LONDON'S GLOBAL UNIVERSITY

A Closer Look: Investigating the role of synaptic diversity in function of retinal neurons

Thesis presented to University College London for the degree of Master of Philosophy (M.Phil)

2022

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Abstract

Retinal ganglion cells (RGCs), the final step in the retinal circuit, relay visual information to the brain. In mouse retina there are 46 genetically distinct RGC types, with each type encoding a distinct aspect of the visual world. While contributions from electron microscopy and single cell transcriptomics have greatly improved our knowledge of RGC type diversity, the physiological function and visual processing significance of many of these cells is yet to be determined. Morphological, molecular, and physiological diversity have been at the forefront of determining cell-type categorization. However, the diversity of excitatory synapses across cell types has not been explored in the same degree of detail. Visual signal propagating through the retina has a relatively slow time course – significantly slower than the time course of excitatory transmission associated with activation of AMPARs or NMDARs. This could imply little functional necessity for varying AMPAR subunit composition between retinal cell classes -- perhaps explaining why diversity of synaptic receptors has been studied less extensively by retinal neurobiologists. Recent single cell transcriptomics has identified mRNA variation in both AMPAR subunits and their auxiliary proteins across RGCs. Elsewhere in the CNS, diversity in AMPAR composition has been widely studied and the regulation of AMPAR composition has been shown to be important in processes including normal fast transmission, plasticity, neurological disorders and excitotoxicity. Much remains to discover about AMPARs and their modulatory proteins in retinal physiology. In this study, we have focused on the auxiliary AMPAR subunit γ-3 in four α-ganglion cell types. Previous studies show a preferential expression of γ-3 in ON-S-α RGCs, but not OFF-S-α RGCs, making it an ideal target for

better understanding the impact of TARPs in the retina. We have examined their post-synaptic currents and light responses in wild type mice and transgenic animals lacking γ-3. ON-S-α RGCs, but not OFF-S-α RGCs, show differences in AMPAR kinetics in γ-3 KO mice. γ-3 markedly slows AMPAR kinetics, causing a delay in the time to the initial spike in response to light stimuli, demonstrating potentially significant functional implications for AMPAR diversity in RGCs. Through quantifying the post-synaptic AMPAR currents in different RGC types, we aimed to reveal another piece of the visual processing puzzle and gain greater understanding of the role of AMPARs in generating light responses.

Impact Statement

Retinal ganglion cells (RGCs) consolidate the light responses generated in the retina and transmit this vital information for later interpretation by the visual cortex. In mice, 46 distinct RGC types have been classified in terms of physiological, functional, and molecular differences. However, the underlying diversity in the synapses of different cell types have yet to be explored.

Excitatory synapses are the fundamental communication unit between neurons, and alteration in the glutamate-type AMPA receptors and NMDA receptors that mediate communication is a key mechanism that which neurons shapes transmission and information processing throughout the CNS. TARPs are AMPAR regulatory proteins which impact the function and expression of AMPARs in synapses. Two particular TARPs, γ-3 and γ-5, have received limited attention since neurons often co-express these together with other TARPs, making it difficult to determine their role in neuronal function. In this respect, retinal ganglion cells present a unique opportunity as mRNA sequencing data suggests preferential expression of γ-3 and γ-5. Electrophysiological experiments in whole-mount retina can preserve the entire circuit and allow use of light stimuli to mimic natural physiological conditions. The present study aimed to provide a greater understanding of RGC diversity, and to cast further light on the role of γ-3 and γ-5 TARPs.

Declaration

I, Brittany Sincox, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Brittany Sincox

July 2022

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Abbreviations

AC	antagonist cocktail
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP5	D-2-amino-5-phosphonopentanoate
BC	bipolar cell
CGCs	cerebellar granule cells
ChAT	choline acetyltransferase
CI	calcium impermeable
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CON A	concanavalin A
CP	calcium permeable
CTD	C-terminal domain
CTZ	cyclothiazide
DS	direction selective
EPSC	excitatory post-synaptic current
GCL	ganglion cell layer
INL	inner nuclear layer
IPL	inner plexiform layer
IS	isradipine
KA	kainate

KO	knock out mouse model
LBD	ligand-binding domain
LTD	long term depression
LTP	long term potentiation
mEPSC	miniature excitatory post synaptic current
mRNA	messenger ribonucleic acid
NBQX	2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
NMDAR	*N*-methyl-D-aspartate receptors
NTD	N-terminal domain
OFF-S-α	off-sustained-α ganglion cell
OFF-T-α	off-transient-α ganglion cell
ON-S-α	on-sustained-α ganglion cell
ON-T-α	on-transient-α ganglion cell
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	orientation selective
PSD	post-synaptic density
qPCR	quantitative polymerase chain reaction
RGC	retinal ganglion cell
R*/rod/s	Rod isomerization per rod per second
sEPSCs	spontaneous excitatory post synaptic currents

STG	stargazin
TARP	Transmembrane AMPAR regulatory protein
TMD	transmembrane domain
TTX	tetrodotoxin

Chapter 1: Introduction

Retinal neurons increase in diversity as signal is propagated through the mouse retina. Light responses begin from one of three photoreceptors and ends at one of 46 RGC cell types. Retinal ganglion cells (RGCs) are the final circuitry element in the retina, and their axons form the optic nerve which transmits essential visual information to the brain. Understanding ganglion cell physiology is essential in understanding our overall visual function. Massive strides have been made in identifying the 46 diverse types of RGCs, and in defining their morphology (Bae et al., 2018), and many aspects of their physiology (Goetz et al., 2022), and genetic compositions (Tran et al., 2019). Their synaptic diversity, however, has yet to be studied in the same level of detail. One reason synaptic diversity is widely studied within the CNS, is because of the importance of dysregulation of synaptic proteins like AMPAR in neurological or neurodegenerative diseases. This same level of attention to synaptic proteins has not yet been applied to retinal neurons and could be invaluable for understanding the type of information encoded by healthy RGCs, and how this changes in disease states.

AMPARs play a central role in excitatory neurotransmission, and their fast kinetics and trafficking are a key factors in central synaptic plasticity (Chater & Goda, 2014; Derkach et al., 2007). Numerous studies have investigated AMPAR diversity in many brain regions, but little is known about the AMPAR diversity present at excitatory synapses in RGCs. Studies have shown differences in calcium permeable (CP-) and calcium impermeable (CI-) AMPARs in different RGC populations (Jones et al., 2012, 2014; Wen et al., 2018). NMDAR subunit specificity and localization in RGC synapses

has also been demonstrated (Zhang & Diamond, 2009). However, most of these studies generalize RGCs into either ON, OFF, or ON/OFF cells. These three subclasses do not encompass the vast diversity of mouse RGCs. To understand the importance of AMPAR diversity and its role in RGC function, it would be useful to compare the AMPAR mediated excitatory currents in specific RGC cell types.

Transmembrane AMPAR regulatory proteins (TARPs) are AMPAR auxiliary proteins that alter gating and expression of AMPARs in excitatory synapses in the central nervous system. TARPs have been widely examined to better understand how AMPARs are regulated - with many studies focusing on their role in fast transmission and plasticity. RGCs in adult mammalian retina have not been shown to exhibit LTP or LTD, but exhibit other forms of plasticity via glutamatergic ion channel regulation at their synapses (Jones et al., 2012). It is hoped that investigating the role of TARPs in RGCs will provides a useful step in understanding their AMPAR regulation.

1.1 Anatomy of the Retina:

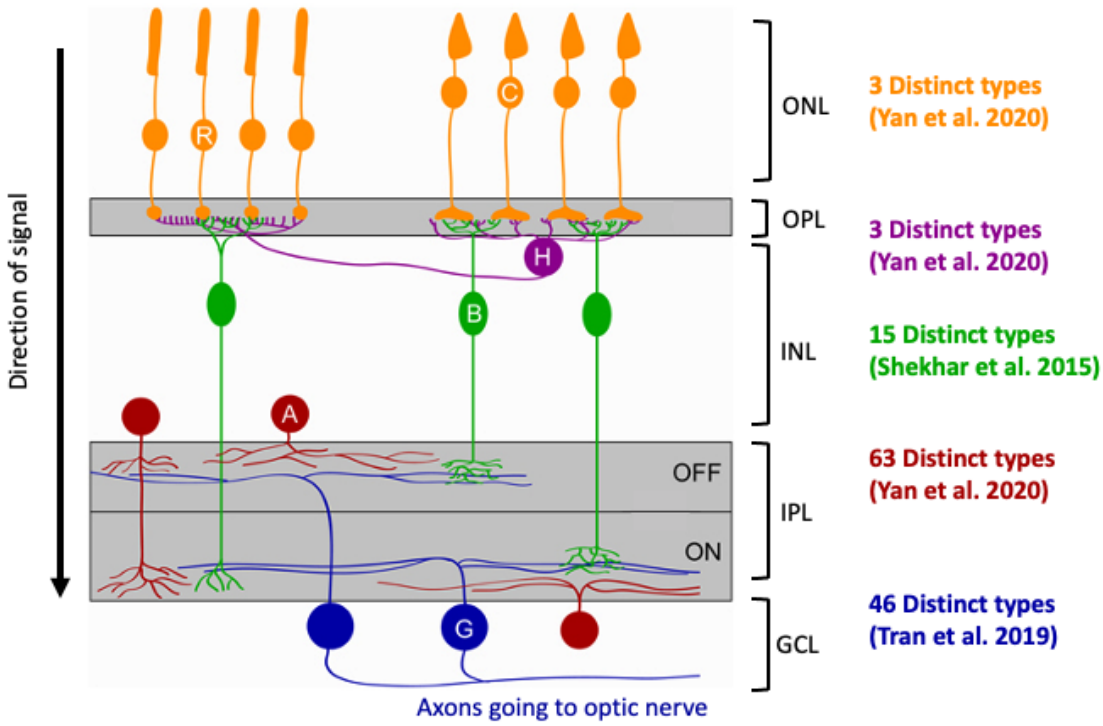


Figure 1: A schematic diagram of the retina modified from Soto and Kerschensteiner, 2015 including the number of genetically distinct cell types in mouse retina observed in recent single-cell transcriptomics studies. Rod (R) and cone (C) photoreceptors (orange) are shown in the outer nuclear layer (ONL) and there are 3 distinct cell types (Yan et al., 2020). The photoreceptors form synapses with 3 distinct horizontal cell types (H, purple) and 15 distinct bipolar cells types (B, green) (Shekhar et al., 2016) in the outer plexiform layer (OPL). 63 distinct amacrine cell types (red) (Yan et al., 2020) and bipolar cell bodies are found in the inner nuclear layer (INL) and form synapses with ganglion cells (G, blue) in the inner plexiform layer (IPL) which is divided into ON and OFF ganglion cell dendrite stratification layers. Finally, 46 distinct ganglion cell types (Tran et al., 2019) and their axons which project to the optic nerve are in the ganglion cell layer (GCL).

