Constructing the rod bipolar cell signalplex using animal models of retinal dysfunction.

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CONSTRUCTING THE ROD BIPOLAR CELL SIGNALPLEX USING ANIMAL MODELS
OF RETINAL DYSFUNCTION

By

Thomas Andrew Ray
B.S., Eastern Kentucky University, 2004

A Dissertation
Submitted to the Faculty of the
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For the Degree of

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Department of Biochemistry and Molecular Biology
University of Louisville
Louisville, Kentucky

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A Dissertation Approved on

November 15, 2013

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ABSTRACT

CONSTRUCTING THE ROD BIPOLAR CELL SIGNALPLEX USING ANIMAL MODELS OF RETINAL DYSFUNCTION

Thomas A. Ray
November 15, 2013

Glutamate mediated neurotransmission from the rod photoreceptors to rod bipolar cells is critical for vision under low light levels. Disruption of this pathway results in the blinding disorder Congenital Stationary Night Blindness (CSNB), which is hallmarked by the inability to see under dim light. Mouse models of CSNB have been critical to identifying proteins required for glutamate signaling and understanding the mechanism of signaling. Currently, our understanding of rod bipolar signaling is incompletely understood because not all of the protein components of the signalplex have been identified. It was known that the glutamate receptor mGluR6 is coupled to the TRPM1 cation channel, via an unknown G protein mediated mechanism and that regulators of G protein signaling (RGS) are required to terminate signaling. During my graduate work, I identified two new protein components of the rod bipolar cell signalplex that are critical to rod-mediated vision. By mapping the mutation in a novel mouse model of CSNB, I identified Gpr179 as critical to rod bipolar cell signaling. Using immunohistochemistry followed by confocal microscopy and protein interaction assays, I identified GPR179 as a signalplex component. Further, we showed that mutations in GPR179 cause CSNB in humans. I found that GPR179 functions as a hub by interacting with TRPM1 and recruiting the RGS protein complex to the signalplex, which serves to enhance the sensitivity of the system. A report identified mutations in LRIT3 cause CSNB in humans (Zeitz et al., 2013). To determine how LRIT3 contributes to rod vision, I created an Lrit3−/− mouse model of CSNB and found that LRIT3 is required for localizing TRPM1 to the signalplex. These findings add to our understanding of how the visual signal is passed through the retina, and more specifically, rod bipolar cell signalplex architecture, G protein signaling mechanisms, and the causes of CSNB.
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CHAPTER I: INTRODUCTION

Our ability to visualize the text before us and consistently and accurately convert contrast differences into meaningful symbols is made possible by the retina, a product of over 600 million years of evolution (Lamb et al., 2007). The earliest photosensitive cells that eventually gave rise to the retina consisted of a single cell photoreceptor capable of phototaxis, shadow detection and entrainment of circadian rhythms that gave our ancient ancestors survival advantages (Lamb et al., 2007). This once rudimentary system for detecting changes in ambient light evolved into a highly specialized multicellular tissue capable of image-formation during the Cambrian explosion 540 million years ago (Lamb et al., 2007). In its current form, the human retina has evolved to function over a wide range of light intensities, provide both high sensitivity and high acuity, and carry out complex image processing.

Humans are heavily invested in their visual system to function during daily life and blinding disorders have a dramatic effect on a person’s quality of life. Because of this, it is of great interest to understand the circuitry of the retina and the mechanism of communication between retinal neurons in the healthy and diseased state. Retinal structure and function has been studied for decades, but there are still cell types to be discovered, circuits to be identified and receptors that allow cells to communicate to be characterized. Apart from gaining insight about vision, the retina is a great model system for studying general neural signaling and neural development. The accessibility of the retina along with the ability to easily assess function makes it a great model system to study neuronal function.
Retina Anatomy

The vertebrate retina is a light sensitive neural tissue at the back of the eye that consists of various synaptic and nuclear layers juxtaposed between an outer retinal pigmented epithelium (RPE) and an inner limiting membrane (ILM) (Cajal, 1892b). The basic organization of the retina is remarkably similar across vertebrate species (Masland, 1986; Ehinger et al., 1988; Marc et al., 1990; Wassle and Boycott, 1991). The first detailed anatomical view of the mammalian retina was produced by Santiago Ramón y Cajal in 1892 using golgi silver nitrate stained retinas (Cajal, 1892b). He showed the retina consists of three nuclear layers; the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL) in which cell bodies reside, and two synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL) in which synapses are formed between various retinal neurons (Cajal, 1892b; Kolb, 1970). A simplistic depiction of retinal anatomy is shown in Fig.1.

The photoreceptor cell bodies reside in the ONL and the outer segments of the photoreceptors are apposed to the RPE (Bok, 1993). Bipolar cells (BCs), amacrine cells and horizontal cells reside in the INL and ganglion cells and displaced amacrine cells reside in the GCL (Cajal, 1892a; Cajal, 1892b; Hughes and Wieniawa-Narkiewicz, 1980). In the OPL, rod and cone photoreceptors form synaptic connections with BCs and horizontal cells (Kolb, 1970). The IPL is divided into two different sublamina, A and B. The OFF ganglion cells make contacts with OFF BCs in sublamina A and ON ganglion cells make contacts with ON BCs in sublamina B (Rodieck, 1973; Famiglietti et al., 1977; Nelson et al., 1978; Wassle and Boycott, 1991). The ganglion cell axons form the optic nerve and transmit signals to the visual centers of the brain. Each neuron type plays a specific role in extracting information from the visual environment and packaging that information into a neural code that is sent to the visual centers of the brain for interpretation.
In addition to the many neurons in the retina, there are three types of glial cells. Glial cells are non-neuronal cells that provide support to neurons. The primary glial cell in the retina is the Müller glial cell, which spans the retina from the RPE to the ILM. Astrocytes envelope retinal vasculature and play an important role in the blood-retinal barrier (Ridet et al., 1997). Microglial cells provide innate immune responses and can migrate to regions of damaged cells or exogenous pathogens (Aloisi, 2001; Lynch, 2009; Zinkernagel et al., 2013).

The major excitatory neurotransmitter in the retina is glutamate and the major inhibitory neurotransmitters are gamma aminobutyric acid (GABA) and glycine. Excitatory and inhibitory neurotransmitters are needed to set up the excitatory vertical pathways through the retina and the lateral inhibitory pathways. These excitatory and inhibitory pathways enable retinal cells to have a center-surround organization. The classical view of center-surround organization is that a ganglion cell is excited or inhibited when a stimulus falls in the middle of its receptive field but the opposite is achieved when a stimulus falls in the surrounding region. Center-surround organization is critical for the extraction of visual features such as object edge detection.
Figure 1: Fluorescent microscopy image and schematic of retinal layers. (Left) Image of mouse retina cross section labeled for cones (purple), horizontal cells (orange), bipolar cells (green), amacrine and ganglion cells (magenta). (Right) Schematic of the retinal layers and the cells that inhabit each layer. The fluorescence microscopy image was adapted and modified from Morgan and Wong (1995) and the retina schematic from http://wonglab.biostr.washington.edu/.
Anatomy and Physiology of the Retina

Retinal Pigment Epithelium

The RPE is a mono layer of pigmented cells that is apposed to the photoreceptor outer segments (OS) and is present in eyes from simple insects to higher vertebrates (Lamb et al., 2007). The RPE contains long apical microvilli that surround the outer segments of the photoreceptors (Bok, 1993; Strauss, 2005). Interactions between the RPE and photoreceptor OS are essential for visual function (Steinberg, 1985; Bok, 1993; Strauss, 2005; Sparrow et al., 2010). The RPE is responsible for delivering nutrients to the photoreceptors and uptake of metabolic end products secreted by the photoreceptors. It is also responsible for the endocytosis of the continuously shed photoreceptor outer segments (Young, 1967; Steinberg, 1985; Bok, 1993; Dornonville de la Cour, 1993; Hamann, 2002). In addition to the RPE providing metabolic support for the photoreceptors, the highly pigmented cells absorb light that passes through the retina. This helps preserve visual acuity by preventing light from scattering back through the retina (Bok, 1993; Boulton and Dayhaw-Barker, 2001).

The RPE plays a major role in supplying the photoreceptors with the visual photopigment, retinal. Retinal (Vitamin A) is a photosensitive aldehyde that undergoes isomerization when it absorbs a photon and it is bound by the opsin G protein-coupled receptors of the photoreceptors. The active form of retinal (11-cis retinal) is used by the photoreceptor opsin receptors for the photoresponse. Once 11-cis retinal absorbs a photon it is converted to the inactive form (all-trans retinal) and is released from the photoreceptors and taken into the RPE where it undergoes isomerization into the active form and subsequent release to be used again by the photoreceptors (Baehr et al., 2003; Besch et al., 2003; Thompson and Gal, 2003).

Photoreceptors
The photoreceptors are the most abundant cells in the retina (Curcio et al., 1990). Light enters the eye through the iris and passes through all of the neural layers before it reaches the photoreceptors. Individual photons are absorbed by photopigments in the outer segments of the photoreceptors and the photoresponse is initiated. The outer segments contain tightly packed disk membranes in which the photopigments reside (Sjostrand, 1953). There are two different classes of photoreceptors, the rod and the cone photoreceptors, named for the 3-dimensional shapes of their outer segments (Schultze, 1866). In the human retina there are approximately 120 million rods and 6.4 million cones or 95% of the photoreceptors are rods and 5% cones (Osterberg, 1937; Curcio et al., 1990). The mouse retina contains a higher percentage of rods, with rods making up ~97% of the photoreceptors (Carter-Dawson and LaVail, 1979). In the primate retina there is one type of rod photoreceptor but three different types of cones: L-type, M-type and S-type, which differ in the type of opsin that is expressed in the outer segments (Brown and Wald, 1963). The mouse retina only contains two types of cones, S-type (Jacobs et al., 1991) and coexpressing S/M-type (Rohlich et al., 1994; Applebury et al., 2000).

The inner segments of the photoreceptors are located in the ONL and contain the photoreceptor nuclei. The axon terminals of photoreceptors form synapses in the OPL with bipolar cells and horizontal cells (Cajal, 1892b; Kolb, 1970). The axon terminals of the photoreceptors contain a specialized structure termed a ribbon synapse (Sterling and Matthews, 2005). The ribbon synapse facilitates tonic neurotransmitter vesicle release and is capable of dynamic adjustment of release over a wide range of light intensities (Schmitz, 2009). The cone photoreceptor axon terminal is termed a cone pedicle, which is a wide synaptic terminal containing between 20 and 50 invaginations, each harboring synaptic ribbons (Wassle, 2004) and are filled by BC and horizontal cell dendrites (Kolb, 1970). The cone pedicle is one of the most complex synapses in the
central nervous system (CNS), making as many as 500 post synaptic contacts (Haverkamp et al., 2000). The rod axon terminal is called a rod spherule and contains a single ribbon synapse with one invaginating rod BC and two horizontal cell dendrites (Kolb, 1970).

**Bipolar Cells**

BCs are the interneurons of the retina that connect photoreceptors to ganglion cells. The BC dendrites project into the OPL and the ON BCs form invaginating synapses with the photoreceptor axon terminals and the OFF BC dendrites make synaptic contacts with cone pedicles. Each bipolar cell makes synaptic connections with one to several different photoreceptors. Overall, the mouse retina has a ratio of approximately 10 photoreceptors per bipolar cell (Jeon et al., 1998). The axons of the BCs project into the IPL and make synaptic connections with amacrine cells and ganglion cells (Cajal, 1892b; Rodieck, 1973; Famiglietti and Kolb, 1975; Nelson et al., 1978; Dacheux and Raviola, 1986; Wassle and Boycott, 1991).

The visual response is split into two different pathways in the OPL, the ON (depolarizing) and the OFF (hyperpolarizing) pathways (Saito and Kaneko, 1983; Attwell et al., 1987; Nayy and Jahr, 1991; Masu et al., 1995). These pathways are mediated by the response of the BCs to a decrease in glutamate release from the photoreceptors. Cones form synapses with both ON and OFF BCs. ON BCs (DBCs) depolarize in response to light increments and OFF BCs (HBCs) hyperpolarize in response to light increments (Saito and Kaneko, 1983; Attwell et al., 1987; Nayy and Jahr, 1991; Masu et al., 1995). The rod photoreceptors synapse with a single class of ON BCs, the rod BCs, which does not signal directly to ganglion cells (Cajal, 1892b; Boycott et al., 1969; Dacheux and Raviola, 1986).

