical analysis confirms the presence of GlyRα4 and GlyRα2 on ooDS RGCs. Our findings confirmed that both GlyRα4 and GlyRα2 are expressed on ooDS RGCs.

Introduction

Retinal Ganglion cells (RGCs) are responsible for receiving information from the bipolar and amacrine cells and transporting this information to the brain (Sanes & Masland, 2015; C. Zhang & McCall, 2012). Ganglion cells have been grouped morphologically by their dendritic ramification patterns in the inner plexiform layer ON and OFF sublaminae, soma diameter, dendritic field size, and branch patterning (C. Zhang & McCall, 2012). There are around 40 RGC types and each utilizes both GABA and glycine receptors. (Sanes & Masland, 2015; C. Zhang, Nobles, & McCall, 2015). Glycine receptors are pentameric ligand-gated chloride ion channels. A typical glycine receptor contains one type of GlyRβ subunit and one of four different types of GlyRα-subunits. These are known as GlyRα1, GlyRα2, GlyRα3, and GlyRα4 (Weiss et al., 2008). As a whole, glycinergic inhibition modulates the light responses of retinal neurons and plays a role in creating specificity, preferentially by crossover inhibition (Wassle et al., 2009). The pattern of expression of each of the GlyRs differs across the inner plexiform layer (IPL) and there is a significant amount of GlyRα subunit diversity across currently identified RGCs (Nobles et al., 2012; Wassle et al., 2009). WT ON/OFF direction selective (ooDS) cells respond preferentially to movement in a preferred direction. There are four types of ooDS cells , each encoding for motion in one of the four cardinal directions (Im & Shelley, 2016). OoDS cells are one of the most common RGCs and represent about 20% of all RGCs in the mouse retina (Sanes & Masland, 2015). Current electrophysiological data has indicated that ooDS RGCs should express GlyRα2 & GlyRα4 (C. Zhang et al., 2014; Haverkamp et al., 2003; Haverkamp et al., 2004). By staining for specific GlyRs, we should find WT ON/OFF direction selective (DS) RGCs express GlyRα2 & GlyRα4 as indicated by electrophysiological data. By comparing the immunohistochemical analysis of GlyRs ON/OFF directional selective (DS) we can strengthen or weaken findings made previously using electrophysiological data.

Table 1: Primary and Secondary Antibodies used for Immunohistochemistry

Primary Antibodies	Concentration	Host	Manufacturer
anti-Lucifer Yellow	1:1000	rabbit polyclonal	Life Technologies
anti-GlyRα2	1:50	goat polyclonal	Santa Cruz Biotech
anti-GlyRα4	1:100	rabbit polyclonal	Chemicon
Secondary Antibodies	**Concentration**	**Host**	**Manufacturer**
anti-goat IgG Cy3	1:200	donkey polyclonal	Jackson ImmunoResearch
anti-rabbit IgG 488	1:200	donkey polyclonal	Jackson ImmunoResearch
streptavidin Cy2 or 633	1:200	*conjugate*	Life Technologies
Hoechst nuclear stain	1:1000	*conjugate*	Life Technologies

Methods and Materials

Animals

For this experiment mice of the TRHR and PVCre/Thy1STP mouse lines were used. In the TRHR and PVCre/Thy1STP mouse lines, some ON/OFF DS ganglion cells express yellow fluorescent protein (YFP), which glows green under the right fluorescent light. This makes the cells easier to find and target. TRHR and PVCre/Thy1STP mice were crossed and backcrossed to Glra2$^{-/-}$, Glra4$^{-/-}$ and Glra2$^{-/-}$4$^{-/-}$ to obtain GlyR global knockout models along with labeled RGCs. All experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research and with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Louisville.

Immunohistochemistry

RGCs from pieces of mouse retina were filled with Lucifer Yellow and neurobiotin, fixed in 4% paraformaldehyde for 12 minutes, washed three times with 0.01 M phosphate buffer saline (PBS), and placed into a 24-well plate. For 60 minutes the retina pieces were incubated in blocking solution consisting of PBX (0.5% Triton-X 100 in PBS) and 10% normal donkey or goat serum. Each retina piece was then reacted with a combination of primary antibodies to stain for the either GlyRα2, GlyRα4, or a combination of the two GlyRαs, and Lucifer yellow overnight at 4°C (Table 1). The primary antibodies were washed off by three washes with PBX. A combination of secondary antibodies and Hoechst stain in normal serum was added to label the primary antibodies and it was left on the tissue overnight at 4°C. The Hoechst stain was used as a label for DNA in the somas and distinguish between the retina nuclear and plexiform layers. The tissue was washed with PBS one last time and mounted onto a slide and covered using VECTASHIELD (Vector Labs, Burlingame, CA) clear mounting medium and a coverslip.