The retina forms a complex network comprised of five main neuron cell classes(Figure 1). The excitatory retinal circuit begins from the photoreceptors, which are able to transform energy (photons) into an electrical signal that is transmitted to bipolar cells, which later form synapses onto RGCs. Amacrine cells and horizontal cells provide lateral inhibitory pathways which shape the overall signal received by RGCs and transmitted to the optic nerve (Diamond, 2017).

1.2 Dimensions of RGC Diversity

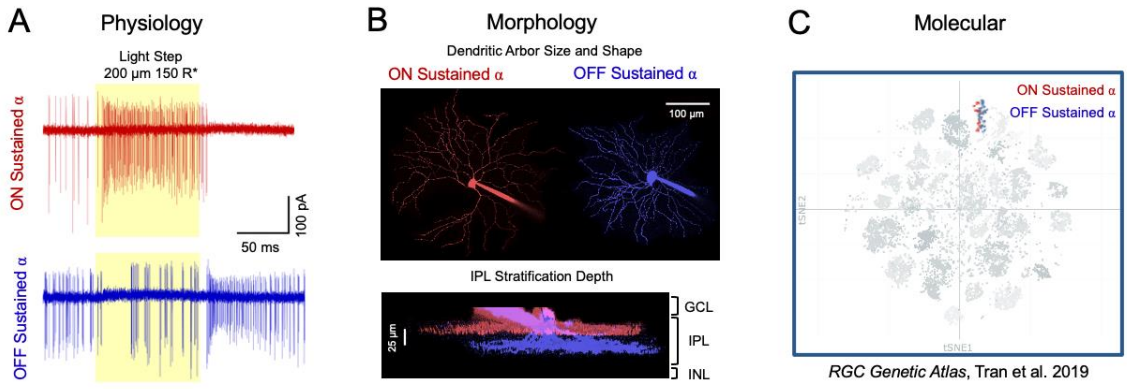


Figure 2: Example of RGC diversity. (A) Extracellular recordings of action potentials in ON-S-α (red) and OFF-S-α (blue) RGCs. 200 µm spots of light at 150 R*/rod/s (R*) intensity are shown from darkness, and the cell's responses highlight the physiological differences between RGC cell types. (B) Alexa-488 filled RGCs were imaged to show the distinct dendritic morphology of ON vs. OFF RGCs. The top image shows the similarities in size and shape of their dendritic arbors. The bottom image overlays these two cells to highlight the differences in dendritic stratification depths in the IPL. (C) A tSNE plot (Tran et al. 2019) statistically groups cells based on the similarities of single-cell mRNA sequencing results. This experiment revealed 46

genetically distinct RGC cell types in mouse retina. ON-S-α (red) and OFF-S- α (blue) clusters are highlighted.

In mouse retina, more than 40 RGC cell types have been classified based on physiological (Baden et al., 2016), morphological (Bae et al., 2018), and molecular differences (Tran et al., 2019). Functionally, RGCs can transmit image forming or non-image forming information based on cell type. ON cells respond to light increments or constant light, OFF cells respond to light decrements or constant darkness, and ON/OFF cells exhibit both responses (Figure 2A). Additionally, there are orientation selective (OS) and directionally selective (DS) RGC cell types in which responses are dependent on the direction and orientation of the stimuli (Hubel & Wiesel, 1968). Non-image forming intrinsically photosensitive-RGCs are involved in necessary functions like pupillary constriction and photoentrainment (S.-K. Chen et al., 2011). Morphologically, many RGC cell types can be differentiated based on a variety of factors including the size of their somas, size of their dendritic arbors, or which layers of the IPL its dendrites are stratified (Figure 2B) (Bae et al., 2018). Molecularly, recent studies using mRNA sequencing data have shown that distinct molecular differences across RGC types can play key roles in neuroprotection against excitotoxicity, and this dataset can also be used by scientists to inform hypotheses about other molecular differences as well (Figure 2C) (Tran et al., 2019). Diversity in RGCs is necessary to encode the variety of visual stimuli received for later interpretation in the brain. While great strides have been made in better understanding the nuance and complexity in RGC diversity, the individual synaptic differences between cell types have much left to be discovered.

1.3 Excitatory Neurotransmission in RGCs

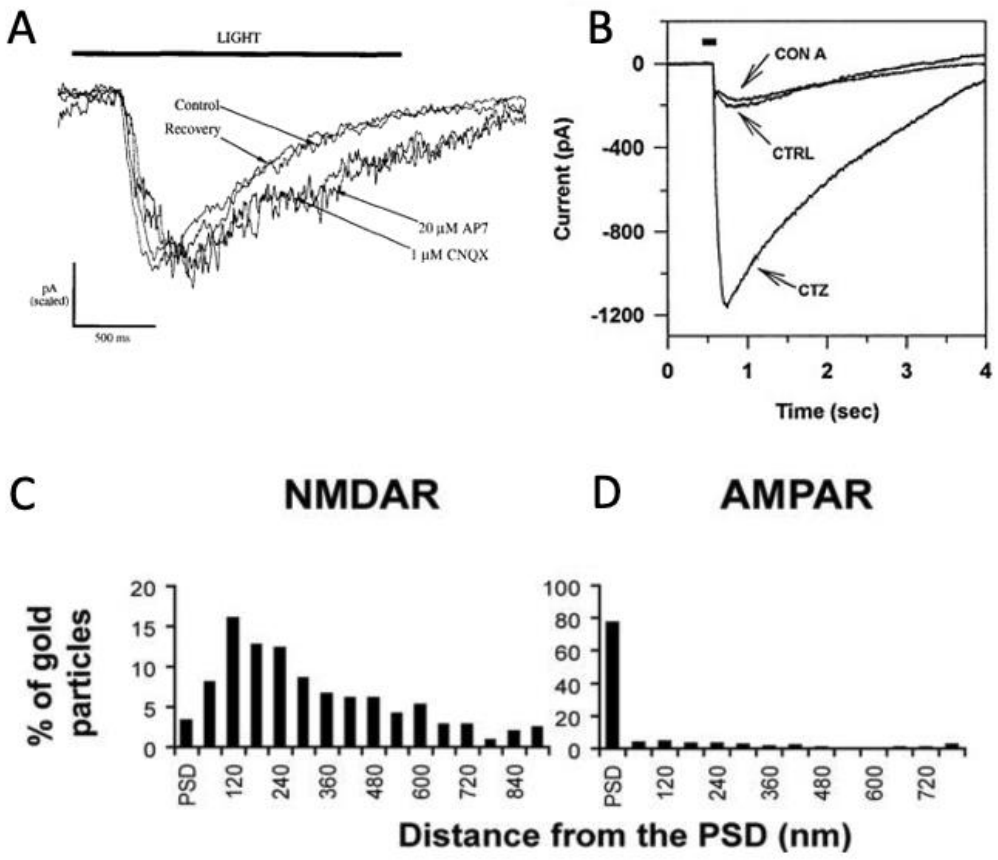


Figure 3: (A) Whole-cell voltage clamp recording of an ON Ganglion cell (Diamond & Copenhagen, 1993). NMDAR receptor antagonist AP7 isolated non-NMDAR mediated EPSCs. AMPAR/Kainate receptor antagonist CNQX was used to isolate NMDAR mediated EPSCs. (B) Glutamate-evoked currents in a whole-cell voltage clamp recording (Lukasiewicz et al., 1997). 300 µg/mL concanavalin A (CON A) and 30 µM cyclothiazide (CTZ) were used to isolate AMPA-preferring and Kainate-preferring receptor responses. (C) Histograms showing the location of immunogold labelling of NMDARs and AMPARs in RGC synapses show preferential expression of AMPARs in the PSD and NMDARs in peri-synaptic locations (Zhang & Diamond, 2006).

Excitatory synapses in RGCs utilize NMDARs, GluA2-lacking CP-AMPARs, GluA2-containing CI-AMPARs, and few (if any) KA receptors to mediate excitatory neurotransmission (Figure 3a, 3b) (S. Chen & Diamond, 2002; Diamond & Copenhagen, 1993; Lukasiewicz et al., 1997). In dark-adapted retinas, the time course of NMDAR and AMPAR mediated EPSCs are similar due to the slow "envelope" style release from presynaptic BCs (Figure 3a).

In rat retina, AMPARs in RGCs are located immediately beneath the presynaptic active zone while NMDARs are expressed mainly in perisynaptic regions (Figure 3c). As such, the mEPSCs of RGCs in these retinas are mediated mainly by AMPARs, while greater excitatory activation leading to a "spillover" of neurotransmitter is mediated by both classes of receptors (S. Chen & Diamond, 2002; Sagdullaev et al., 2006; Zhang & Diamond, 2006, 2009). A similar type of 'AMPAR only synapse' has also been identified in the cerebellum (Clark & Cull-Candy, 2002). RGCs are presynaptically partnered by at least one, or more usually multiple, types of BCs which release glutamate at BC-RGC synapses. The number of synapses formed by each BC type varies, leading to "weighted" inputs of excitatory activity for different BC types (Dunn & Wong, 2014; Masland, 2012).