The difference in the ON and OFF type of BCs is accounted for by the type of glutamate receptor the BC expresses on its dendrites. The OFF BCs or HBCs utilize
ionotropic glutamate receptors of the AMPA/Kainate type (Slaughter and Miller, 1983) and the ON BCs or DBCs use the metabotropic glutamate receptor 6 (mGluR6) (Masu et al., 1995). BCs that produce a sustained or transient response are differentiated by the expression of rapidly or slowly inactivating glutamate receptors (Awatramani and Slaughter, 2000; DeVries, 2000).

In addition to BCs being divided into ON and OFF classes by physiology, they are also classified based on morphology. In the mammalian retina twelve different types of bipolar cells have been identified based on morphology (Fig. 2) (Ghosh et al., 2004; MacNeil et al., 2004; Wassle et al., 2009; Helmstaedter et al., 2013). Eleven of the BC types make contacts with cones and only the rod BCs make contacts with rods. Each of the different classes of cone BCs is thought to parse specific information from the cone’s output and relay that information to the ganglion cells (Masland, 2012). All of the ON bipolar cells stratify in the inner half (sublamina b) of the IPL and OFF bipolar cells stratify in the outer half (sublamina a) of the IPL (Famiglietti et al., 1977; Nelson et al., 1978).

**Horizontal Cells**

Horizontal cells form lateral connections in the OPL. At least two types of horizontal cells have been described in the primate retina, the HI and HII (Fig. 2) (Kolb et al., 1980; Kolb et al., 1994). The HI horizontal cell has two distinct arbors. The dendritic arbor associated with the cell soma makes synaptic contacts with cone pedicles and the remote axonal arbor makes synaptic connections with rods (Kolb, 1970; Masland, 2011). The HII horizontal cell has a broad, radial dendritic spread that makes synaptic contacts with cones (Kolb et al., 1980; Ahnelt and Kolb, 1994b, a; Masland, 2011). The mouse retina only contains the HI type horizontal cell (Peichl and Gonzalez-Soriano, 1994).

The horizontal cells form inhibitory contacts with rod spherules and cone pedicles and are electrically coupled to one another by gap junctions (Kolb, 1970; Klaassen et al.,
2011; Sun et al., 2012). It also has been suggested they form inhibitory connections with BC dendrites (Herrmann et al., 2011). Horizontal cells have center surround organization and are thought to be responsible for setting up receptive fields in the retina through lateral inhibitory synapses (Werblin and Dowling, 1969; Mangel and Miller, 1987; Mangel, 1991). In addition to inhibition, horizontal cells provide lateral gain control to the outer retina by averaging the brightness within a local region and subtracting a proportional value from rod and cone output (Klaassen et al., 2011). This ensures that the signal passed through the retina will be within the operating range of downstream neurons (VanLeeuwen et al., 2009; Masland, 2011).

**Amacrine Cells**

Amacrine cells reside in the INL and provide lateral connections in the IPL. Amacrine cells are the most diverse cell type in the retina with ~50 distinct types being identified by morphology and physiology in the mouse (Fig. 2) (MacNeil and Masland, 1998; MacNeil et al., 1999; Lin and Masland, 2006). They can be organized into four general classes based on their dendritic spread; narrow field (30-150µm), small-field (150-300µm), medium-field (300-500µm), and wide-field (>500µm) (Kolb and Nelson, 1981). Amacrine cells are also classified based on their stratification within the IPL (Mariani, 1990; Kolb et al., 1992).

The functional roles of amacrine cells in the retina are as diverse as their structures. The starburst amacrine cells serve to provide direction selectivity to a subset of ganglion cells (Euler et al., 2002; Fried et al., 2005). The AII amacrine cells serve as a link between the rod BC and the ganglion cells (Strettoi et al., 1994; Trexler et al., 2005). The A17 amacrine cells make reciprocal synapses with rod BCs and provide feedback inhibition onto the rod BC axon terminal (Nelson and Kolb, 1985). However, the majority of amacrine cells still have unresolved functions.

**Ganglion Cells**
Ganglion cells are the output neurons of the retina and their axons exit the retina through the optic disc and bundle together to form the optic nerve. There are approximately 15 to 20 different types of ganglion cells in the mammalian retina based on morphology and physiology (Fig 2) (Masland, 2001a; Rockhill et al., 2002; Wassle, 2004; Masland, 2011). Each type of ganglion cell is responsible for sending a unique feature of the visual field to specific retinorecipient nuclei in the brain (Roska and Werblin, 2001; Berson, 2008).

The predominant type of ganglion cell in the human retina is the midget ganglion cell which contains a single dendrite extending from the cell soma and accounts for ~80% of ganglion cells in the retina (Perry et al., 1984). Midget ganglion cells receive input from one cone photoreceptor in the central retina and are capable of relaying color information (Wiesel and Hubel, 1966; Valois, 1973; Lennie, 1984). Parasol ganglion cells have dendritic trees much larger than the midget cells and they receive input from several photoreceptors of mixed spectral input, disabling them from relaying color information (Schiller and Logothetis, 1990; Wassle and Boycott, 1991). The midget and parasol ganglion cells are the predominant ganglion cell types in the primate retina (Schiller and Logothetis, 1990; Merigan and Maunsell, 1993; Dacey, 2004).

There are several other types of ganglion cells, many of which we do not understand their role in vision. Direction selective ganglion cells (DSGCs) respond preferentially to object movement in one of four directions, posterior, anterior, superior and inferior (Oyster and Barlow, 1967; Elstrott et al., 2008; Briggman et al., 2011; Sun et al., 2011). Their discovery was quite revolutionary because it showed retinal neurons were capable of complex visual processing, previously thought to be carried out only in the higher visual centers of the brain (Hubel and Wiesel, 1959; Barlow and Hill, 1963; Barlow et al., 1964).
Apart from the photoreceptors, the only other known photosensitive cells in the retina are the intrinsically photosensitive retinal ganglion cells (ipRGCs). The ipRGCs express the melanopsin photopigment on their dendrites and cell soma. ipRGCs are responsible for non image forming light responses such as circadian photoentrainment, the pupillary light reflex and light suppression of locomotor activity (reviewed in (Sand et al., 2012)). There is emerging evidence that ipRGCs have roles in image formation and are responsible for pattern recognition (Ecker et al., 2010; Hicks, 2011). The ipRGCs project to several different regions of the brain. Some projections target the suprachiasmatic nucleus of the hypothalamus, which helps the solar cycle synchronize with circadian rhythms (Gooley et al., 2001; Berson et al., 2002; Berson, 2003; Hirota and Fukada, 2004). Another target is the intergeniculate leaflet, which also helps with circadian photoentrainment (Harrington, 1997; Hattar et al., 2002; Morin et al., 2003). A third connection is made to the olivary pretectal nucleus, which mediates the pupillary light reflex (Trejo and Cicerone, 1984; Clarke and Ikeda, 1985; Hattar et al., 2002; Morin et al., 2003).

**Müller Cells**

Müller cells are radial glial cells and are the primary glial cells in the retina. They span the entire thickness of the retina and provide light guidance, structural support and nutrient support for their associated neurons (Bringmann et al., 2006; Agte et al., 2011; Reichenbach and Bringmann, 2013). Müller cells orient in the direction of incoming light in the retina and behave as optical fibers to guide light to the photoreceptors (Franze et al., 2007). It has been demonstrated in retinal slices that a thin laser beam traverses all retinal layers if it hits a Müller cell endfoot, but significant intraretinal scattering is observed if the laser hits an area devoid of Müller cells (Agte et al., 2011). In the human retina there is approximately one Müller cell per cone photoreceptor allowing maximum possible resolution (Agte et al., 2011; Reichenbach and Bringmann, 2013). There are
several rod photoreceptors per Müller cell that optimizes for maximum sensitivity to light (Agte et al., 2011; Reichenbach and Bringmann, 2013).

Each Müller cell provides a structural core that encapsulates a column of retinal neurons (Hollander et al., 1991; Reichenbach and Robinson, 1995). The structural core not only ensures the delivery of light to associated photoreceptors, but Müller cells have a symbiotic relationship with retinal neurons ensuring their viability. Müller cells produce and secrete neurotransmitter precursors to be taken up by retinal neurons (Pow and Crook, 1996; Bringmann et al., 2009). They take up glutamate in the inner retina contributing to rapid removal of glutamate and termination of the excitation (Higgs and Lukasiewicz, 1999; Matsui et al., 1999). Müller cells also protect retinal neurons from reactive oxygen species, which are frequently generated in the retina from light and oxygen consumption. In response to oxidative stress Müller cells secrete glutathione, an antioxidant, to protect the retinal neurons (Schutte and Werner, 1998). Müller cells also serve a critical role in ensuring photoreceptor viability and retinal ion homeostasis (Reichenbach and Bringmann, 2013).
Figure 2: The diverse cell types of the retina. Shown are representative drawings of the many different types of cells found in mammalian retinas. Adapted from (Masland, 2001b).
**Convergence and Divergence**

Retinal cell type distribution across the retina plays an important role in shaping the visual field of animals. Cell type distribution reflects the lifestyle of an animal and varies across species and within species with different lifestyles, allowing the retina to capture information most critical to the animal’s survival (Hughes et al., 1977; Stone, 1983; Talbot and Marshall, 2011). Nocturnal plains dwelling animals typically have a horizontal band extending naso-temporal across the retina with high ganglion cell density (Hughes, 1971; Provis, 1979). This “visual streak” is thought to allow detection of moving objects in the periphery. In contrast, most predatory animals, including humans, tend to have photoreceptors and ganglion cells concentrated around the central retina resulting in higher acuity vision (Hughes, 1975; Stone, 1978; Wassle et al., 1989).

Human visual acuity, color vision and sensitivity are the result of the cell type distributions of the fovea and the connections they make (Wassle et al., 1989; Curcio and Hendrickson, 1991). Near the center of the retina is a yellow-pigmented region, known as the macula. The yellow pigmentation is due to the presence of the carotenoids lutein and zeaxanthin (Beatty et al., 1999; Beatty et al., 2004), which absorb short wavelength light and provide a level of protection against short wavelength light damage beyond what is provided by the lens (Snodderly et al., 1984a; Snodderly et al., 1984b). Within the macula is the fovea, consisting of the highest density of cone photoreceptors and lowest density of rods and is devoid of ganglion cell bodies and retinal vessels to improve visual acuity (Curcio and Hendrickson, 1991; Provis et al., 1998). Primates are the only mammals that possess a fovea, although it has evolved independently in some non-mammalian vertebrates (Inzunza et al., 1991; Moore et al., 2012).

When a signal is passed through the retina it can undergo convergence and divergence at each synaptic layer. The convergence of signal comes from several
photoreceptors pooling their input onto a single ganglion cell. The rod system has highest convergence, which increases the sensitivity of the system at the cost of visual acuity by pooling the input from a large area of the retinal surface. In the cat, as many as 75,000 rods connect to 5,000 rod BCs which connect to 250 All amacrine cells to a single alpha ganglion cell (Wassle et al., 1989). The cone pathway through the retina is less convergent as a whole and the low convergence in the fovea accounts for the high visual acuity. The other factor increasing visual acuity is the ability of cones to pack in close proximity. In the fovea, cones are primarily connected to midget bipolar cells, which connect to midget ganglion cells, setting up a 1:1 cone to ganglion cell ratio (Kolb and Dekorver, 1991). In general, convergence is greatest in the outer retina and lowest in the central retina (Schein, 1988; Curcio et al., 1991; Wa¨ssle et al., 1994). The cell topography of the retina allows humans to have high acuity and good color vision at the center of the visual field and high sensitivity and reduced spatial resolution in the periphery.

**Rod Visual Circuit**

The rod visual circuit is believed to have developed evolutionarily after the cone pathway (Lamb, 2009). Electron microscopy (EM) revealed that rod bipolar cells do not make direct synaptic contact with ganglion cells. Instead, they make synaptic contacts with a specific class of amacrine cells, the All amacrine cell (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975; Strettoi and Masland, 1996) and utilized the preexisting cone BCs to connect to GCs (Strettoi et al., 1992).