Confocal Imagery and Colocalization Analysis

The filled RGCs were imaged, using an Olympus Fluoview FV1000 confocal microscope with Fluoview software (Olympus, Tokyo, Japan) with a 60x oil immersion objective (NA 1.4). Depending on the size of the dendritic field 3-5 images were taken of each cell, taking care to capture as much of the dendritic field as possible without capturing the soma. Images of ooDS RGC dendrites and GlyR puncta were deconvolved, using constrained iterative deconvolution in cellSens (Olympus, Tokyo, Japan). Using the Imaris (Bitplane, Zurich, Switzerland) surfaces and distance transformation tools, coincident GlyR puncta (color channel 1) on or within the dendritic processes (color channel 2) were counted. The length of the dendritic processes within the image was measured using the filaments tool. The coincident puncta divided by the dendritic length estimated puncta density.

To determine which puncta were colocalized onto the dendrite, the puncta channel (channel 1) was flipped along the x axis and on the y axis and coincident GlyR puncta were recounted. Then, the flipped or “random” (RAN) puncta density was subtracted from the “original” (ORI) puncta density and the result estimated the “corrected” puncta density.

The ORI and RAN puncta density counts were compared using a paired one-tailed t-test.

Results

OoDS RGCs Express GlyRα4-Positive Puncta

To assess the expression of GlyRα4 positive puncta on the ooDS RGC dendrites we compared the original puncta density to an estimate of random puncta coincidence. The average ooDS GlyRα4-positive puncta density was 1.05± 0.31 puncta·µm^{-1}, which was significantly more than the random GlyRα4-positive puncta density, which was 0.82 ±0.24 puncta·µm^{-1} (Figure 1) $p < 0.05$ (paired one-tailed T-test). When corrected for our estimate of randomly associated GlyRα4-positive puncta, the corrected density of GlyRα4 puncta expression on ooDS RGCs was 0.23±0.04 puncta·µm^{-1}. This corrected GlyRα4-positive puncta value suggest positive expression of GlyRα4 on ooDS RGCs.

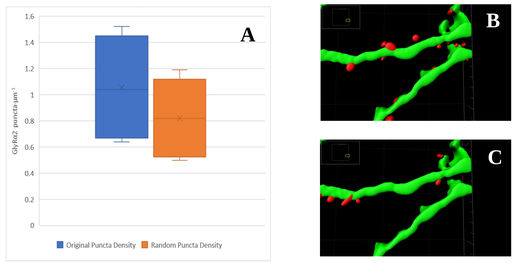

Figure 1: OoDS RGCs Express GlyRα4: A) Dendrites on ooDS RGCs have a significantly higher GlyRα4 puncta density co-localization than coincidence co-localization in the flipped (RAN) orientation. B) GlyRα4 puncta on ooDS RGC dendrites in original orientation. C) GlyRα4 puncta on ooDS RGC dendrites in randomized orientation.

OoDS RGCs Express GlyRα2-Positive Puncta

We also assessed GlyRα2 positive puncta on the ooDS RGC dendrites and compared the original density to an estimate of random puncta coincidence. The average ooDS RGC GlyRα2-positive puncta density was 0.92±0.73 puncta·µm^{-1}. This was significantly more than the random average GlyRα2-positive puncta density, which was 0.72±0.69 puncta·µm^{-1} (Figure 2) $p < 0.05$ (paired one-tailed T-test). When corrected for our estimate of randomly associated GlyRα2-positive puncta, the corrected density of GlyRα2 puncta expression on ooDS RGCs was 0.21±0.05 puncta·µm^{-1}. The similarity between the GlyRα2-positive puncta and chance puncta coincidence suggests GlyRα2 expression is present ooDS RGCs.

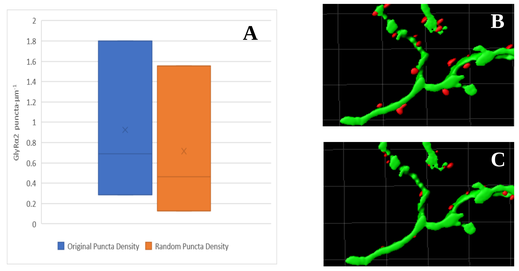

Figure 2: OoDS RGCs Express GlyRα4: A) Dendrites on ooDS RGCs have a significantly higher GlyRα2 puncta density co-localization than coincidence co-localization in the flipped (RAN) orientation. Min and max lines not available due to small sample size B) GlyRα2 puncta on ooDS RGC dendrites in original orientation. C) GlyRα2 puncta on ooDS RGC dendrites in randomized orientation.

Discussion

The established immunohistochemical approach we used to identify GlyR puncta expression was able to positively identify GlyRα2 and GlyRα4 on ooDS RGCs. This supports the conclusion that GlyRα2 and GlyRα4 are both expressed on ooDS RGCs. Our immunohistochemical data combined with previous electrophysiological data provides strong support of the theory that ooDS RGCs that these cells express GlyRα4 and GlyRα2.

The expression of GlyRα4 and GlyRα2 on ooDS RGCs leads us to believe that it is likely that GlyRα4 and GlyRα2 play an important role in modulated inhibition in ooDS RGCs.

Only 3 cell images were used to determine GlyRα2 expression and 5 cell images were used to determine GlyRα4 expression. While the number of cell images we were able to incorporate into our experiment is not ideal, we were limited in our ability to gather more data by the COVID-19 pandemic. Repeating the experiment with a larger sample size would increase our confidence in the results of the experiment.

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