1.4 Structure and function of TARPs and AMPARs

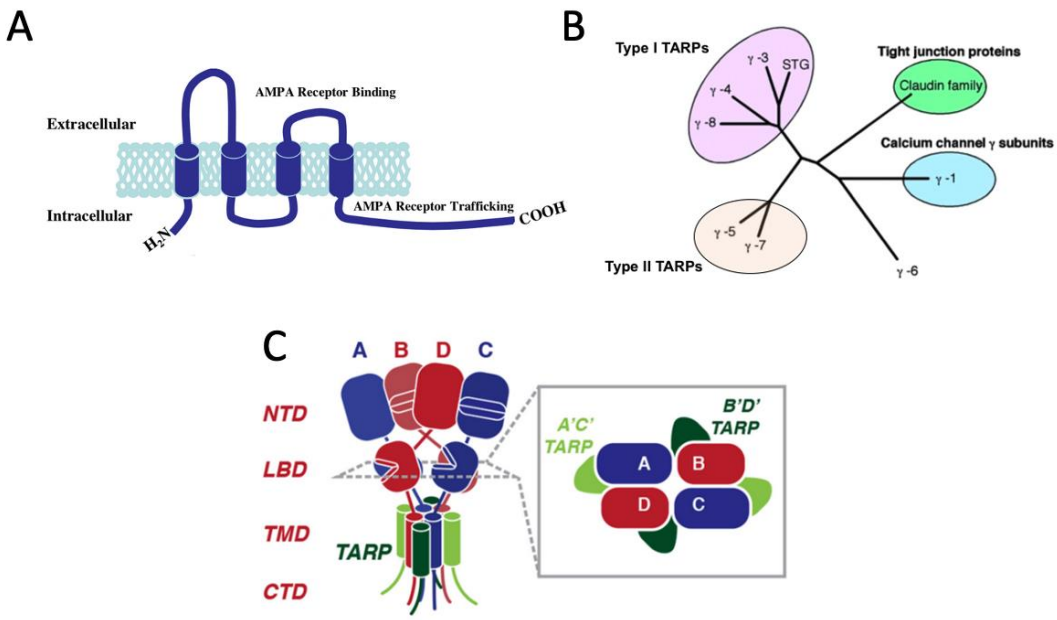


Figure 4: A brief overview of the structure, function, and phylogenetic history of TARPs Modified from Payne 2007, Herguedas et al. 2019, and Tomita et al. 2003. (A)The general TARP structure is a transmembrane protein comprised of 4 α helices, with an extracellular loop and a long intracellular tail at the C-terminus which is associated with AMPAR trafficking. (B) The calcium channel γ subunits are described in a phylogenetic tree to display their structural similarities based on a common ancestor. (D) A schematic of TARP and AMPAR association. The AMPAR (red, blue) is shown to contain 4 domains, and a top-view shows the arrangement of a hetero-tetrameric AMPAR with four TARPs attached at the transmembrane domain.

AMPARs are glutamate receptors composed of four subunits which form an ion channel. They can form heterotetramers, containing two or more different types of GluA(1-4) subunits, or homomers containing one type of GluA subunit. Each individual subunit is comprised of an extracellular N-terminal domain (NTD) and

ligand-binding domain (LBD), a transmembrane domain (TMD), and an intracellular C-terminal domain (CTD) (Figure 3). The subunit composition of AMPARs influences channel gating and ionic properties. GluA2-lacking AMPARs are CP while RNA editing of GluA2 subunits changes a glutamine in the pore-lining region to a much larger, positively charged arginine, rendering GluA2-containing AMPARs CI (Pellegrini-Giampietro et al., 1997; Sommer et al., 1991). The most common composition of an AMPAR in the brain is a heteromeric CI-AMPAR containing GluA2, and either GluA1 or GluA3 subunits (Lu et al., 2009). However, this differs widely in different brain regions and cell classes.

TARPs are AMPAR auxiliary subunits which have varying effect on AMPAR trafficking, receptor pharmacology, and gating kinetics. TARPs are small proteins comprised of 4 α helices and an intracellular C-tail (Figure 4a) (Greger et al., 2017; Payne, 2008). The six TARPs can be divided into three distinct groups: γ-2 and γ-3 are Type 1a TARPs, γ-4 and γ-8 are Type 1b TARPs, while γ-5 and γ-7 are Type 2 TARPs. These three TARP groups have distinct protein structure differences (Figure 4b) (Tomita et al., 2003) as well as distinct AMPAR modulatory differences which are later described in chapter 1.5. An AMPAR-TARP complex can have anywhere between 1-4 associated TARPs, and the number of associated TARPs appears impact the extent of AMPAR modulation in a "dose-dependent" manner. Thus, many studies have focused on understanding the possible compositions of AMPAR-TARP complexes (Greger et al., 2017). Up to four γ-2 or γ-3 proteins can assemble in AMPAR-TARP complexes, but only a maximum of two γ-4 TARPs can (Hastie et al., 2013). The number of γ-5, γ-7, and γ-8 proteins that can associate in AMPAR-TARP complexes have yet to be examined using single molecule counting and remains unclear, but functional data can

inform hypotheses about these three TARPs in AMPAR-TARP complexes (Cull-Candy & Farrant, 2021, p.; Greger et al., 2017). Cryo-EM studies have provided further insight into AMPAR-TARP structure complexes, and more specifically how the TARP structures interact with AMPARs to modulate their function (Twomey et al., 2016; Zhao et al., 2016). It has been inferred that the extracellular loop on γ-2 TARPs interacts with the LBD region of AMPARs based on functional studies (Dawe et al., 2016; Payne, 2008), but these Cryo-EM studies did not indicate this type of interaction and rather inferred stronger interactions between TARPs and the TMD region in AMPARs. There is still much left to be discovered about AMPAR-TARP complexes, and hopefully further studies in TARP-TMD interactions can reveal more functional details.

Many neuronal cell types will have overlapping TARP expression, and this redundancy can be protective to maintain proper AMPAR function (Menuz et al., 2008, 2009). As such, most TARP KO mice – with the exception of γ-2 – generally have little gross phenotypic change (Bissen et al., 2019). However, not all cells express every TARP, and TARP types are differentially distributed in the brain. γ-2 has highest expression levels in the cerebellum, γ-3 in the forebrain, γ-4 in the prefrontal developing brain, and γ- 8 in the hippocampus (Fukaya et al., 2005; Tomita et al., 2003). This subsequently allows for even more diversity in the composition of AMPARs in excitatory CNS synapses. However, this pattern is an oversimplification as neurons in most brain regions studied appear to express more than one type of TARP.

1.5 How do TARPs modulate AMPARs?

The prototypical auxiliary subunit, stargazin (TARP γ-2) was first discovered from experiments on cerebellar granule cells (CGCs) in γ-2 lacking (stargazin) mice(Letts et al., 1998). Type 1 TARPs were first categorized by their ability to rescue AMPA currents in stargazin CGCs. Thus, it was determined that γ-2, γ-3, γ-4, and γ-8 proteins acted as AMPAR auxiliary subunits and increased AMPAR expression at the cell surface (Tomita et al., 2003). Type 1 TARPs increase expression of AMPARs by improving trafficking with PSD-95-like-MAGUKs (L. Chen et al., 2000) and prevent the diffusion of AMPAR in the membrane by aiding in anchoring at synapses (Bats et al., 2007). They also have been shown to increase AMPAR responses to glutamate(Yamazaki et al., 2004) in addition to decreasing desensitization and prolonging channel opening(Kott et al., 2007, 2009; Tomita et al., 2005). More recently, the Type 1 TARPs have been further divided into Type 1a (γ-2, γ-3) and Type 1b (γ-4, γ-8). While both sub-groups have similarities in the way they modulate AMPARs, Type 1b TARPs tend to have a greater impact on AMPAR kinetics than their Type 1a counterparts (Greger et al., 2017).

The effects of type 2 TARPs are less well understood compared with type 1 TARPs. Previous studies on CGCs investigating the role of γ-7 in CGCs suggest that it selectively enhances the expression of synaptic CP-AMPARs, while suppressing CI-AMPARs (Studniarczyk et al., 2013). γ-5 was not originally thought to be a TARP and was thus used as a negative control by some previous work (Tomita et al., 2004; Turetsky et al., 2005). It was later found that γ-5 is highly expressed in Bergmann Glia (Fukaya et al., 2005), a cell type containing only CP-AMPARs(Iino et al., 2001), and was found to function as a TARP by modulating CP-AMPAR currents (Soto et al., 2009).

However, γ-5 does not appear to control trafficking of CI-AMPARs since it failed to rescue AMPA currents in stargazin CGCs (Tomita et al. 2003). This is due to a difference in the C-terminal motif responsible for PDZ binding which is highly conserved in type 1 TARPs (Bats et al., 2007; Kato et al., 2007). Overall, there is still much left to be discovered about the role of type 2 TARPs in AMPAR modulation.

1.6 Do AMPARs participate in synaptic plasticity in RGCs?

The fast trafficking rates and modulation of AMPARs are key components of synaptic plasticity in the brain (Chater & Goda, 2014; Derkach et al., 2007). Certain neurons can induce synaptic plasticity via AMPARs by regulating subunit composition, the number of AMPARs present, phosphorylation state, or the auxiliary subunits associated with them (Sprengel, 2006). There are numerous studies covering synaptic plasticity, most notably in the hippocampus, but there are many glutamatergic synapses where there is no clear evidence for receptor mediated plasticity. Currently, there is no substantial evidence to suggest that LTP and LTD, which are the most widely studied forms of synaptic plasticity in the hippocampus, exist in adult mammalian retina. However, studies have shown specificity of CP and CI-AMPAR subunit composition in different RGC populations in addition to chemical and light-induced NMDAR-mediated plasticity of CP-AMPARs (Jones et al., 2012). NMDAR subunit specificity and localization in RGC synapses has also been demonstrated (Zhang & Diamond, 2009). In cultured RGC neurons, OFF and ON/OFF RGC types showed increased CP-AMPAR expression in high pressure glaucoma-mimicking environments, while ON-A RGCs were resilient to CP-AMPAR increase (Wen et al., 2018). Although these examples differ from “classical” forms of synaptic plasticity,

they support the idea that cell-specific glutamatergic receptor regulation occurs at RGC synapses.