The Rods can transmit their signal to the ganglion cells through two different pathways (Fig. 3). In the primary rod pathway, rods signal to rod BCs, which make synaptic contacts with All and A17 amacrine cells (Famiglietti and Kolb, 1975; Dacheux and Raviola, 1986; Raviola and Dacheux, 1987). The A17 amacrine cells provide GABAergic feedback inhibition onto the rod BC synapses to modulate their output (Kolb
and Nelson, 1983; Dacheux and Raviola, 1986; Raviola and Dacheux, 1987; Protti and Llano, 1998; Zhang et al., 2002; Chavez and Diamond, 2008). The All amacrine cell is connected with other Alls and the axon terminals of cone ON BCs by gap junctions (Famiglietti and Kolb, 1975; Strettoi et al., 1992; Strettoi et al., 1994; Mills and Massey, 1995; Veruki and Hartveit, 2002; Petrides and Trexler, 2008). It is through the cone ON BCs, via the All, that the rod signal reaches the ON ganglion cell (Kolb, 1979). The Alls also make inhibitory synapses onto select OFF BC axon terminals and OFF ganglion cells, allowing the rod signal to integrate into the ON and OFF pathways (Tsukamoto et al., 2001; Murphy and Rieke, 2008; Munch et al., 2009). In the secondary rod pathway the rod photoreceptors are connected to the cone photoreceptors through gap junctions and the cone ON BCs are used to transmit the signal to the ganglion cells (Raviola and Gilula, 1973; Nelson, 1977; Schneeweis and Schnapf, 1995).
Figure 3: Rod Visual Circuits. The primary rod pathway: rod photoreceptors synapse with the rod BCs, the rod BCs transmit signal to the All amacrine cell, the All amacrine cell is coupled to the cone ON BCs through gap junctions or the OFF BC axon terminals through glycinergic synapses, the cone ON BC signal to the ON ganglion cell (GC) and the OFF BC signal to the OFF GC. Secondary rod pathway: the rods feed directly into cones via gap junctions, the signal is then passed through the cone ON and OFF BCs to the ganglion cells.
**Phototransduction**

Phototransduction is the process by which a light signal is converted into an electrical signal, which is passed through the retina by the retinal neurons. Phototransduction begins with the absorption of photons by 11-cis-retinal bound to one of the various retinal opsins. Opsins are light sensitive G protein-coupled receptors (GPCRs) and each type of photoreceptor expresses a specific type of opsin. The rod photoreceptors express rhodopsin (RHO) and are most sensitive to 498 nm light, the L-type cones express long-wave-sensitive opsin 1 (OPN1LW) and are most sensitive to 564 nm light, the M-type cones express medium-wave-sensitive opsin 1 (OPN1MW) and are most sensitive to 533 nm light and the S-type cones express short-wave-sensitive opsin 1 (OPN1SW) and are most sensitive to 437 nm light (Brown and Wald, 1963).

The ligand of the visual pigments of most vertebrates is 11-cis-retinal (Dartnall and Lythgoe, 1965). Absorption of a photon causes a conformation change in the opsin bound chromophore 11-cis-retinal (Wald, 1968). The conformation change from 11-cis-retinal to all-trans retinal subsequently activates the associated opsin (Wald, 1968). Activated opsin can then bind and activate the heterotrimeric G protein, transducin, resulting in a GDP for GTP exchange on Gαt (reviewed in Chen (2005)). Active Gαt binds and activates the cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE), which is anchored to the disk membrane by isoprenylation (Anant et al., 1992; Qin and Baehr, 1994). Activated PDE catalyzes the hydrolysis of cGMP, the second messenger in phototransduction (Zhang and Cote, 2005; Lugnier, 2006; Conti and Beavo, 2007). The decrease in cGMP causes cyclic nucleotide gated (CNG) ion channels to close, resulting in hyperpolarization of the cell (Fesenko et al., 1985; Haynes et al., 1986; Zimmerman and Baylor, 1986; Matthews, 1987; Haynes and Yau, 1990).

Deactivation of phototransduction is the result in deactivation of activated opsins and transducin. Deactivation of opsin begins with phosphorylation of 6 to 7 amino acids
on the cytoplasmic carboxy terminus (Hurley et al., 1998; Mendez et al., 2000; Kennedy et al., 2004) and is completely inhibited when arrestin binds the phosphorylated cytoplasmic domain and inhibits interaction with transducin (Xu et al., 1997; Chen et al., 1999; Mendez et al., 2000; Doan et al., 2009). The intrinsic rate of G\(\alpha\)GTP to GDP conversion is relatively slow and is accelerated by interaction with RGS9 (He et al., 1998). RGS9 is a part of a multi-protein complex that includes R9AP (membrane anchor) and G\(\beta\)5 (Hu and Wensel, 2002). Hydrolyzing G\(\alpha\)GTP to G\(\alpha\)GDP inhibits PDE activation and allows levels of cGMP to recover and effectively gate the CNG ion channels open.

Deactivation of phototransduction in rods is slower (~200 ms) than the activation steps (~130 ms) (Krispel et al., 2006; Nikonov et al., 2006; Arshavsky and Burns, 2012). This is an important concept because the bipolar cell light response is similar to the deactivation of a G protein cascade and not the activation. In mouse rod photoreceptors, the RGS9 complex rate limits the deactivation of the photoreponse (Krispel et al., 2006). Overexpression of the RGS complex results in a faster photoresponse recovery (~74 ms) (Krispel et al., 2006; Chen et al., 2010; Gross and Burns, 2010). In cones, which express more RGS proteins than rods (Cowan et al., 1998; Zhang et al., 2003), deactivation of rhodopsin becomes the rate-limiting step in photoresponse deactivation (Gross and Burns, 2010; Matthews and Sampath, 2010).

Hyperpolarization of the photoreceptor modulates the L-type voltage gated calcium channel (L-VGCC) and results in channel closure (Katz and Miledi, 1967; Smith and Augustine, 1988; Schmitz and Witkovsky, 1997; Bech-Hansen et al., 1998). The reduction in calcium entry decreases the release of the neurotransmitter glutamate into the synaptic cleft, in a stimulus intensity dependent manner (Trifonov, 1968a; Penn and Hagins, 1969; Ball and Gregg, 2002; Barnes and Kelly, 2002). Photoreceptors release neurotransmitter in a graded manner with a linear relationship to intracellular calcium
levels (Thoreson et al., 2004; Heidelberger et al., 2005; Thoreson, 2007). RIBEYE, the main protein component of ribbon synapses, forms complexes with proteins that regulate L-VGCCs to tightly correlate synaptic vesicle release to calcium entry (Schmitz et al., 2000; Alpadi et al., 2008; Haeseleer, 2008).
Figure 4: Phototransduction in the rod photoreceptor. (A) Activation of rhodopsin by a single photon causes G protein activation. Active Gα binds and activates PDE, allowing it to hydrolyze cGMP. cGMP gated ion channels then open causing the cell to hyperpolarize. (B) Adapted from Arshavsky and Burns (2012)
ON Bipolar Cell signaling

BCs are hypothesized to have evolved from photoreceptors due to the fact they share many common features including; microtubule arrangement, an output synapse with similar structure containing a ribbon synapse, and a similar G protein signal transduction cascade (Lamb et al., 2007). While there are many similarities between these cell types, the ON BC signaling cascade is less well understood and it is emerging that the ON BC signaling cascade is markedly different from the signaling mechanism in the photoreceptors (Peachey et al., 2012b; Shen et al., 2012).

Signaling from the photoreceptors to the bipolar cells requires glutamate receptors at the dendritic tips of the bipolar cells. The ON bipolar cells express the metabotropic glutamate receptor 6 (mGluR6) and the OFF bipolar cells express different variations of ionotropic glutamate receptors (iGluR) of AMPA and kainate types (Slaughter and Miller, 1981; Saito and Kaneko, 1983; Masu et al., 1995; Puller et al., 2013). A decrease in glutamate release at the photoreceptor terminals cause the OFF BCs to hyperpolarize due to closure of the iGluR cation channel (Kaneko and Saito, 1983; Saito and Kaneko, 1983). The ON BCs depolarize in response to a reduction in glutamate by opening a non-specific cation channel, TRPM1, linked to the mGluR6 glutamate receptor (Slaughter and Miller, 1981; Masu et al., 1995; Morgans et al., 2009; Shen et al., 2009; Koike et al., 2010b; Morgans et al., 2010).

In the dark, photoreceptors tonically release glutamate from their axon terminals into the synaptic cleft (Trifonov, 1968b; Copenhagen and Jahr, 1989; Ayoub and Copenhagen, 1991). Glutamate binding to mGluR6 activates the receptor, releasing the active heterotrimeric G proteins Gα and Gβ3γ13 dimer (Dhingra et al., 2000; Huang et al., 2003; Okawa et al., 2010; Dhingra et al., 2012). The activation of the G proteins results in the closing of the TRPM1 cation channel by an unknown mechanism (Morgans et al., 2009; Shen et al., 2009; Koike et al., 2010a; Koike et al., 2010b). When the
photoreceptors hyperpolarize in response to light increments, glutamate release into the synaptic cleft is decreased (reviewed in Stryer (1991)). The reduction in glutamate bound by mGluR6 causes a decrease in activated G proteins allowing the TRPM1 channel to open and the cell to depolarize (Audo et al., 2009; Morgans et al., 2009; Shen et al., 2009; Koike et al., 2010b; Morgans et al., 2010). The RGS proteins Gβ5, RGS7 and -11 are thought to be critical in deactivating the G proteins allowing the TRPM1 cation channel to open (Rao et al., 2007; Cao et al., 2012). Several other proteins with unidentified functions also are critical to normal cascade function and include nyctalopin, GPR179 and LRIT3 (Gregg et al., 2003; Audo et al., 2012; Peachey et al., 2012b; Zeitz et al., 2013a). Figure 5 shows a schematic of DBC signaling.
Figure 5: Schematic of depolarizing bipolar cell signaling. In the dark glutamate is tonically released into the synaptic cleft where it was bound by mGluR6, activating a G protein cascade that results in the closure of the TRPM1 channel. In response to a light increment glutamate release into the synapse is decreased. The mGluR6 receptors are inactive because it is not bound to glutamate and the RGS proteins deactivate any remaining active G proteins and the TRPM1 channel opens, causing the cell to depolarize.
Photoreceptor Light Sensitivity

The retina is capable of operating over a range in illuminance of a factor of $10^{11}$ (Burkhardt and Gottesman, 1987; Burkhardt, 1994; Stockman and Sharpe, 2006). To achieve this, the retina uses two different types of photoreceptors with differing sensitivities to light (Fig. 6). The rod photoreceptors are the most sensitive and function under scotopic lighting conditions (luminance $10^{-2}$ to $10^{-6}$ cd/m$^2$) (Hood et al., 1979). A cloudy night sky without moon is equivalent to $\sim 10^{-4}$ cd/m$^2$ and a clear night sky with full moon is equivalent to $\sim 10^{-2}$ cd/m$^2$ (Middleton and Mayo, 1952). In humans, a light stimulus of only a few photons is capable of creating a visual percept (Hecht et al., 1942; Van Der Velden, 1946; Sakitt, 1972). Individual rods are capable of reliably responding to single photon stimuli and relaying this signal to retinal interneurons (Hecht et al., 1942; Field et al., 2005). Due to the rods only being active under scotopic conditions there is no color vision because rods only have one photopigment. Visual acuity of rod-mediated vision is poor because of the high degree of signal convergence.

At the upper limit of rod sensitivity and lower limit of cone sensitivity is mesopic lighting conditions, with luminance from $10^{-2}$ to 1 cd/m$^2$ (Hood et al., 1979). Mesopic conditions are equivalent to a clear night sky with a full moon to a clear sky just after sunset (Middleton and Mayo, 1952). Under mesopic conditions color vision is poor, but visual acuity is improved due to the use of the less convergent cone visual pathway. Under photopic lighting ($1$ to $10^6$ cd/m$^2$) conditions only the cone photoreceptors are functional (Jones et al., 1993; Burkhardt, 1994). The photopic visual range is equivalent to just after sunset on a clear day to a bright sunny day. Color vision and high visual acuity are possible because vision is primarily foveal (Middleton and Mayo, 1952; Hood et al., 1979).
Figure 6: Photoreceptor Illumination Sensitivity. The diagram correlates typical ambient light levels to photoreceptor sensitivities. Modified from (Stockman and Sharpe, 2006)
Electroretinogram

The activity of retinal neurons can be assessed non-invasively by the electroretinogram (ERG). The ERG measures the gross potential of the corneal surface in response to a light stimulus and produces waveforms that reflect electrical activity of specific neural layers. In 1933, Ragnar Granit reported that the ERG is composed of three components that last the entire duration of the light stimulus and can be isolated by deepening the state of anesthesia of the test animal (Fig. 7 and Granit (1933)). Granit won the Nobel Prize in Physiology and Medicine in 1967 for his work on the ERG. The first waveform produced by the ERG is the negative deflecting a-wave that reflects the hyperpolarization of the photoreceptors in response to an increase in luminance (Brown and Murakami, 1964; Brown, 1969; Penn and Hagins, 1969). The second wave produced by the ERG is the corneal positive b-wave that is produced by the depolarization of the bipolar cells (Slaughter and Miller, 1981; Stockton and Slaughter, 1989; Gurevich and Slaughter, 1993). Only the ON BCs contribute to the b-wave of the ERG. The OFF bipolar cells contribute to the repolarization of the ERG after the b-wave peak at light offset (Bush and Sieving, 1994). The c-wave is a slow forming positive wave that originates from RPE and glial cells (Noell, 1954; Steinberg et al., 1970).