1.7 Single cell-RNA sequencing reveals preferential expression of γ-3 and γ-5 TARP mRNA in RGCs

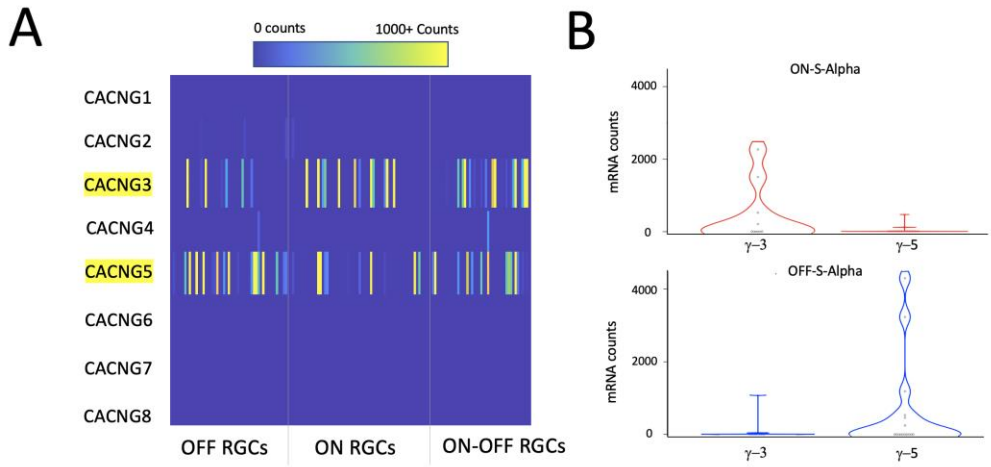


Figure 5: (A) Single-cell RNA sequencing data of genes CACNG(1-8) found from a database created in Goetz et al. 2022. Sequencing data is reported using a color grading scale, where each vertical line corresponds to an individual cell. Cells have been grouped together into OFF, ON, and ON-OFF RGCs in the x-axis. >1000 counts indicate a high level of mRNA expression in a cell. (B) Violin plots displaying mRNA counts of γ-3 and γ-5 TARPs in ON-Sustained-α and OFF-Sustained-α RGCs.

Genes CACNG(1-8) were scanned for mRNA expression in the single-cell sequencing dataset created in Goetz et.al. 2022. This study obtained mRNA sequencing data from individual RGCs that were first identified using light typing procedures, allowing us to understand functional information of the sequenced cells. CACNG1 and CACNG6, genes associated with non-TARP proteins with similar

structures to TARPs, were used as negative control. A small number of retinal ganglion cells were found to express CACNG2 (γ-2) and CACNG4 (γ-4), whereas CACNG7(γ-7) and CACNG8 (γ-8) showed no expression. However, genes corresponding to γ-3 and γ-5 TARPs were preferentially expressed in RGCs as shown in Figure 5A. Thus TARPs γ-3 and γ-5 were identified as ideal targets for TARP expression studies in RGCs. Further mRNA analysis indicates no obvious pattern for which TARP is selectively expressed in a specific cell type based on a generalized functional assessment of ON, OFF, or ON/OFF cells. However, when grouped into narrower sub-sections of α-RGCs, an expression pattern appeared. ON-S-α RGCs showed high expression of γ-3 and low expression of γ-5, the inverse being true for OFF-S- α RGCs(Figure 5B). This either-or expression pattern could have underlying behavioral impacts in these cells but needs further electrophysiological analysis to determine.

1.8 Role of α-RGCs in mouse retinal function

Currently, 4 types of α-RGCs have been identified in mouse retina and have distinct light responses: ON-Sustained, ON-Transient, OFF-Sustained, and OFF-Transient. Their visual signals have large receptive fields, low levels of surround inhibition, are highly sensitive, and are not direction selective. Additionally, their action potentials have a distinctly faster time course compared to other RGCs (Krieger et al., 2017; Pang et al., 2003). α-RGCs are morphologically characterized by their relatively large somas, neurofilament expression, and large mono-stratified dendritic arbors. Each α-RGC stratifies in a different IPL layer, allowing for easy morphological distinction from each other (Bae et al., 2018). α-RGC are a highly sensitive type of RGC. They can detect light from darkness when only a few photons are present (Ala-Laurila et al., 2020; Ala-

Laurila & Rieke, 2014; Smeds et al., 2019), and can even detect shadows in the dimmest of light conditions when only a few photons are missing from the visual field (Westö et al., 2021). This heightened sensitivity and robust ability to identify these cells have made them ideal for studying retinal function, yet little is known about their individual synapses.

1.9 What is known about the role of γ-3 TARPs in AMPAR function

γ-3, like other type 1 TARPs, have been shown to modulate AMPAR kinetics by increasing single-channel conductance and increasing duration of single channel openings (Shelley et al., 2012; Tomita et al., 2005). γ-3 has high expression levels in the cerebral cortex (Tomita et al., 2004) and potentially in RGCs (as shown in Fig 5) (Goetz et al., 2022). While γ-3 KO mice show no gross phenotypic changes or impairments to AMPAR trafficking, combination knockouts of TARPs such as γ-3/γ-2 double KO mice die shortly after birth, suggesting that γ-3-only KO mouse functionality may be preserved due to the presence of multiple TARPs in many neuronal cell types (Menuz et al., 2009). γ-3 may have some role in development due to high expression levels at birth which decrease overtime (Tomita et al., 2003), but whether it serves a unique overall role is still uncertain. Many studies have focused on neurons in the brain. These cells often have co-expression of other Type 1 TARPs including γ-2 and γ-8 which can compensate and restore AMPAR function in the absence of γ-3, thus impeding the ability to use functional methods to identify the role of this protein. The retina, however, presents a unique opportunity since a number of RGCs may have sole expression of γ-3, and retinal function in these knockout mice has yet to be explored in detail.

1.10 Project Aims

Previously collected single-cell RNA sequencing of ganglion cells show a trend with γ-3 and γ-5 TARP expression in the retina. Previous research suggested cell-specific regulation of AMPARs in RGC synapses, and current experiments suggested specific TARP expression in the retina. Therefore, this project aimed to identify the role of γ-3 TARP in the retina due to the availability of KO mouse models. Our electrophysiological studies confirmed the presence of γ-3 in ON-S-α RGCs, and that γ-3 containing AMPARs have a slower kinetics than γ-3 lacking receptors. Furthermore, we aimed to determine if the loss of this TARP resulted in profound changes in overall retinal function due to changes in AMPAR expression. While we are unable to confirm the role of γ-3 in wider physiological function of ON-S-α RGCs, we have observed changes in light responses that would be of interest to explore behaviorally in the future.

Chapter 2: Methods

2.1 Mouse Retina Preparation

All electrophysiology experiments were performed on whole-mount adult mouse retinas. Wild-type C57BL/6J mice were used as controls. γ-3 knock-out mouse were obtained from JAX laboratories (JAX stock #005781). Mice were dark adapted for a minimum of 4 hours and all dissections were performed in darkness using IR equipment. Each retina was detached from the pigmented epithelium and segmented into dorsal and ventral sections before being mounted to a poly-lysine coated coverslip (Corning, Corning, NY) for stabilization. Retinal pieces were stored in darkness in an external solution of 290 mOsm AMES Medium (US Biologicals, Salem, MA) pH 7.3 at 30°C until experiments began.

2.2 Electrophysiology

Patch-clamp recordings were obtained using 4-6 MΩ pipettes (World Precision Instruments, Sarasota, FL) pulled on a two-stage horizontal puller (Zeitz Instruments, Planegg, Germany). In cell-attached recordings, pipettes were filled with AMES Medium (290 mOsm, pH 7.3) used in the external solution bath. Whole-cell patch pipettes contained an internal solution of 120 mM $CsMeSO_4$, 14 mM Tris-phosphocreatine, 6 mM TEA-Cl, 5 mM HEPES, 4 mM Mg-ATP, 2 mM NaCl, 2 mM EGTA, 2 mM QX-314-Br, 0.4 mM Na-GTP (mOsm 285, pH 7.3). Whole-cell recordings were made at a -70 mV holding potential (-60 mV programmed on MultiClamp software but corrected for a junction potential of -10 mV).

Excitatory post-synaptic currents (EPSCs) were recorded in AMES solution containing an Antagonist Cocktail (AC) comprised of 100 µM hexamethonium bromide, 50 µM D-AP5, 10 µM SR-05531, 1 µM strychnine, and 1 µM TTX. 5 µM and 7 µM concentrations of L-type calcium channel blocker, Isradipine (Is), were added to decrease presynaptic activity of retinal bipolar cells. 50 µM NBQX was added in addition to AC at the end of recordings. Recordings were sampled at 10 kHz and filtered at 4kHz (Bessel Filter in Multiclamp software). 3 mV test pulses were included at the start of every 10 second epoch.

2.3 Analysis of mEPSCs

Whole cell recordings were imported into a customized analysis software (IGOR Pro; WaveMetrics, Lake Oswego, OR) and a standardized threshold of two times the standard deviation of the first derivative was used to initially screen for events. All detected events were manually sorted until each cell had a minimum of 200 individual mEPSCs. Any recordings that did not contain at least 200 usable mEPSCs were discarded. Any mEPSCs that did not correspond to an individual presynaptic release event or whose rise/decay times were not measurable by the software were discarded.

20-80% Rise times, decay times, and amplitudes were analyzed by an algorithm within the custom Igor program as previously described (Diamond & Jahr, 1997). For comparative analysis, cumulative probability histograms with 25 bins were used to allow each cell to have equal weight in comparison regardless of the number of events collected in each individual cell.

2.4 Immunohistochemistry

In whole cell recording, a 5 MΩ glass pipette with an internal solution of 120 mM $CsMeSO_4$, 14 mM Tris-phosphocreatine, 6 mM TEA-Cl, 5 mM HEPES, 4 mM Mg-ATP, 2 mM NaCl, 2 mM EGTA, 2 mM QX-314-Br, 0.4 mM Na-GTP, and 1% Neurobiotin (mOsm 285, pH 7.3; to fill target cells). Retina tissue was immediately fixed for 15 minutes using 1X PBS containing 4% PFA.

γ-3 and γ-5 antibodies were purchased from LS-Bio. The γ-3 antibody targets the N-terminus of the protein, and the γ-5 antibody targets aa 150-200. Both of these aa regions are located intracellularly. Whole-mount mouse retinal tissue was isolated and fixed using a 1X phosphate-buffered solution (PBS) containing 4% paraformaldehyde and blocked for 1.5 hours using 10% normal donkey serum (NDS). Both primary antibodies were applied in a (1:400) ratio and incubated for 5 days at 4°C in 1X PBS with 0.1% triton to allow the antibodies to penetrate deep into the tissue. Secondary antibodies were then applied for 2 days in the same conditions as the primary antibodies with the addition of (1:400) 488-conjugated streptavidin binds to neurobiotin and identifies the recorded cell. Tissue was imaged using a Zeiss LSM 800 confocal microscope. Retina slices were stained with TARPs in addition to combinations of RNA-binding protein with multiple splicing (RBPMS) to identify ganglion cells (Rodriguez et al., 2014), the neurofilament SMI-32 to identify α-ganglion cells (Feng et al., 2015), or used ChAT-Cre Td-Tomato mice to identify IPL layers.

2.5 IHC Image Analysis

IHC image acquisition was completed in Zen 2 on a Zeiss LSM 800 microscope. Neuron tracing was completed in Neutube (Feng et al., 2015) and images were then analyzed in FIJI (Schindelin et al., 2012).

Chapter 3: Project Hypothesis

mRNA sequencing data indicate a preferential expression of γ-3 TARP in ON-S-α, but not OFF-S-α RGCs. However, there is no existing evidence that γ-3 is present in these synapses and are associated with AMPARs. Therefore, our working hypothesis was that, if γ-3 is present in RGC synapses and associated with AMPARs, the mEPSC responses in γ-3 lacking mice might be changed in ON-S-α, but not OFF-S-α RGCs. If a lack of γ-3 causes a change in mEPSCs, then the characteristic light responses within ON-S-αs might change as a result.

Chapter 4 Results: Comparison of mEPSCs in two RGC cell types

4.1 Use of light-typing protocols and morphology analysis to identify RGCs

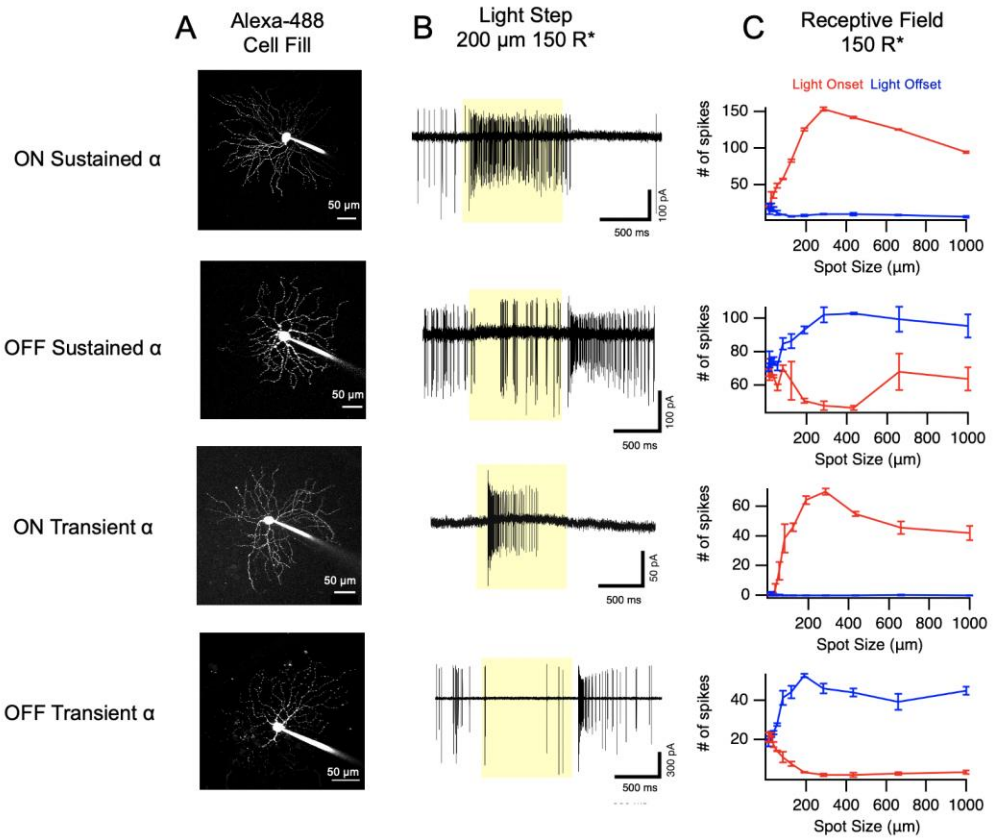


Figure 6: Identifying RGCs in Whole-Mount retina. A. α-RGCs have relatively large somas and dendritic arbors that help distinguish them from the other 46 RGC types. Cells are filled with Alexa-488 during whole-cell recordings and imaged to confirm morphology post-experiment. B. 200 µm spots of light at 150 R*/rod/s (R*) intensity are recorded in cell-attached patch clamp to categorize the large RGC into one of four α-RGCs. Each α-RGC has a distinct firing pattern that can easily distinguish types. C. A receptive field test is completed by showing 10-1000 µm spots of light at 150 R*/rod/s intensity and the number (#) of spikes after light onset (red) or offset (blue) are counted. α-RGC are typically characterized by large receptive fields with relatively low surround inhibition, meaning that relatively high spike rates can still

be seen at the cell's preferred spot side and large spot sizes. Cells with high surround inhibition, by contrast, will have little to no spiking at large spot sizes.

The first step in the RGC-typing workflow is to exclusively target the relatively large somas that are characteristic of α-RGCs. To further distinguish, each α-RGCs has distinct firing patterns that depend on cell type. In ON cells, the main characteristic is an increased firing rate in response to light increments, and the opposite is true for OFF cells. All experiments are performed in darkness using 200 µm 150 R*/rod/s spots of light, which is a mesopic light range that activates both rods and cones. A light spot can initially identify whether a cell is ON or OFF and sustained or transient (Figure 6b). ON/OFF cells exhibit both properties, where they may increase their firing rate at both light onset and light offset making them easily filtered out in the typing protocol. However, some non-α-RGCs are direction selective (DS) or orientation selective (OS), meaning that they respond best to light that is orientated or moving in a specific direction. Therefore, light stimuli that can create moving light bars and change light orientation direction are needed to distinguish these cells from α-RGCs (not shown). Finally, a receptive field test is performed that shows a range of spot sizes from 10-1000 µm to see both center and surround responses. If the cell shows weak surround inhibition, a high spike rate at light onset or offset, and is non-DS we can feel confident in the correct identification of an α-RGC.

Within the α-RGCs, the different cell types were identified based on cell-attached light step responses. Thus, ON-S and ON-T were distinguished from one another by the fact that ON-T cells exhibit a short duration of spiking with little to no baseline spiking. On the other hand, ON-S typically spiked during the entire light stimulus and occasionally

have a baseline firing rate even in darkness. OFF-S and OFF-T-α RGCs can occasionally be difficult to distinguish since OFF-T-α cells with a high baseline firing rate can appear sustained if not further examined. In these cases, the cell is distinguished post-experiment using dendritic stratification distance from the soma as later described in Figure 7. These distinct characteristics measured by cell-attached and whole-cell patch clamp recordings allowed us to feel confident in our cell-typing for future analysis of mEPSCs.

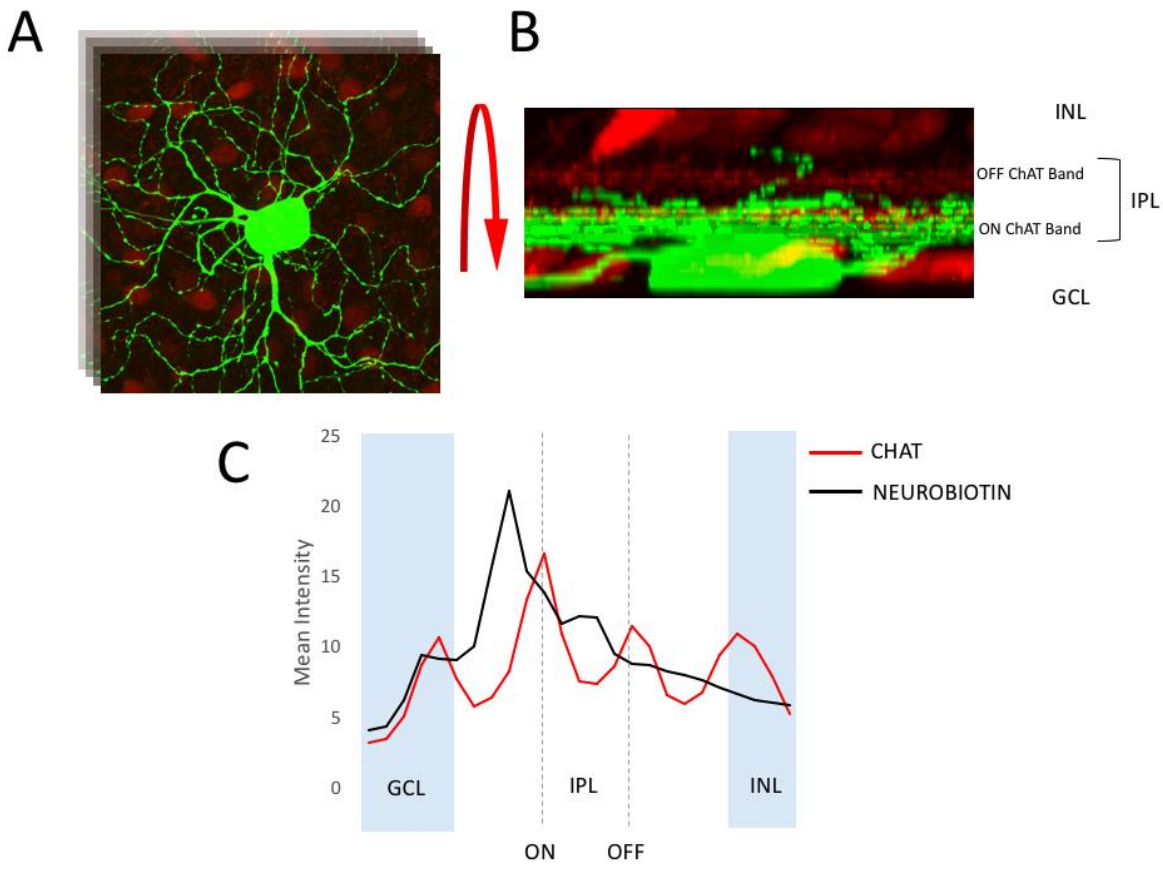


Figure 7: Morphological analysis of a fixed and stained ON DS RGC, post recording. (A) The cell was filled with neurobiotin (green), and a z-stack image was taken to visualize dendritic stratification. ChAT positive cells (red) are included to later confirm morphology in view B. (B) The Z stack was rotated to view the stratification

of dendrites into the IPL. Two distinct "ChAT bands" are shown in red and labeled to add clarity to dendritic stratification. The ChAT positive cells create anatomical landmarks that divide the IPL into majority ON RGC dendrites and majority OFF RGC dendrites. (C) Intensity levels of both ChAT and neurobiotin plotted against each other to show the location of neurobiotin signal in relation to the corresponding ChAT bands. The cell's dendritic arbor is located mainly in the space between the GCL and ON ChAT band, which is morphologically characteristic of an ON RGC.