The ERG can be measured under different lighting conditions to isolate specific neural pathways in the retina. The scotopic ERG is used to measure rod responses. By dark adapting the retina and using flash responses below cone threshold the rods and rod BC responses can be isolated. The photopic ERG is used to measure cone and cone BC responses. To measure the photopic ERG the test is performed in the presence of an adapting background that saturates the rods.

The ERG has been an invaluable tool for assessing retinal function and identifying defects in humans and mice (Peachey and Ball, 2003). A reduction in the a-wave of the ERG is indicative of defects in phototransduction or a reduction in the
number of photoreceptor outer segment disks. A reduction in the b-wave of the ERG may indicate a disruption in neurotransmission from the photoreceptors to the bipolar cells (Peachey and Ball, 2003). This disruption may be caused by a defect on the pre or post-synaptic side of the synapse. The absence of the b-wave in the ERG has been associated with the non-progressive disease congenital stationary night blindness (CSNB). It has been reported that between 2.9 and 4.8% of the human population has a negative ERG and the underlying cause of the vast majority of negative ERGs is CSNB (Koh et al., 2001; Renner et al., 2006; Kim et al., 2012).
Figure 7: Components and waveforms of the Electroretinogram. The ERG response to a 2 sec stimulus (yellow). The ERG waveforms (solid line) are labeled a, b, c, d and the individual components (broken lines) are labeled PI, PII and PIII. Modified from (Granit, 1933)
Congenital Stationary Night Blindness

Congenital stationary night blindness (CSNB) is a non-progressive disease that results in the disruption of rod signal transmission through the inner retina. Human patients with CSNB often have reduced visual acuity and reduced visual function under low light conditions (Miyake et al., 1986; Miyake et al., 1987; Audo et al., 2008). CSNB in humans is classified into two groups. Mutations that result in a complete ablation of the ERG b-wave are said to cause the complete form of CSNB (cCSNB) (Riggs, 1954; Miyake et al., 1986). A number of genetic mutations have been identified in genes that cause cCSNB. They include; Nyx (Pardue et al., 1998; Bech-Hansen et al., 2000; Pusch et al., 2000; Gregg et al., 2007), Grm6 (Masu et al., 1995; Dryja et al., 2005; Zeitz et al., 2005; Pinto et al., 2007), Trpm1 (Audo et al., 2009; Morgans et al., 2009; Koike et al., 2010b; Nakamura et al., 2010), Gpr179 (Audo et al., 2012; Peachey et al., 2012b) and Lrit3 (Zeitz et al., 2013b).

The incomplete form of CSNB (iCSNB) is characterized by a reduced b-wave as measured by ERG (Miyake et al., 1987). iCSNB is the result of disruptions in neurotransmitter release from the photoreceptor terminals and genes involved include Cacna1f (Strom et al., 1998), Cacnb2 (Ball et al., 2002; McCall and Gregg, 2008) and Cebp4 (Zeitz et al., 2006).

A study by Bijveld et al., (2013) characterized the visual defects of people with cCSNB and iCSNB. cCSNB patients report difficulty in performing tasks at night such as driving, cycling, and maneuvering in dimly lit social settings such as movie theaters and pubs (Bijveld et al., 2013a). Many cCSNB patients report being blind at night and dependent on others in dark circumstances (Bijveld et al., 2013a). iCSNB patients report to having difficulty at the aforementioned tasks at a reduced rate and none felt they were always blind at night and dependent on others (Bijveld et al., 2013a).
The screening of mice with the ERG has been critical to identifying spontaneous mutant mice with DBC dysfunction. Mapping and cloning the causative mutations has led to the identification of novel genes critical to the glutamate signaling cascade in DBCs (Pardue et al., 1998; Gregg et al., 2007; Peachey et al., 2012b). The screening of human patients with previously unidentified causes of CSNB for the orthologous mouse gene has identified genes that cause CSNB (Bech-Hansen et al., 2000; Pusch et al., 2000; Audo et al., 2009; Nakamura et al., 2010; Audo et al., 2012; Peachey et al., 2012b).

cCSNB mouse models known to date have a normal retinal morphology as visualized at the light or electron microscope level (Masu et al., 1995; Pardue et al., 1998; Tagawa et al., 1999; Dhingra et al., 2000; Pardue et al., 2001; Morgans et al., 2009; Koike et al., 2010b). iCSNB mouse models have abnormal retinal morphology highlighted by ectopic bipolar and horizontal cell dendrites that extend into the ONL (Ball and Gregg, 2002; Dick et al., 2003; Chang et al., 2006; Bayley and Morgans, 2007).

G Protein-Couple Receptor Signaling

GPCRs are critical for phototransduction and signaling through the inner retina. The retina is a very attractive system for studying GPCR signaling mechanisms because of the availability of mutant mice, accessibility and ability to functionally assess specific signaling events. GPCRs are the target of approximately 30% of pharmaceutical drugs (Salon et al., 2011). The more knowledge gained about these diverse signaling pathways the better we are able to develop effective therapies for diseases that impair vision. All GPCRs are membrane proteins that contain an extracellular amino terminus followed by a 7 transmembrane (TM) domain and a cytosolic carboxy terminus. GPCRs represent the largest class of signaling proteins in the human genome with more than 800 members (Fredriksson et al., 2003).
GPCRs have been classified into three main families based on shared homology. Class A are the rhodopsin-like receptors, Class B are the secretin-like receptors and Class C are the metabotropic glutamate-like receptors (Foord et al., 2005). The simplistic model of GPCR signaling is that binding of an extracellular ligand produces a conformational change in the receptor that activates heterotrimeric G proteins (Gαβγ) inside the cell. The ligands for GPCRs are a diverse group including small compounds, ions, peptides and amino acids (Muller et al., 2008). The majority of GPCRs initiate intracellular signaling through heterotrimeric G proteins but it is beginning to be understood that some operate through G protein-independent mechanisms (Galandrin et al., 2007; Altier and Zamponi, 2008).

The extracellular domain of GPCRs consists of the amino terminus and the extracellular loops (ECLs) ECL1, ECL2 and ECL3. There is large diversity in sequence length and composition among the receptors amino terminus (Lagerstrom and Schioth, 2008). Class A GPCRs form a ligand-binding pocket with the ECLs that can either be occluded or water-accessible (Palczewski et al., 2000; Hanson et al., 2012). Class B GPCRs have an amino terminus ligand binding domain composed of an α-helix and two β-sheets stabilized by disulfide bridges (Parthier et al., 2009). Class C GPCRs typically possess a large bilobed amino terminal ligand-binding domain coined the venus flytrap (VFT) domain (Chun et al., 2012). The VFT ligand binding domain shares sequence homology with bacterial periplasmic binding proteins (PBP) (Zhang et al., 2008). An accepted hypothesis for the origin of Class C GPCRs is that they are the result of a fusion between a rhodopsin-like receptor and a PBP (Pin et al., 2003).

The 7TM helix region of GPCRs serves to relay extracellular information to the inside of the cell (Muller et al., 2008; Venkatakrishnan et al., 2013). The crystallization of GPCRs bound to agonist and ligand free GPCRs provided detail about the 7TM movement that occurs when the receptor is activated (Audet and Bouvier, 2012).
Movement of the 7TM region cause a conformational change that allows the binding of heterotrimeric G proteins by the intracellular regions (IC) of the GPCR (Audet and Bouvier, 2012).

The IC loops of GPCRs typically bind downstream signaling effectors such as G proteins, kinases and arrestins (Rasmussen et al., 2011; Katritch et al., 2012). The IC regions undergo dramatic conformational changes in response to ligand binding (Park et al., 2008; Scheerer et al., 2008; Choe et al., 2011; Lebon et al., 2011; Rasmussen et al., 2011; Standfuss et al., 2011; Xu et al., 2011). The conformational change allows the binding of the receptor’s associated GDP bound heterotrimeric G proteins (Latek et al., 2012). The conformation of the GDP Goβγ when bound to the GPCR facilitates GTP for GDP nucleotide exchange resulting in release of the activated GoGTP and dissociation from the Gβγ complex (Chung et al., 2011; Rasmussen et al., 2011).

Some GPCRs are capable of functioning as monomeric receptors while others require assembly into dimers or larger complexes (Bouvier, 2001; Chabre and le Maire, 2005; Milligan, 2006; Bayburt et al., 2007; Whorton et al., 2007; Rondard et al., 2008; Rondard et al., 2011; Yanagawa et al., 2011). In addition to forming complexes with other GPCRs, they may also associate with other transmembrane proteins to form signaling complexes (Altier and Zamponi, 2008; Altier, 2012). This is believed to enhance signaling kinetics.

The rhodopsin GPCR signaling pathway is the prototypical GPCR pathway. The first crystal structure of a GPCR was of bovine rhodopsin (Palczewski et al., 2000), since then the crystal structure of 18 different class A GPCRs has been solved (Venkatakrishnan et al., 2013). As a result, much of what we know about GPCR signaling pertains to class A GPCRs and a one-size-fits all approach has typically been used when studying GPCRs from other classes. While this approach has proven useful, it has become clear with the growing diversity of GPCRs that not all will fit that mold. It
is important to identify these novel mechanisms of GPCR signaling to not only enhance our understanding of GPCR signaling but to unlock answers that may prove therapeutically beneficial.

**What we can learn from the retina**

The retina is a model system that allows us to learn about more than just vision. It is the most accessible part of the CNS allowing us to more easily study the development of complex neural circuitry. The retinal neurons offer a glimpse of neural signal processing that we understand must also take place in the brain, but are not as easily measured. The retina can be easily genetically manipulated and the results of these manipulations can be functionally assessed with visual tests, allowing perhaps a more accurate depiction of gene function in neural circuits than in other regions of the CNS.
CHAPTER II: GPR179 IS MUTATED IN THE NOB5 MOUSE

**Aim 1:** Identify the mutation responsible for the nob phenotype in the nob5 mouse

**Rationale:** Spontaneous mutant mice are important tools for identifying genes involved in disease. The nob5 mouse lacks the ERG b-wave and is predicted to have a defect in the glutamate-signaling cascade of DBCs. By identifying the mutation in the nob5 mouse we can better understand how the glutamate receptor, mGluR6, gates the TRPM1 cation channel closed in the DBCs. As a testament to our incomplete knowledge of the glutamate signaling cascade in the DBCs, there are patients with cCSNB that do not have mutations in the known cCSNB causing genes (Nakamura et al., 2010; Bijveld et al., 2013b). Therefore, it is probable that there are cCSNB patients with a mutation in the nob5 ortholog. Although there currently is no cure for cCSNB and patients have relatively minor lifestyle inconveniences (Bijveld et al., 2013a), identifying the cause of their disease can provide closure and the knowledge that they do not have a progressive retinal disease in addition to the scientific value of understanding a novel GPCR pathway.

**Hypothesis:** The nob5 mouse has a mutation in a gene expressed in the ON BCs (DBCs) of the retina.

**Introduction**

The ERG is a common tool used to evaluate visual function in mice and can be used as a high-throughput method for identifying retinal dysfunction (Dalke et al., 2004). The screening of mice using the ERG has identified spontaneous mouse mutants with DBC dysfunction (Maddox et al., 2008; Peachey et al., 2012a; Peachey et al., 2012b).
In some of these cases, the mutated genes were novel (Bech-Hansen et al., 2000; Peachey et al., 2012b) and required various molecular biology techniques to locate the mutation within the genome. In other cases, the mutations were in genes already known to cause the nob phenotype but offer unique insights into protein properties that the knockout model does not display (Maddox et al., 2008; Peachey et al., 2012a).

The nob5 mouse is a spontaneous mutant that arose on a C3H/HeJ mouse strain. The C3H/HeJ mouse strain carries a homozygous mutation in the rod phosphodiesterase gene, Pde6b, and was an early model for retinal degeneration (Sidman and Green, 1965; Pittler and Baehr, 1991). This form of retinal degeneration progresses quickly and causes blindness by weaning age, because of a complete loss of photoreceptors (Farber and Lolley, 1974; Lolley and Farber, 1976). The retinal degeneration in the C3H/HeJ mouse strain allowed the nob5 phenotype to go unnoticed until the C3H line was crossed to a congenic C3H line lacking the Pde6b mutation. It was then that an ERG screen by Gianluca Tosini at Morehouse College of Medicine discovered the nob5 mutant.