Although electrophysiology is a powerful tool for identifying RGCs, with over 40 types of RGCs in the retina, we considered additional confirmation of cell type would be useful given that RGC types differ in soma size, dendritic arbor size, and stratification depth in the IPL. In Figure 7, the potential ON DS cell was viewed in a Z-projection to confirm morphology. The majority of the neurobiotin was found in the ON ChAT band, further suggesting that this was an ON-Type RGC. A few of the dendrites can be seen to project into the OFF ChAT band, which is a characteristic feature of DS and OS cells. By utilizing the structure references available in the Eyewire Museum (Bae et al. 2017), the neurobiotin fills were further compared for typing confirmation. A similar analysis can be completed to further confirm the four cell types of interest in this study in situations where light typing protocols alone make it difficult to draw conclusions on the exact RGC type being examined. For example, some OFF-T-αs with high baseline spike rates can be difficult to differentiate from OFF-S-αs based on cell-attached light response recordings alone, but OFF-S-αs characteristically stratify in a different IPL layer than OFF-T-αs. Thus, knowledge of the dendritic stratification depth was helpful for cell typing post-recording in these cases.

4.2 Isradipine can successfully isolate mEPSCs in RGCs

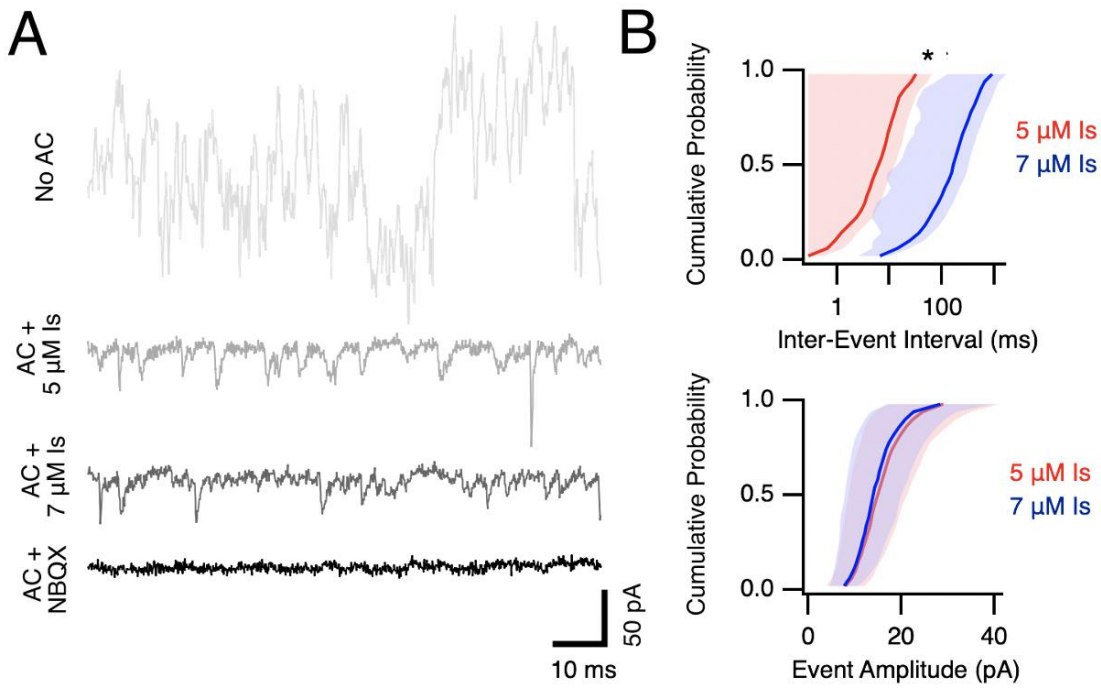


Figure 8: (A) Whole cell voltage recording of an OFF-S-α RGC at -60 mV in darkness. AMPAR currents were isolated using an antagonist cocktail (AC) comprised of 100 µM hexamethonium bromide, 50 µM D-AP5, 10 µM SR-05531, 1 µM strychnine, and 1 µM TTX. In AC only recording conditions, presynaptic activity was too high to reliably measure mEPSCs (not shown). Two concentrations of calcium channel blocker, Isradipine (Is), were used to find the ideal concentration for recording mEPSCs. (B) Cumulative probability histograms of the inter-event intervals and the event amplitudes in both isradipine concentrations. Cumulative probability histograms were compared using a Wilcoxon Rank Test. Inter-event interval analysis showed statistically significant differences ($p < 0.05$).

Miniature excitatory post-synaptic currents (mEPSCs) measure post-synaptic responses from the spontaneous release of small quantities of excitatory

neurotransmitters pre-synaptically. By blocking all non-AMPAR receptors in the post-synaptic terminal, AMPAR currents can be isolated and quantified in whole-cell voltage clamp. Bath application of tetrodotoxin (TTX) is typically a critical component in isolating mEPSCs. TTX is a sodium channel blocker that eliminates spiking which allows for pre-synaptic single vesicle release. However, bath application of TTX has little effect on the EPSCs of RGCs since many pre-synaptic bipolar cells are non-spiking neurons. Therefore, use of TTX alone is unable to reduce pre-synaptic vesicle release enough to measure quantal events. We needed to design an approach that would allow us to measure mEPSCs without relying solely on TTX. Instead, we have used the L-type calcium channel blocker, isradipine, to achieve these quantal events. mEPSCs were successfully recorded in whole cell voltage clamp using isradipine to isolate release of single transmitter packets onto RGCs (Figure 8a). Increased isradipine concentration reduced frequency of mEPSCs, which were blocked in the presence of AMPAR antagonist, NBQX. Initially, 3 µM and 5 µM isradipine concentrations were used (not shown) but found that 3 µM isradipine could not sufficiently reduce presynaptic activity in all targeted RGCs. To confidently conclude that 5 µM isradipine allowed for the recording of mEPSCs, we needed to significantly reduce the inter-event interval while not changing the average amplitudes to determine if these events corresponded to the release of single quanta. 5 µM and 7 µM Isradipine were added serially which both reduced the event frequency to a level that allowed the isolation of clear and well defined single events. 7 µM Is allowed for a significant increase in inter-event interval (Figure 8b) but saw no change in the average event amplitude across these two concentrations, indicating that RGC mEPSCs could be successfully recorded using both 5 µM and 7 µM isradipine. 5 µM was used in subsequent

experiments as the recordings could be completed in a shorter period of time, resulting in better recording quality and avoiding a decrease in health of the cells over time.

4.3 γ-3 slows AMPAR kinetics in ON-S-α RGCs

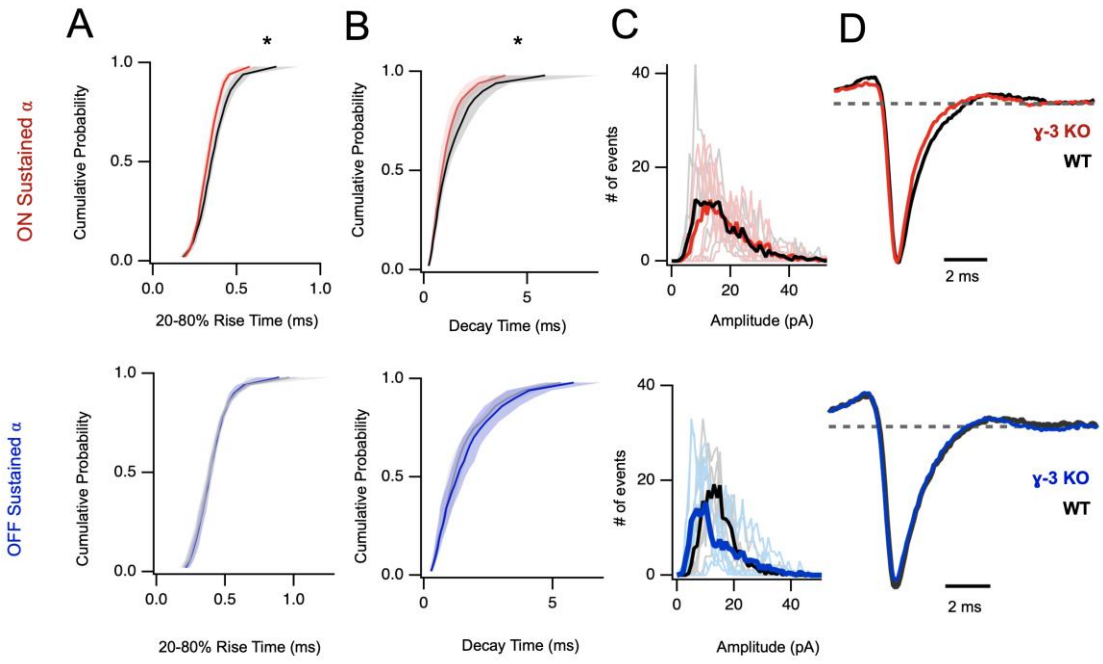


Figure 9: (A) Analysis of mEPSCs in RGCs. ON-S-α cells (n=8), but not OFF-S-α (n=7), showed a decrease in the 20-80% rise time and (B) decay times in γ-3 KO conditions. Rise and decay times were compared using Wilcoxon Rank Tests and found statistical significance if $p < 0.05$. (C) All event amplitudes were compiled into histograms, which showed an unchanged average amplitude in both cell types. (D) The average mEPSCs of all cells are overlayed, showing that γ-3 KO mEPSCs are faster in ON-S-α, but not OFF-S-α RGCs. The average KO mEPSC is normalized to the WT amplitude to emphasize kinetic changes.

mEPSCs in ON-S-α and OFF-S-αs were recorded in an antagonist cocktail with 5 µM Isradipine as previously described in WT and γ-3 KO mice. Events were isolated using

a standardized threshold of two times the standard deviation of the first derivative. Events were then manually filtered and rise times, decay times, and amplitudes were measured. We predicted that ON-S-As, but not OFF-S-αs, would have changes in their mEPSCs due to the expression of ɣ-3. We found that in ON-S-αs, but not OFF-S-αs, ɣ-3 lacking AMPARs had significantly faster rise times (Figure 9a) and decay times (Figure 9b) but showed no change in their average amplitude (Figure 9c). This suggests that in the WT, the presence of ɣ-3 allows for a greater charge transfer to occur in response to a quantal event. It should be noted that in the mEPSC average waves, there is a slight increase in baseline prior to the event start (Figure 9d). This was due to some events that arose arising shortly after the decay of a previous event, leading to a disrupt in the average baseline. Given that ON-S-αs are known for their high sensitivity to light onset, we wanted to see if this change in AMPAR response times affected the light response of these cells.

4.4 γ-3 KO ON-S-α RGCs have decreased spike latency

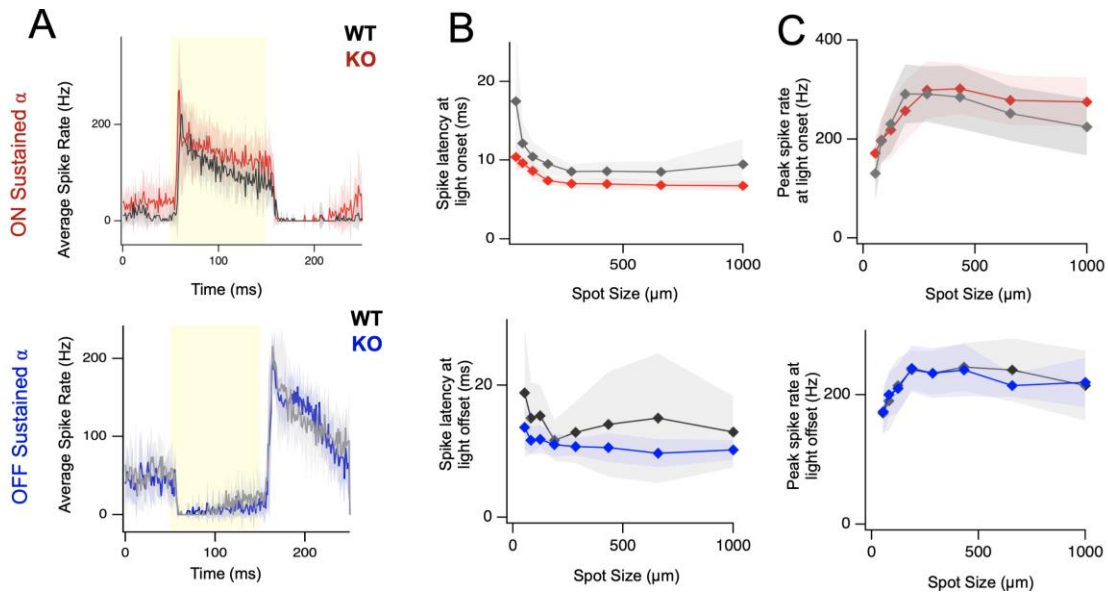


Figure 10: (A) Cell-attached recording in response to a 284 μm 150 R* spot of light shown as the average spike rate across all cells. (B) Spike latency is defined as the time to reach 1⁄2 maximum spike frequency. γ-3 KO mice showed a decrease in spike latency across all light spots in ON-S-α (n=11), but not OFF-S-α (n=7) RGCs in a Wilcoxon Rank Test (significant difference if p < 0.05). (C) The peak spike rate of each cell is defined as the maximum spike frequency at light onset or offset, which was unchanged in γ-3 KO mice in both cell types.

Utilizing the data from the typing protocol described in Figure 6, we wanted to see what physiological changes occurred in γ-3 lacking RGCs. Unfortunately, the time course of our stimulus was shorter than needed to determine baseline firing rate and steady state firing rates of these two cell types. However, we are able to examine other factors that are important to the sensitivity of these cells: spike latency – the time it takes from light onset to first spike -- and peak firing rate. We found that in

ON-S-αs, the ɣ-3 lacking mice had significantly faster spike latency, which was unexpected since the total charge transfer of AMPARs in these cells was reduced in the absence of ɣ -3. This could be caused by upstream changes in the retinal circuit due to the global knockout of ɣ-3. Recent studies that created amacrine cell mRNA databases reveal high expression of ɣ-3 across most amacrine cell types. However, amacrine cells also provide excitatory input to OFF-S-αs, which showed no change in their spike latency.

Chapter 5 Results: We were unable to determine colocalization of TARPs in RGC dendrites by means of ɣ-3 and ɣ-5 antibodies

5.1 Quantifying the puncta expression of on RGC dendrites

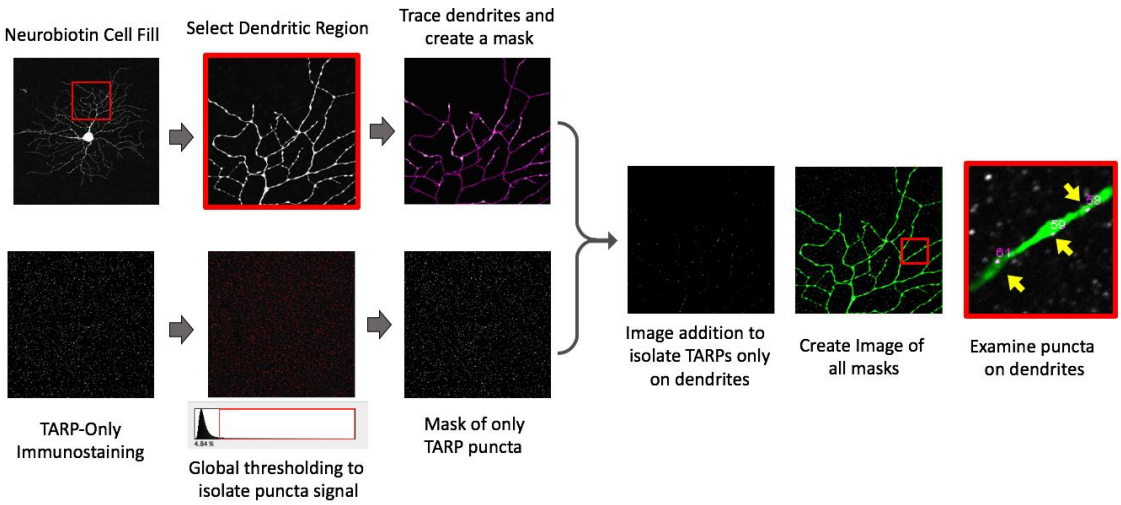
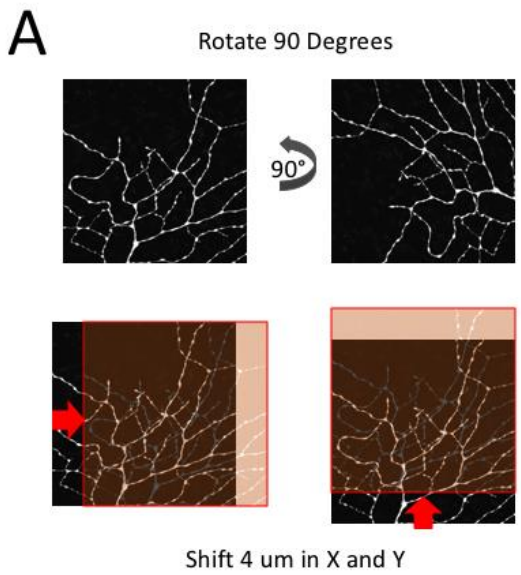


Figure 11: Analysis of γ-3 TARP colocalization on dendrites using FIJI analysis software. Neurobiotin cell fills were first isolated, traced, and filled to create a mask of only a specific dendritic region. TARP-only images were then thresholded using a conservative global thresholder to isolate puncta signal from secondary fluorescent noise. An image calculator removed all TARPs that were not expressed within the cell mask, and the remaining puncta were individually verified for expression on dendritic regions.

In order to determine if a TARP was present on a Neurobiotin cell fill, the cell regions of interest were traced in Neutube (Feng et al., 2015) and filled in FIJI to create a mask. TARP-only images were globally thresholded to remove 97% of the total signal. This conservative thresholding method was used to ensure that the signals seen were only

the brightest puncta response. FIJI's image calculator was used to create a new image containing only TARPs that expressed within the cell mask. A 3D Image counter in FIJI was applied to the subtracted image and provided statistics on the number of puncta found the corresponding size. Puncta that corresponded to ~1 μm^3 and above in size were then counted. All of the original images plus the newly identified puncta were overlaid to examine puncta distribution on any part of the cell.



B

Image Orientation	# of puncta found
Original	134
Rotate 90	172
4 um shift right	152
4um shift down	148

Figure 12: Further puncta analysis to assess the specificity of TARP-dendrite colocalization. (A) Dendritic mask images were manipulated via rotation or shift in X and Y planes, and the previously described puncta count analysis in Figure 11 was performed. (B) The number of puncta in the dendritic mask manipulations were compared to the original identified puncta.

In order to verify if puncta colocalized with RGC dendrites, mask manipulations were performed as shown in Figure 12. This analysis was used to assess the specificity of

the puncta on our original cell orientation and whether or not the signals were indeed colocalized. Common manipulations in this type of analysis include rotating one mask by 90 degrees, or incrementally moving one mask along the X or Y axis by up to 4 µm (Dunn & Wong, 2012). This additional analysis was used to verify the significance of TARP expression within dendrites, and in the future could be used to look at expression within the soma. Typically, if colocalization decreased post-manipulation, it could be concluded that puncta are corresponding to proteins expressed on neurites and are not signals from background noise or incorrect/non-specific labelling. In the example cell illustrated in Figure 12, colocalization post-manipulation increased. This is uncharacteristic of previous literature on this type of analysis and implies that the antibodies may have been ineffective (Dunn & Wong, 2012; Jacoby et al., 2018).