The nob5 mouse has a normal a-wave but lacks a b-wave in the scotopic ERG (Fig. 8A). The scotopic ERG measures responses through the rod photoreceptor and rod BC pathway. These data indicate that the rod photoreceptors are functioning normally, but the rod BCs are not. The nob5 mouse has a normal a-wave in the photopic ERG but lacks a b-wave (Fig 8B). The light adapted or photopic ERG measures responses mediated by cones. These data indicate the cone photoreceptor phototransduction is normal but cone DBC signaling is completely absent. The ERG of the nob5 mouse (Fig. 8A,B) is similar to other cCSNB mouse models (Masu et al., 1995; Pardue et al., 1998; Pinto et al., 2007; Maddox et al., 2008; Morgans et al., 2009), which lead us to the hypothesis that the causative mutation would be in a gene expressed in the DBCs and critical for the function of the mGluR6 to TRPM1 cascade.
Figure 8: Scotopic and photopic ERGs of WT and nob5 mice. (A) Scotopic ERGs of WT and nob5 mice show nob5 mice have a normal ERG a-wave in response to increasing flash intensity but are lacking a b-wave. (B) Photopic ERGs show the nob5 mice lack a cone mediated b-wave response. Taken together these data indicate that there is likely a defect in DBC signaling in the nob5 mice.
The phenotype in the nob5 mice displays an autosomal recessive inheritance pattern. To determine if the underlying mutation was in a gene known to cause DBC dysfunction, complementation crosses were performed at the Cleveland Clinic with established models of DBC dysfunction Grm6\textsuperscript{nob3}, Nyx\textsuperscript{nob} and Trpm1\textsuperscript{−/−}. The F1 offspring were assessed for retinal function using the ERG. All offspring had normal ERGs indicating the mutant gene in nob5 mice was not Grm6, Nyx or Trpm1.

To localize the nob5 mutation within the genome a mapping cross was set up at the Cleveland Clinic. The nob5 mice were outcrossed to C57BL6/J mice producing nob5\textsuperscript{+/−} F1 offspring. The F1 offspring were subsequently intercrossed to produce F2 offspring with new allele combinations. The F2 offspring were phenotyped by ERG. DNA was pooled from 20 mice displaying the nob5 phenotype and genotyped at approximately 103 simple sequence repeat (SSR) polymorphic markers across the genome (Taylor et al., 1994). Genomic regions that were heterozygous for both mouse strains (C3H/HeJ and C57BL6/J) were ruled out as containing the nob5 gene. This strategy mapped the nob5 allele to a 4.1 megabase (MB) region (Chr11:94563438-98683655) on chromosome 11.

**Results**

**Linkage mapping reduced the nob5 critical region to 1.3 Mb**

The SSR low-resolution genome mapping localized the nob5 phenotype between MIT Markers D11MIT288 (Chr11:94563438) and D11MIT14 (Chr11:98683655) on chromosome 11. The region between D11MIT288 and D11MIT14 spans 4.1Mb and is equivalent to 3.5 centimorgans (cM) (http://cgd.jax.org/mousemapconverter/). To reduce the region containing the nob5 mutation, which was thought to arise on a C3H background, crosses were set up in which the nob5 mouse was outcrossed with C57BL6/J mice. These two strains can be easily differentiated with various SSR and single nucleotide polymorphism (SNP) markers, which allows for the detection of
chromosomal crossovers to narrow the critical region. The SSR and SNP genotyping markers were identified using the National Center for Biotechnology Information's dbSNP database and confirmed by Sanger sequencing. SNPs were genotyped using a high throughput method referred to as high resolution melting (HRM), which relies on melting curve analyses on an ABI 7900 HT real-time PCR machine. SSR markers were genotyped by running amplified samples on agarose gels. Because the nob5 critical region is small we expect only 5% of mice to have a crossover in the critical region. Therefore we used a two-step genotyping and phenotyping strategy.

In total ~1000 F2 mice were generated from a backcross of F1 (nob5 x C57BL/6J) mice to nob5 mice. All 1000 mice were genotyped with D11MIT14 and D11MIT288 markers to identify animals with chromosomal recombination events within the nob5 critical region. These animals with chromosomal crossovers then were genotyped using a combination of nine different MIT and SNP markers spanning the critical region to further localize the recombination event (Fig. 9B). ERGs were performed on the mice with crossovers that should narrow the critical region.

Of the ~1,000 backcross progeny, 39 had crossovers between the D11MIT288 and D11MIT14 markers, reducing the nob5 critical region to ~1.3 MB. The ~1.3 MB region was situated in a highly gene rich region on chromosome 11 (Zody et al., 2006) containing ~80 predicted genes (Fig. 9B). Due to the fact that 1000 mice were needed to reduce the region to a 1.3 MB region of high gene density, it became clear further refinement using this genetic approach was not feasible. Therefore two strategies were used to identify the causative mutation in the nob5 mouse, a candidate gene search and next-generation sequencing of the region.
Figure 9: Mapping panel of *nob5* critical region. (A) Chromosome map of mouse chromosome 11 with the protein coding gene density (top) mapped to the chromosome. (B) Chromosome map of *nob5* critical region including genotyping markers used to narrow the region. (Bottom) The 1.3 Mb region between markers D11MIT54_2 and D11MIT67_4 contained ~80 predicted genes.
Genes located within the critical region were evaluated for possible roles in DBC signaling. Genes that had known roles in signaling, such as ion channels or had expression in neuronal systems were considered candidates. Genes for which knockout mice had already been made and showed a lethal phenotype were excluded as candidates because the nob5 mouse is otherwise healthy.

To identify mutations in candidate genes RNA was isolated from nob5 mouse retinas and converted into cDNA. Primers were made that flanked the open reading frame (ORF) and were used to amplify the ORF and clone it into a pCR4-TOPO vector (Invitrogen). The ORFs were then sequenced and compared to the reference genome. Genes thought to be good candidates included Npepps, Arhgap23, Pip4k2b, Srcin1 and Cacnb1, however, none had mutations in the ORFs.

Next generation sequencing identified an insertion in Gpr179

Therefore, to identify the mutant gene we decided to sequence the entire 1.3 MB region using next generation sequencing. This method requires capturing and amplifying genomic DNA from the critical region using microarray sequence capture (Albert et al., 2007). The sequence capture array was designed and made off site by Roche NimbleGen and was designed to capture a 1.5 MB region of DNA that included the entire nob5 critical region plus upstream and downstream flanking regions, but excluded highly repetitive regions. I used the UCSC genome browser to ensure the probes on the array design would capture all of the gene containing regions within the nob5 critical region.

Sequencing of the captured DNA was performed on an Illumina GAIIx sequencing machine by Ambry Genetics. Mouse liver genomic DNA was isolated from a nob5 mouse and a C3H control and shipped to Ambry Genetics for enrichment of the target region and sequencing using 54bp paired-end sequencing. The bioinformatics report was prepared by Ambry Genetics and delivered to the lab, along with a hard drive
containing the raw FASTQ sequence files. The bioinformatics report contained two points of interest. There were no unique non-synonymous point mutations found in the nob5 sample compared to the control and there was a possible transposable element insertion in the nob5 sample that was not found in the control sample. The sequence files identified two ~30 bp of junction fragments on the 5 prime and 3 prime ends of a possible insertion site at Chr11:97212072-97212086, which is within intron 1 of the Gpr179 gene.

To confirm the insertion and determine its size, primers were created that flanked the putative insertion and PCR was used to amplify the DNA fragment in the nob5 and control samples. The PCR results showed that DNA from the nob5 mouse contained an insertion that is approximately 6.5 kb (Fig. 10A,B). Sequence analyses of the PCR fragment indicated it was a retrotransposable element of the ERV2 class, although complete sequencing was not possible because of its repetitive nature.
Figure 10: A transposable element disrupts \textit{Gpr179} expression. (A) PCR amplification of the insertion followed by agarose gel electrophoresis revealed the insertion was approximately 6.5 kb in length. (B) Schematic of \textit{Gpr179} exon map and insertion site. Subsequent sequencing of the insertion fragment revealed it was a transposable element of the ERV2 class. (C) Quantification of \textit{Gpr179} expression at the exon 1-2 boundary using a taqman assay. \textit{Gpr179} expression was normalized to 18S RNA and is relative to WT gene expression. Error bars indicate mean and standard deviation for three mice. \textit{Gpr179} expression is significantly reduced in the \textit{nob5} mouse (p<0.0005). From Peachey et al., 2012b.
The transposon insertion dramatically decreases Gpr179 gene expression

To understand how the transposable element affected Gpr179 gene expression in the nob5 sample two methods were used. First, using primers nested in exon 1 and exon 11, I attempted to clone a cDNA fragment made from WT and nob5 retinal RNA. PCR amplification using cDNA from WT resulted in the expected fragment size of ~6.8 kb, whereas the nob5 sample did not produce a PCR product. Forward and reverse primers were then created across the gene designed to amplify smaller fragments of the predicted cDNA to see if any of the Gpr179 mRNA was expressed and processed in nob5 retinas. Primers placed in exon 2 through exon 11 yielded PCR fragments of the expected size in the WT and nob5 sample. Forward primers placed in exon 1 and reverse primers placed in any exon downstream of the transposon insertion (exons 2-11), failed to produce a PCR fragment in the nob5 samples. These experiments confirm that Gpr179 is expressed in the retina and that gene expression is altered in the nob5 retina.

To quantitatively measure Gpr179 gene expression in the nob5 sample I used two different TaqMan Assays (Life Technologies). The first (Mm00615352_m1) spanned the exon1/2 boundary of Gpr179 and had an amplicon length of 83 bp. The second (Mm04204651_m1) spanned the exon 5/6 boundary and had an amplicon length of 103 bp. The results of the experiment show that gene expression is virtually undetectable in the nob5 mouse using the exon1/2 TaqMan assay (Fig. 10C). The exon5/6 TaqMan assay revealed that Gpr179 expression downstream of exon 1 showed only a slight increase compared to WT (data not shown).

These experiments indicate that exons downstream of intron 1 are expressed and processed but intron 1 is not processed properly. The transposable element insertion presumably interferes with processing of intron 1 because it likely contains many splice acceptor and splice donor sites that may result in a large number of different
mRNAs. These splice variants would likely create transcripts that undergo nonsense-mediated decay. Exons downstream of exon 1 appear to be processed correctly. It seems unlikely that the loss of exon 1 would cause a hypomorphic mutation because exon 1 contains the translation start site, the signal sequence and the majority of the protein’s extracellular amino terminus. It would be unlikely that GPR179, a 7 TM spanning protein could have any function without these critical domains.

**In situ hybridization localizes Gpr179 mRNA to the INL of the retina**

My RT-PCR experiments had determined that Gpr179 is expressed in the retina, but it was not known by which cells. To localize Gpr179 gene expression I used *in situ* hybridization (ISH) on fixed retinal slices. Antisense digoxigenin (DIG) labeled RNA probes were created by cloning a ~800 bp cDNA fragment encoded by Gpr179 into a pSPT18 vector and performing *in vitro* transcription using T7 RNA polymerase. The ISH was performed on fixed retinal slices and visualized by probing for the DIG RNA with horseradish peroxidase (HRP) labeled antibodies followed by Cy3 tyramide signal amplification (TSA). Immunostaining with PKC antibodies, which labels the rod BCs, was performed to give a cellular reference to identify in which cells the RNA was expressed.

The Gpr179 antisense probes annealed in the INL of the retina where the bipolar cell bodies reside (Fig. 11 A). The antisense probes did not anneal to any of the other nuclear layers. As a positive control, antisense probes were designed against Grm6 mRNA, the glutamate receptor expressed in the DBCs with a known ISH expression profile (Kim et al., 2008). The antisense Grm6 DIG probes labeled the INL of the retina as identified by co-labeling with PKC and gave a similar staining pattern as Gpr179 (Fig. 11A,B). These data suggest Gpr179 is expressed in the DBCs of the retina.
Figure 11: GPR179 is expressed in bipolar cells. *In situ* hybridization localizes Gpr179 mRNA expression. (Top) Antisense Gpr179 probes (red) hybridize to the INL and expression overlaps with the rod BC bodies (green). Gpr179 expression mirrors the mRNA expression of Grm6 (bottom), a gene that is solely expressed in the DBCs of the retina.
GPR179 is expressed on the DBC dendritic tips in human retinas

To localize GPR179 protein expression in the retina, immunostaining was performed using a commercially available antibody designed against a peptide (HPA017885 Sigma) that is located in the amino terminus of the extracellular domain of human GPR179. The human peptide shares 84% sequence identity with the orthologous mouse peptide. Immunostaining was performed on WT C57BL6/J retinal sections while co-labeling with peanut agglutinin (PNA), which binds to a lectin on cone pedicles in the OPL (Blanks et al., 1988; Haverkamp et al., 2001). The staining for GPR179 showed a staining pattern inconsistent with the known pattern of proteins expressed in the DBCs (not shown). Further, the pattern in WT and nob5 was indistinguishable suggesting the antibody was not recognizing mouse GPR179 (data not shown).