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5.2 γ-3 and γ-5 TARP antibodies may be an unreliable tool for confirming expression in the retina

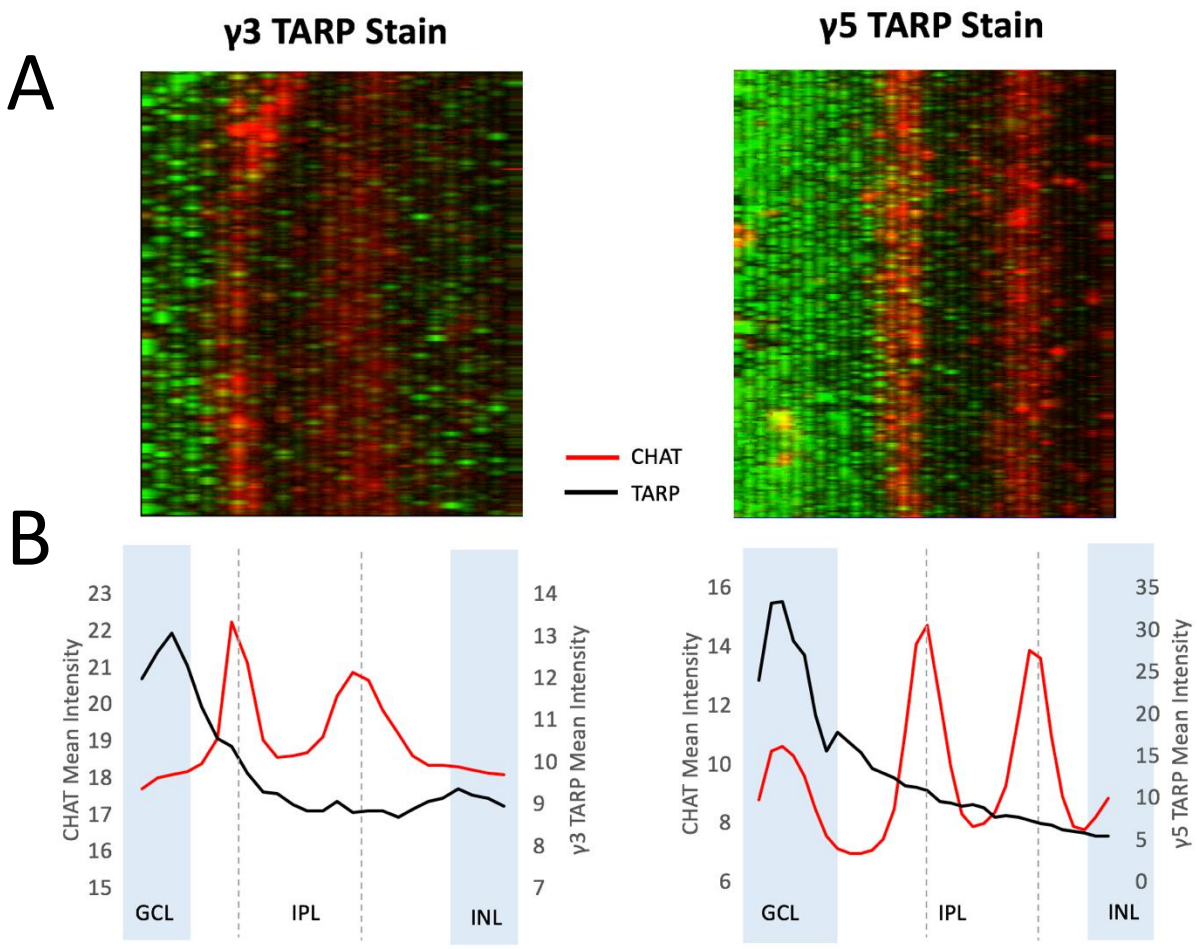

Figure 13: (A) Antibody stains of γ-3 and γ-5 TARPs in whole mount wild-type mouse retina. TARPs (green) are co-stained with Choline Acetyltransferase (ChAT, red) antibodies. (B) The intensity levels of each TARP (black) are plotted against the intensity of ChAT (red) expression in three different retina layers to measure expression patterns. The GCL and INL are labeled using light blue bands. The grey dotted vertical lines correspond to the two ChAT peaks in the IPL, which serve as physical boundary lines for the ON and OFF RGC dendrite stratification layers.

We aimed to determine if TARPs are expressed preferentially in somatic vs dendritic regions of RGCs and potentially see if there was a preference in ON vs OFF RGC dendrites. As ChAT expression in the retina is a reliable “landmark” of retinal layers (with two distinct bands appearing within the IPL and general somatic expression in the GCL and INL (Jeon et al., 1998)) we hoped to verify the locations of TARPs based on the relative location to ChAT expression. In particular we hoped to use the ON ChAT band proximal to the GCL, and the distal OFF-ChAT band as reliable anatomical guides to identify where ON and OFF RGC dendrites stratify.

γ-3 and γ-5 antibodies showed high fluorescence levels in the GCL but expression decreased as it progressed into the tissue. This raised potential concern for penetration issues, and overall efficacy of the antibody. Additionally, the antibody used for γ-3 and γ-5 were both rabbit-based, and subsequently could not be co-stained to determine co-localization. Hence, they were less than ideal for verifying if a cell’s AMPARs were TARPless. Staining in transverse retinal slices as opposed to whole mount preparations could potentially address the penetration issues and determine if the lower expression in the INL was accurate since all retina layers were exposed directly to the antibodies. However, our results from slice preparations suggested there was non-specific binding of γ-3 and γ-5 antibodies (not shown) making these an unreliable tool for quantifying the colocalization of these particular TARPs in RGC dendrites.

Chapter 6: Discussion

The experiments presented in this thesis sought to examine synaptic diversity in two α-RGCs, using whole-cell patch clamp in γ-3 KO and WT mice to isolate AMPAR responses in whole-mount retina. Additionally, the use of mRNA and IHC to explore expression patterns of TARP proteins in the retina was also explored.

mRNA seq data revealed the preferential expression of γ-3 and γ-5 TARPs in RGCs. Furthermore, ON-S-α RGCs showed high expression of γ-3 and low expression of γ-5 – the opposite of OFF-S-A RGCs. These two cell types can be accurately identified, have opposing light responses, and are well characterized in the field of retinal physiology– making them an enticing target for deeper understanding of overall function. With a focus on TARP γ-3 due to its role in modulating AMPAR kinetics, we sought to investigate the mEPSC and light response changes in the absence of γ-3. We found that γ-3 has a role in slowing AMPAR kinetics in ON-S-α RGCs. The slower kinetics may cause an increase in the amount of time needed to cause an influx of current great enough to propagate an action potential, which may have led to a longer spike latency at light onset in WT mice. These data show that small changes in AMPAR kinetics can lead to physiological changes in overall neuronal function and brings to light additional questions on how general AMPAR diversity in RGCs could impact overall cell function.

6.1 mRNA sequencing data revealed multiple expression patterns of excitatory proteins in RGCs

When analyzing mRNA sequencing data with cell-type specific molecular information, we found a number of unique expression patterns of AMPAR receptor subunits and their regulatory proteins (Figure 5). Most notably, γ-3 and γ-5 were found to be preferentially expressed in RGCs – which are some of the least well studied TARPs. While we did not find distinct expression patterns within the generalized category of ON and OFF cell types, we did find that α-RGCs showed preferential expression of only one of the two TARPs. Access to mRNA sequencing data allowed us to narrow down our experimental scope and will be a useful tool for further exploring expression patterns.

While the scope of this thesis focused on the impact of γ-3, the role of γ-5 TARPs in overall retinal function is an additional avenue to be studied. When looking through available mRNA datasets, we also observed a lack of GluA1 expression across nearly all RGC types, with the exception of one cell: F-Mini OFFs (Tran et al., 2019). GluA1 plays a critical role in LTP and LTD elsewhere in the CNS, and the lack of LTP and LTD in the retina could explain this sparse expression. However, the presence of this subunit in one cell type could raise questions about the F-mini OFF's function and overall role in the retinal circuit. These are just a few additional examples of how valuable mRNA sequencing can be in informing future hypotheses in synaptic diversity.

6.2 Subtle changes in AMPAR kinetics can impact overall physiological function in ON-S-α RGCs

γ-3 lacking ON-S-A RGCs showed faster mEPSC kinetics and decreased spike latency at light onset, showing that relatively small kinetic changes in receptor function can have an overall impact on cellular responses. We did not determine if the total charge transfer changed significantly between γ-3 lacking and γ-3 containing ON-S-A RGCs, however this type of analysis would have been beneficial. One could assume that the faster kinetics in γ-3 lacking AMPARs leads to faster visual responses. However, if a smaller amount of total charge was transferred with each quantal event, it could take longer to activate the perisynaptic NMDARs necessary to activate an action potential. Consequently, further analysis is needed to draw a robust conclusion. Additionally, later studies using mRNA sequencing in amacrine cells revealed expression of γ-3 in these cells providing inhibitory input to RGCs. Cell-attached recordings were completed without blocking any inhibition, so changes in cell responses upstream could be contributing to the changes in spike latency – not just AMPAR kinetics in RGCs. However, OFF-S-α RGCs – which also receive inhibition from amacrine cells – showed no change in spike latency at light offset. This leads to an ambiguous result that requires further experimentation to clarify.

Viral knockdowns as opposed to global knockouts could be a helpful tool to navigate this issue. Using an intravitreal injection method, we could preserve some of the upstream signaling methods that may be impacted by the loss of γ-3 in a global

knockout. Viral knockdowns will also be essential for studying the effects of γ-5 in the retina, since a γ-5 global knockout was not readily available for experiments.

Additionally, a change in light stimuli is essential to gain further information about the overall physiological changes of these cell types. ON cells would need an increase in stimuli length so we can examine steady-state firing rates, with longer time in between stimuli to better examine recovery and baseline firing rates. Similar changes would need to be made to examine OFF-Sustained-αs. Light decrements from a mean light level as opposed to light increments from darkness would be an essential change to better isolate the functional changes in OFF-S- αs. This would allow us to examine each cell type with greater functional significance.

6.3 TARP antibodies did not provide fruitful results

Initially, the use of IHC to confirm the presence of TARPs in RGC dendrites was expected to provide insight into the presence of multiple TARPs in a cell type and see if these proteins were expressed in synaptic regions. Multiple trials were performed under a variety of staining conditions in both whole mount and sliced retina and appeared to result in non-specific binding. The analysis method used for determining colocalization has been previously shown to be a powerful tool for measuring cellular expression (Dunn & Wong, 2012) but the unreliability of the antibodies used were unable to provide us with interpretable results.

6.4 Conclusions

The findings of this study verify the presence of γ-3 TARPs in ON-S-A RGCs that was previously reported in mRNA sequencing data (Goetz et al., 2022). The absence of γ-3 caused changes in ON-S-α, but not OFF-S-α RGCs, a finding that aligned with expression patterns seen using mRNA sequencing techniques. While further experiments are needed to conclude exactly how γ-3 impacts the wider physiology of ON-S-α RGCs, this project shows a clear example of synaptic diversity between two α-RGC types and that subtle changes in AMPA receptor function can influence overall retinal function.

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