To determine if the antibody used above gave the predicted staining in human, immunostaining was performed on transverse human retinal sections. GPR179 staining in the human retinas showed a punctate staining pattern in the OPL typical of other proteins that are part of the DBC signal transduction cascade (Fig. 12). Because the antibody only stained puncta, I could not be determine if the staining was pre synaptic or post synaptic, but taken with the ISH data and ERG phenotype of human patients with mutations in GPR179, I predict it will be located post-synaptically. This was confirmed recently by immuno-electron microscopy of human retinas (Klooster et al., 2013). These data also indicate that the staining in the mouse retina by the human GPR179 antibody is non-specific. To study GPR179 protein expression in the mouse retina an antibody had to be developed that recognized the mouse protein.
Figure 12: GPR179 is expressed in the OPL in human retina. GPR179 labeling (green) and DAPI (blue) in fixed transverse retinal sections from human retina gives a punctate staining pattern typical of proteins in the glutamate signaling cascade of the DBCs.
GPR179 staining is absent in the OPL of the nob5 retina

Due to the robust labeling of the human antibody in human retina, we used a portion of the orthologous mouse peptide sequence as the antigen (KVQETPGEHLDRPVKLQR) for generation of our mouse antibody. Double labeling using antibodies against PKC, a rod bipolar cell marker, and the GPR179 antibody specific to mouse shows that in WT mouse transverse retinal sections GPR179 shows punctate staining at the tips of the rod bipolar cells in the OPL (Fig. 13), consistent with the pattern seen in the human retina (Fig. 12), and with other proteins expressed at the tips of the DBCs including TRPM1, GRM6 and NYX (Gregg et al., 2007; McCall and Gregg, 2008; Koike et al., 2010b; Orlandi et al., 2012; Peachey et al., 2012b; Zeitz et al., 2013b). Immunostaining of the nob5 retinas with PKC and the mouse GPR179 antibody revealed the absence of GPR179 expression in the OPL of the nob5 retina (Fig. 12) and (Peachey et al., 2012b). These data indicate that GPR179 is expressed in the DBCs and localizes to the DBC dendritic tips. The mutation in Gpr179 in the nob5 mouse disrupts GPR179 protein expression and further supports the hypothesis that GPR179 is required for DBC function.
Figure 13: GPR179 immunostaining in WT and nob5 retinal slices. (A) WT retinas were labeled with antibodies against PKC (red) and GPR179 (green). WT retinas show robust labeling of GPR179 in the OPL. (B) In contrast, nob5 retinas are devoid of GPR179 staining in the OPL.
GPR179 is expressed at the tips of the DBCs

To determine a more precise localization for GPR179 expression I co-labeled retinas with antibodies against GPR179 and several that label other cell type and have dendrites or axon terminals in the OPL. The horizontal cells form invaginating synapses with rod and cone photoreceptors, along with the ON bipolar cells (Kolb, 1970). To determine if GPR179 was expressed in the horizontal cells of the retina I double labeled retinal sections for GPR179 and calbindin, a horizontal cell marker. The staining shows GPR179 expression is juxtaposed to calbindin staining in the OPL, but they do not colocalize (Fig. 14A). This result indicates that GPR179 is not expressed in the horizontal cells (Fig. 14A).

To determine if GPR179 is expressed in the Müller cell processes in the OPL retinas were double labeled for GPR179 and glutathione synthetase (GS) a Müller cell marker. Müller cells have processes throughout the INL and OPL that aid in uptake and release of neurotransmitters, ions and other metabolic molecules (Bringmann et al., 2006; Bringmann et al., 2009; Agte et al., 2011). The immunostaining shows that GPR179 expression is restricted to the tips of the DBCs in the OPL and does not colocalize with GS (Fig. 14B). There also is no GPR179 staining in the vicinity of the Müller cell endfeet (Fig. 14B).

GPR179 was expected to be expressed post-synaptically on the DBCs. The ISH data along with the normal ERG a-wave and absent b-wave suggested the protein was expressed in the DBCs. To further test this hypothesis I double labeled fixed transverse retinal sections with the presynaptic marker RIBEYE, which stains ribbon synapses, and GPR179 (Fig. 14C). RIBEYE is expressed pre-synaptically in the OPL and forms a “horseshoe” shaped pattern at the axon terminals of rod BCs. The immunostaining shows that GPR179 and RIBEYE do not colocalize, but appose one another, indicating
that GPR179 is expressed post-synaptically (Fig. 14C) and (Orlandi et al., 2012; Peachey et al., 2012b).
Figure 14: GPR179 is expressed post-synaptically at the tips of the DBCs. (A) WT retinas are labeled with antibodies against GPR179 (green) and calbindin (red). Visualization of labeled proteins shows GPR179 and calbindin staining do not colocalize. (B) WT retinas are double labeled with antibodies against GPR179 (green), PKC (red) and GS (blue). The PKC staining localizes the GPR179 puncta at the tips of the rod BCs in the OPL. GPR179 labeling does not colocalize with GS labeling indicating that GPR179 is not expressed in the müller cells. (C) WT retinas are double labeled with antibodies against GPR179 (green) and RIBEYE (red). The RIBEYE staining localizes GPR179 to the postsynaptic side of the synapse.
If GPR179 is expressed on the tips of the DBCs it should colocalize with mGluR6. Double labeling transverse retinal sections with antibodies against GPR179 and mGluR6 followed by confocal microscopy shows robust colocalization in the OPL (Fig 15). Because mGluR6 is known to colocalize with TRPM1 (Koike et al., 2010b) and TRPM1 with nyctalopin (Pearring et al., 2011), GPR179 is expected to colocalize with nyctalopin and TRPM1. These data support the hypothesis that GPR179 is expressed at the tips of the DBCs and is part of the postsynaptic signalplex, which is the supramolecular complex of proteins involved in signal transmission.
Figure 15: GPR179 colocalizes with mGluR6. (A) WT transverse retinal sections were labeled with antibodies against GPR179 (green) and mGluR6 (red) and visualized using confocal microscopy. GPR179 puncta appear to overlap with all mGluR6 puncta. (B) A 2.4 µm line was drawn through a puncta and fluorescence was measure across the line. The GPR179 signal colocalizes with mGluR6. Scale bar represents 5 µm.
Morpholino Knockdown of GPR179 greatly reduces ERG b-wave

To determine the effect of disruption of GPR179 protein translation on zebrafish vision, we used morpholinos to knockdown GPR179 expression. Morpholinos are antisense oligomers made complementary to an mRNA sequence that interferes with either protein translation or RNA processing (Bill et al., 2009).

GPR179 blocking morpholinos (Gene Tools, Philomath, OR) were injected into single cell embryos and ERGs were recorded 4-6 days post injection. The zebrafish injected with GPR179 blocking morpholinos show a significantly decreased b-wave compared to those injected with scrambled morpholino at all flash intensities (Fig. 16) and (Peachey et al., 2012b). Morpholinos against nyctalopin were injected as a positive control (Bahadori et al., 2006) and show a reduced b-wave, similar in magnitude to the morpholinos directed against GPR179 (Fig. 16) (Peachey et al., 2012b).
Figure 16: Morpholino knockdown of GPR179 in zebrafish. (A) ERG responses to various flash intensity stimuli from zebrafish injected with scrambled morpholinos (left). ERG responses to various flash intensity stimuli injected with morpholinos against GPR179 (right). ERG b-wave is severely decreased in zebrafish injected with morpholinos against GPR179 compared to scrambled morpholinos. (B) Morpholinos against nyctalopin cause a similar decrease in b-wave amplitude (Peachey et al., 2012b)
Human CSNB patients identified with mutations in *Gpr179*

To determine if human cCSNB patients had mutations in *GPR179* our collaborators screened all 11 exons of *Gpr179* in 44 patients with cCSNB of unknown cause. Two unrelated probands were identified with putative inactivating mutations (Fig. 17B,C) (Peachey et al., 2012b). The first proband was a compound heterozygote that carried two frameshift mutations, pLeu63Serfs*12* and pSer329Leufs*4*, terminating translation (Fig. 17B). The second proband had the pSer329Leufs*4*, the same as proband 1 on one allele and a missense mutation pTyr220Cys on the other allele (Fig. 17C). The missense mutation changes an amino acid in a highly conserved region of the protein and is predicted to be a null allele (Fig. 17D). Neither of the probands had other known health problems. In addition to the two patients our collaborators identified, Catherine Zeitz’s group identified five cCSNB patients with mutations in *Gpr179* (Audo et al., 2012).
Figure 17: Human cCSNB patients with mutations in Gpr179. (A) ERGs from patient with cCSNB and mutations in Gpr179. (B) Proband 1 is a compound heterozygote with two frameshift mutations. (C) Proband 2 is a compound heterozygote with frameshift mutation and a missense mutation. (D) The missense mutation changes an amino acid in a highly conserved region of the protein. From (Peachey et al., 2012b).
**GPR179 knockout**

The \textit{Gpr179}\textsuperscript{nob5} mouse is an insertion mutation and there is the possibility that a small amount of normal splicing does occur, making this a hypomorphic allele. To determine if the \textit{Gpr179}\textsuperscript{nob5} mouse is a null we will compare the phenotype to a \textit{Gpr179} knockout mouse. A \textit{Gpr179} knockout mouse is currently in the pipeline. Embryonic stem cells containing a conditional knockout allele were available from the University of California Davis Knockout Mouse Project (KOMP) Repository. The \textit{Gpr179}\textsuperscript{tm1e(KOMP)Mbp} have conditional potential because LoxP sites were engineered into the gene and flank exon 2. The deletion of exon 2, which encodes 109 nucleotides in the gene will cause a frameshift mutation, resulting in a truncated protein, which would most likely undergo nonsense-mediated decay. The ES cells were purchased and used to create a mouse line at the University of Cincinnati. The colony is currently being established at the University of Louisville. We currently have 1 viable mouse carrying the knockout allele that we are breeding. Once the knockout allele is made homozygous, an ERG will be performed to confirm GPR179 is critical for DBC function.

**Discussion**

It was previously thought that the major components in the DBC signaling cascade had been identified such as the glutamate receptor mGluR6 and the cation channel TRPM1. Much of the recent effort has been to identify the molecules that are activated by the mGluR6 receptor such as G proteins and second messengers that are responsible for gating of the TRPM1 channel (Okawa et al., 2010; Dhingra et al., 2012; Shen et al., 2012). In this chapter I have shown that a novel GPCR, GPR179, is the likely cause of cCSNB in human patients and is mutated in a new model of cCSNB (Peachey et al., 2012b). The discovery of a second GPCR in the system adds another level of complexity to the signaling mechanism and appears to be quite unique among GPCR cascades.
GPR179 is classified as a class C orphan GPCR based on sequence identity (Rondard et al., 2011). Despite this categorization, it does not share the typical characteristics of other Class C receptors. *In silico* analyses of GPR179 shows it lacks the extracellular venus fly trap (VFT) ligand binding domain that the vast majority of class C GPCRs possess (Fig. 18), including mGluR6 (Soding, 2005). The VFT domain is required for ligand binding and plays a role in receptor dimerization (Ray et al., 1999; Awatramani and Slaughter, 2000; Kennedy et al., 2004). The extracellular amino terminus of GPR179 contains a calcium-binding EGF-like domain of unknown function. This domain is commonly found in membrane bound or extracellular proteins that require calcium for their tertiary protein structure, which is thought to facilitate protein interactions (Selander-Sunnerhagen et al., 1992).

The carboxy terminus IC domain of GPR179 is unique among Class C receptors. The IC domain is large (~1700 amino acids) and composed of a highly repetitive amino acid sequence CPWE that is conserved across species. This sequence does not share homology with other known proteins; therefore, the function is unknown. The tertiary structure of the carboxy terminus of GPR179 is predicted to be disordered and contain several internal repeats (Letunic et al., 2012). The IC loops of GPR179 are predicted to couple to G_{i/o} G proteins (Sgourakis et al., 2005a; Sgourakis et al., 2005b), potentially allowing it to activate G proteins in the DBCs.

Interestingly, none of the *Gpr179* mutations reported to date in patients with cCSNB reside in the carboxy terminus (Audo et al., 2012; Peachey et al., 2012b), which comprises 73% of the amino acid sequence. Perhaps mutations in the carboxy terminus of GPR179 that inhibit function are more rare because the it is highly redundant and has enough structural flexibility that a missense or nonsense mutation is unlikely to adversely affect function. This suggests that the extracellular amino terminus is critical to function because mutations in the extracellular amino terminus cause cCSNB.
Figure 18: Protein domains of GPR179. GPR179 is predicted to be a 7 TM protein with an extracellular calcium-binding EGF like domain. The large cytoplasmic carboxy terminus of GPR179 contains intermittent regions of low complexity. Domains predicted using SMART 7 (Letunic et al., 2012).
There are a number of important directions for future studies on GPR179. One direction that needs to be explored is the expression profile of Gpr179 in other tissues. Despite the fact that nob5 shows no other phenotypes and human patients with mutations in Gpr179 are healthy (Audo et al., 2012; Peachey et al., 2012b), it is possible that Gpr179 is expressed in other tissues and cell types. Finding Gpr179 in other cell types may allow a tissue culture system to be utilized with endogenous expression of Gpr179, allowing its function to be more easily studied.

In conclusion, we mapped the mutation responsible for DBC dysfunction in the nob5 mouse. Using next generation sequencing we identified a new gene, Gpr179, critical to glutamate signaling in the DBCs. We found human patients with cCSNB carrying mutations in GPR179, validating our hypothesis that Gpr179 is critical to DBC function and we localized mRNA and protein expression to the DBCs using ISH and immunohistochemistry. A Gpr179−/− mouse was created to confirm Gpr179 is the cause of DBC dysfunction in the nob5 mouse and that the nob5 mouse is not a hypomorph.

Methods

Animals

All procedures involving animals were performed in accordance with the policies on the use of animals in research and each local Institutional Animal Care Use Committees. Descriptions of all mice used have been published previously (Masu et al., 1995; Pardue et al., 1998; Gregg et al., 2007; Pearring et al., 2011; Cao et al., 2012; Peachey et al., 2012b) and each line was either generated on, or backcrossed onto a C57BL6/J background for at least 6 generations. All mice were housed in local AAALAC approved facilities under a 12 h light/12 h dark cycle. Mice were euthanized by CO2 inhalation followed by cervical dislocation.

DNA isolation
For mouse genotyping, 5mm tail biopsies were collected from mice and placed in 0.7 mL microcentrifuge tube, and lysed with 200 µL of DirectPCR (Viagen Biotech) containing 2 µL proteinase K (20 mg/ml). Samples were incubated for at least 4 h at 55°C in a dry bath with brief vortexing every h. After the tail sample was adequately digested, proteinase K was inactivated by heating to 85°C for 30 min. Samples were then ready for PCR.

For next generation sequencing, DNA was isolated from mouse liver. Liver samples were collected from mice and placed into a 1.5 mL microcentrifuge tube and digested in 300 µl of cell lysis solution (Qiagen) plus 3 µl proteinase K (20mg/ml). Samples were incubated at least 4 h and vortexed until fully digested. Protein was precipitated from solution using protein precipitation solution (Qiagen) and DNA was recovered by ethanol precipitation.

**General Polymerase Chain Reaction**

Polymerase Chain Reaction (PCR) was generally performed using Phire II or Phusion taq polymerase (Thermo Scientific). Reactions were made to 20 µl consisting of 0.5 µM of each forward and reverse primers, 200 µM of dNTPs, 4 µl of 5X reaction buffer, and either 0.4 µl of Phire II or Phusion DNA Polymerase. Template DNA amount and water varied by application. Reactions were run on a Bio Rad C1000 Touch Thermal Cycler.

**High Resolution Melting analysis**

High Resolution Melting (HRM) analyses were performed according to Applied Biosystems High Resolution Melting Getting Started Guide (Applied Biosystems). Briefly, primers were designed by the web program Primer3 (Untergasser et al., 2012) to flank a SNP and produce a 60-100bp amplicon product. Reactions were set up in 96-well Fast reaction plates with a 10 µl reaction volume consisting of 5 µm of each forward and reverse primers, 5 µl of MeltDoctor™ 2X HRM Master Mix (Applied Biosystems), 1
μl of genomic DNA and PCR grade water to 10 μl. The reaction was run on a 96-well Fast plate on an Applied Biosystems 7900 HT Fast Real-Time PCR system. After PCR amplification DNA was denatured at 95°C and annealed at 37°C before high resolution melting at 1% ramp rate on a 96-well Fast block. Melting curves were analyzed using HRMv2.0.1 software.

**Next Generation Sequencing**

Samples were prepared using the Illumina protocol “Preparing Samples for Paired-End Sequencing (Part# 1005063 Rev. A June 2008). Genomic DNA samples were sheared to an average size of ~300bp using sonication. DNA fragment ends were repaired and phosphorylated using Klenow, T4 DNA Polymerase and T4 Polynucleotide Kinase. An adenine base was added to the 3’ end of the blunted fragments, followed by ligation of Illumina Paired-End adapters via T-A mediated ligation. The library was hybridized to the sequence capture array on a Nimlegen Hybridization System 4 for ~64 h at 42°C. The array was washed to remove non-hybridized DNA and captured DNA was eluted using the Nimblegen Elution System. The eluted DNA was amplified by PCR using Illumina Paired-End primers and size selected for 300bp fragments via gel electrophoresis. The library size and concentration were determined using an Agilent Bioanalyzer.

**RNA isolation from Retina**

Mice were euthanized by CO₂ inhalation and cervical dislocation. Eyes were extracted and enucleated and the retinas were removed and stored at -80°C in RNALater (Ambion) until needed. RNA was isolated according to manufacturers protocol for the PureLink® RNA Mini Kit (Ambion). Briefly, retinal tissue was removed from RNALater and homogenized in 10-20 volumes of Tri Reagent (Ambion) by pipetting. The homogenized mixture was incubated for 5 min at room temperature then 200 μl chloroform per ml of homogenate was added and the mixture vortexed, and incubated
for an additional 5 min at room temperature; followed by centrifugation at 12,000 x g for 10 min at 4°C. The aqueous phase volume was transferred to a new 1.5 ml centrifuge tube and 200 µl of 100% ethanol was added to the sample and vortexed for 15 sec. The sample was placed in a filter cartridge (Ambion) and centrifuged at 12,000 x g for 30 sec. Flow through was discarded and 500 µl wash solution was applied to the filter cartridge and centrifuged at 12,000 x g for 30 sec. The wash step was repeated and the filter cartridge was transferred to a new microcentrifuge tube and 100 µl of Elution buffer was applied to elute the RNA. The RNA was collected by centrifugation for 30 sec at 12,000 x g. The RNA was stored at -80°C.

**Antibodies**

The following primary antibodies and their concentrations were used: sheep anti-GPR179 (peptide KVQEETPGEDLDRPVLQKR), 1:2,000 (Peachey et al., 2012b); mouse monoclonal anti-ctbp2/Ribeye (BD Bioscience), 1:1,000; guinea pig anti-mGluR6 1:1,000 (Koike et al., 2010b); sheep anti-TRPM1 1:1,000 (Cao et al., 2011); rabbit anti-GFP (MBL), 1:800 and Rhodamine Peanut Aglutinin (PNA) conjugate 566 (Vector Labs), 1:1,000; goat anti-Gβ5 (peptide MATDGLHENETLASLKC), 1:1,000 (Morgans et al., 2007); goat anti-R9AP 1:1,000 (Hu and Wensel, 2002); mouse anti-DIG HRP, 1:1,000 (Perkin Elmer); mouse anti-GFP, 1:1,000 (Molecular Probes); sheep anti-RGS11 (peptide CSPALQSTPREAPATSSPEGADGE), 1:1,000 (Martemyanov et al., 2005); sheep anti-RGS7 1:1,000 (Morgans et al., 2007); mouse anti-RIBEYE 1:1,000 (BD Transduction Labs); rabbit anti-PKC 1:1,000 (Sigma Aldrich).

Secondary antibodies (Invitrogen, 1:1,000) appropriate to each primary antibody included: donkey anti-sheep Alexa-488, donkey anti-rabbit Alexa-680, donkey anti-rabbit Alexa-546, donkey anti-mouse Alexa-647 and donkey anti-guinea pig Cy3 (Millipore, 1:1,000). In lieu of an antibody specific to nyctalopin, we used Tg(Gabbr1-YFP/nyx)Tgg
transgenic mice that express a yellow fluorescent protein (YFP) tagged nyctalopin (Gregg et al., 2007). They are labeled WT in the figures.

**Retinal dissections for in situ hybridization**

Mice were sacrificed by carbon dioxide gas inhalation followed by cervical dislocation. The eyes were enucleated and the cornea, iris and lens were removed and the eyecup was rinsed in ice cold PBS-Diethylpyrocarbonate (Diethylpyrocarbonate (DEP) was added to PBS at a dilution of 1:1,000, solution was mixed vigorously, incubated overnight then autoclaved). Eyecups were immediately fixed in 4% formaldehyde PBS-DEPC for 1 h at room temperature. Eyecups were then washed twice in PBS-DEPC then cryoprotected in 15% sucrose PBS-DEPC at 4°C until sucrose fully penetrated and were then moved into a 30% sucrose PBS-DEPC solution at 4°C overnight.

**DIG labeling RNA**

DIG RNA labeling was performed as described in the Roche DIG RNA Labeling Kit (SP6/T7). In brief, purified template DNA (1 µg) was added to a sterile, RNase-free PCR tube. The volume was brought to 13 µl with sterile, RNase free DEPC treated water. To the reaction tube, 2 µl of 10X NTP labeling mixture, 2 µl of 10X Transcription buffer, 1 µl of Protector RNase inhibitor, and either SP6 or T7 RNA Polymerase (2 µl) were added and mixed gently. Reaction was incubated at 37°C for 2 h on a PCR thermal cycler. DNase I (2 µl) was added to the reaction tube and incubated for 15 min at 37°C. RNA was analyzed by native agarose gel electrophoresis. The reaction volume was brought to 100 µl with sterile, RNase free, DEPC treated water containing 10 mM DTT and stored at -80°C.

**In situ hybridization**

Day One
Slides were removed from -80°C storage and dried at 65°C in hybridization oven for 30 min. Sections were fixed for 20 min at -20°C in methanol then washed three times for 5 min each with PBST-DEPC (PBS-DEPC + 0.1% tween twenty). Slides were incubated in proteinase K solution (1.2µg/ml Proteinase K in 50mM Tris-HCl pH 7.5 + 5mM EDTA) for 12 min, washed in PBST for 5 min and fixed again with 4% formaldehyde PBS-DEPC for 5 min. Sections were rinsed in PBST-DEPC then washed 2 more times for 5 min. Sections were then acetylated in acetylation solution (73.75mL H$_2$O-DEPC, 1mL Triethanolamine, 135µL HCl and 188 µL acetic anhydride). Sections were washed in PBST-DEPC 3 times for 5 min then incubated in PBST-DEPC + 3% H$_2$O$_2$ for 30 min. Sections were air dried for ~10 minutes. Subsequently hybridization solution was removed from the freezer and brought to 65°C. DIG RNA probes were thawed on ice. Probes were diluted 1:100 in 100 µL of hybridization solution and heat denatured for 10 min at 85°C. Sections were placed in hybridization chamber that was dampened with 50% formamide/5X SSC. Probe solution was removed from heat and 100 µL was placed directly on slide and a glass coverslip was added to slide. Sections were incubated overnight in a hybridization oven at 65°C.

Day Two

The coverslip was removed by immersing the slide in 2X SSC and slides were incubated in fresh 2X SSC for 20 min at 65°C, then were washed in coplin jars containing 0.2X SSC at 65°C over a period of 3-4 h, changing the solution every 30 min. Slides were then washed 3 times for 5 min in maleic acid buffer supplemented with 0.1% Tween-20 (MABT, pH 7.5) then incubated for 1 h in MABT + 5% normal donkey serum. Mouse anti DIG HRP antibody was diluted 1:1000 and added to slides and incubated overnight in humidity chamber at 4°C. If double labeling with a cell marker antibody, it was applied to the solution at this time.

Day Three
Slides were washed 3 X 10 min in PBST. Cy3-TSA staining solution (Roche) was prepared at 1:100 dilution and immediately added to slides and incubated for 50 min. Sections were washed 3 X 10 min in PBST. If fluorescent staining for a cell marker, dilute fluorescent antibody was incubated for 1 h at room temperature then was 3 X 10 min in PBST. Slides were mounted with glass coverslips using Immu-Mount (Thermo Scientific) and imaged on a FV-1000 confocal microscope.

Immunohistochemistry

Mice were euthanized with CO$_2$ followed by cervical dislocation. Their eyes were enucleated and the lens was removed. Eyecups were washed in phosphate buffered saline (PBS) then fixed for 30 min in a 4% paraformaldehyde PBS solution (pH 7.4). Eyecups were washed 3 times with PBS then cryoprotected in increasing concentrations of sucrose in PBS (10%, 15% for 1 h each and 30% overnight). Eyecups were embedded in 2:1 OCT/20% sucrose PBS solution frozen in a liquid nitrogen cooled bath of isopentane. Eyecups were sectioned (18 µm) using a Leica 1850 cryostat. Sections were mounted on glass slides (SuperFrost) and allowed to dry for 10 minutes at room temperature before being stored at -80°C. Sections were air dried at 37°C for 15 min and washed with PBS and PBS containing 0.05% Triton X-100 (PBX) for 5 min each, then blocked in blocking solution (PBX plus 5% normal donkey serum) for 1 h. Sections were incubated overnight at room temperature in the presence of the primary antibody diluted in blocking solution, then washed 3 times for 10 min each with PBX followed by incubation in secondary antibody in blocking solution for 1 h at room temperature. Sections were washed 2 X 10 min in PBX and 1 X PBS. Slides were cover-slipped using Immu-Mount (Thermo Scientific) and slides were imaged using an Olympus FV1000 confocal microscope. Images were universally adjusted for brightness using Photoshop (Adobe).
CHAPTER III: CONSTRUCTING THE DEPOLARIZING BIPOLAR CELL SIGNALPLEX

**Aim 2:** Determine signlaplex architecture and the role of GPR179 by identifying the interplay of DBC signalplex proteins in various mouse models of DBC dysfunction.

**Rationale:** All known mouse models of cCSNB have normal retinal structure (McCall and Gregg, 2008) but the synapse proteome is often affected by the loss of one or more proteins (Maddox et al., 2008; Jeffrey et al., 2010; Cao et al., 2011; Pearring et al., 2011; Orlandi et al., 2012; Xu et al., 2012). Many of the proteins expressed at the dendritic tips of the DBCs are dependent on expression of other proteins for trafficking or localization (Jeffrey et al., 2010; Cao et al., 2011; Pearring et al., 2011; Xu et al., 2012). Proteins co-dependent on one another for trafficking and localization often interact. To understand the arrangement of signalplex proteins in the DBCs, we can use immunohistochemistry and confocal microscopy to determine their interdependency for localization. Elucidating signalplex architecture is a step toward understanding the signaling mechanism that couples mGluR6 to the TRPM1 channel.

**Hypothesis:** GPR179 is a protein hub and is responsible for the recruitment and localization of signalplex proteins in the DBCs.

**Introduction**

The ability of cells to respond to external stimuli is fundamental to life and is possessed by all cells from the simplest of single cell organisms to all cells in vertebrates. At the most fundamental level, this ability requires the expression of a membrane receptor and its associated intracellular proteins. In neuronal systems, it is imperative that the postsynaptic neuron is able to faithfully relay the message from the
presynaptic neuron, or when necessary, parse the neuronal code to relay a subset of the presynaptic message. To achieve this, the postsynaptic neuron must express a highly ordered and efficient signaling complex that captures the external signal and instructs the cell to respond in an appropriate manner.

The ~12 types of BCs in the retina respond to light induced decreases in glutamate release from the photoreceptors in four general ways, a transient hyperpolarizing response, a sustained hyperpolarizing response, a transient depolarizing response and a sustained depolarizing response (Werblin and Dowling, 1969; Kaneko, 1970). Each of these responses requires a unique set of receptors and signaling proteins expressed at the plasma membrane of the postsynaptic dendrite (Masu et al., 1995; DeVries and Schwartz, 1999; DeVries, 2000). Although physiologists have described the BC responses in detail, the molecular architecture of signaling proteins that give rise to these responses is largely unknown.

Rod BCs respond to a decrease in glutamate release from the photoreceptors with a transient depolarizing response (Dacheux and Raviola, 1986). Because ~95% of the photoreceptors in the human retina are rods (Osterberg, 1937; Curcio et al., 1990) and rods only synapse with rod BCs, understanding how information is processed in the rod BCs denotes an understanding of how the majority of the ON BCs respond to light. Determining the interdependency of signalplex proteins should offer clues as to how the proteins are arranged at the synapse and how they may carry out signal transduction.

The glutamate receptor on the rod BCs is mGluR6 and binds glutamate, which activates a G protein cascade by promoting GαoGDP to GαoGTP exchange. Activation of Gαo results in dissociation of the Gβγ complex. It is understood that one of the G protein subunits, Gαo or Gβγ, is responsible for gating the TRPM1 channel closed, by either direct interaction or though an effector protein. Recently, Shen et al. (2012)
published that the Gβγ dimer is responsible for gating the TRPM1 channel through direct interaction or an effector protein.

The RGS proteins, which inactivate GαGTP are known to be critical to DBC function but little is known about their molecular arrangement. The R7 family of RGS proteins (RGS6, RGS7, RGS9 and RGS11) are GTPase activating proteins (GAP) and are expressed almost exclusively throughout the nervous system. They are critical for setting the speed of G-protein signaling cascades in general because they speed up GαGTP to GαGDP hydrolysis (Koelle and Horvitz, 1996; Gold et al., 1997; Berman and Gilman, 1998; Burchett, 2000; Ross and Wilkie, 2000). The molecular arrangement of these proteins including interaction with a membrane anchor is critical to their function (Porter and Koelle, 2010). They are thought to organize into heterotrimeric complexes consisting of a membrane anchor protein, Gβ5 and the RGS protein (Cabrera et al., 1998; Anderson et al., 2009). There are two RGS membrane anchors expressed in DBCs, R9AP and R7BP (Cao et al., 2008; Jeffrey et al., 2010).

Several RGS knockout mouse models have been created to understand the arrangement and function of RGS proteins in the DBCs. The \( R9AP^{-/-}, R7BP^{-/-} \) and \( RGS7^{-/-}/RGS11^{-/-} \) mouse models were all intended to disrupt DBC function but only the \( RGS7^{-/-}/RGS11^{-/-} \) mouse succeeded (Cao et al., 2008; Cao et al., 2009; Jeffrey et al., 2010; Cao et al., 2012). From these models we learned RGS7 and RGS11 are the main GAP proteins in the DBCs (Cao et al., 2012) but the membrane anchors R9AP and R7BP are not the only RGS anchors (Cao et al., 2008; Jeffrey et al., 2010). In the \( R9AP^{-/-} \) retina, RGS11 did not localize properly at the dendritic tips of the DBCs but RGS7/Gβ5 did (Jeffrey et al., 2010). R7BP deletion did not affect the localization of RGS7, RGS11 or Gβ5 (Cao et al., 2008). These data indicate that in the DBCs there is new mechanism for RGS protein localization to the DBC dendritic tips or R7BP and R9AP are redundant to one another.
To better understand the architecture of the DBC signaling proteins I used mutant mice with DBC dysfunction to examine synaptic protein localization using immunohistochemistry and confocal microscopy. The proteins mGluR6, GPR179, TRPM1, RGS11, RGS7, nyctalopin and R9AP were examined in all of the available mouse models of DBC dysfunction. Proteins that were co-dependent for localization at the DBC dendritic tips were examined for interaction using Duolink and Membrane Yeast Two-Hybrid (MYTH) assay. From these data, a model of signalplex protein arrangement was built.

Results

GPR179 localizes to the DBC dendritic tips in mouse models of DBC dysfunction

To determine if GPR179 is localized at the dendritic tips of the DBCs in the absence of other signalplex proteins, it was visualized by immunohistochemistry followed by confocal microscopy in cCSNB mouse models. Fixed transverse retinal sections were prepared from WT, Grm6\(^{-/-}\), Trpm1\(^{-/-}\), RGS7\(^{-/-}\)/RGS11\(^{-/-}\) and Nyx\(^{noc}\) mice and were labeled with antibodies against GPR179 and the lectin peanut agglutinin (PNA). PNA labels the cone pedicles in the OPL and co-localizes with postsynaptic signalplex proteins on cone ON BCs (Blanks et al., 1988; Haverkamp et al., 2001). Visualization of the labeled proteins using confocal microscopy revealed that GPR179 is properly localized to the DBC dendritic tips in the Grm6\(^{-/-}\), Trpm1\(^{-/-}\), RGS7\(^{-/-}\)/RGS11\(^{-/-}\) and Nyx\(^{noc}\) retinas (Fig. 19). These data indicate that GPR179 is not dependent on mGluR6, TRPM1, RGS7/RGS11 or nyctalopin for proper localization at the DBC dendritic tips. It also indicates that protein localization of GPR179 is independent of normal DBC function.
Figure 19: GPR179 expression in various mouse models of DBC dysfunction. (A-E) GPR179 (green) localizes to the DBC dendritic tips and co-localizes with PNA (red) in the mouse models of DBC dysfunction (B) Grm6<sup>−/−</sup>, (C) Nyx<sup>nob</sup>, (D) Trpm1<sup>−/−</sup> and (E) RGS7<sup>−/−</sup>/RGS11<sup>−/−</sup>. (F) GPR179 expression is absent in the Gpr179<sup>nob5</sup> retina.
The $Gpr179^{nob5}$ mouse retina retains key signaling components including mGluR6, TRPM1 and nyctalopin

To determine the dependency of signalplex proteins on the expression of GPR179, I used the $Gpr179^{nob5}$ mouse model, which lacks expression of GPR179 at the DBC dendritic tips (Fig. 17 and Peachey et al. (2012b)). Because we do not have a suitable antibody against nyctalopin for immunohistochemistry, I crossed the $Gpr179^{nob5}$ with the $Tg(Gabr1-YFP/nyx)^{Rgg1}$ mouse, which expresses a yellow fluorescent protein (YFP) tagged nyctalopin (Gregg et al., 2007) to visualize nyctalopin expression in the $Gpr179^{nob5}$ mouse. Fixed transverse retinal sections from $Gpr179^{nob5}$ mice were prepared in conjunction with WT retinas and labeled with antibodies against mGluR6, TRPM1 and YFP-nyctalopin. The confocal microscopy revealed that mGluR6, TRPM1 and nyctalopin are properly localized on the DBC dendritic tips in the absence of GPR179 (Fig. 20). These data indicate GPR179 expression is not critical for the localization of mGluR6, TRPM1 and nyctalopin at the DBC dendritic tips.
Figure 20: Expression of major signaling components in the $Gpr179^{nob5}$ retina. Expression of TRPM1 (green), mGluR6 (blue) and EYFP-Nyx (red) in WT (A) and $Gpr179^{nob5}$ (B) retinas. Protein expression is not different compared to WT. Scale bar is 5 µm. From (Ray et al., 2013)
The absence of TRPM1 does not affect other signalplex proteins

Localization of signalplex proteins was examined in $Trpm1^{-/-}$ retinas. The proteins mGluR6 and nyctalopin are known to be expressed and localize correctly in the $Trpm1^{-/-}$ DBCs (Pearring et al., 2011) and I showed that GPR179 expression and localization is not dependent on normal TRPM1 expression (Fig. 19). To determine if other signalplex proteins are dependent on TRPM1 expression for proper localization, immunohistochemical staining for RGS7, RGS11, R9AP, and Gβ5 was performed on fixed transverse retinal sections from $Trpm1^{-/-}$ mice and visualized by confocal microscopy. In the $Trpm1^{-/-}$ retinas RGS7, RGS11, R9AP, and Gβ5 all localized correctly to the DBC dendritic tips (Fig. 21), suggesting that they localize independently of TRPM1. Therefore, TRPM1, although critical for DBC function, does not play a critical structural role in the assembly of signalplex proteins.

Nyctalopin is critical for TRPM1 localization

From previous studies we know that TRPM1 is dependent on expression of nyctalopin for proper localization (Pearring et al., 2011) and the two proteins interact in vivo (Cao et al., 2011; Pearring et al., 2011). The mechanism of this dependency has not been resolved, therefore, I investigated the expression of mGluR6, GPR179, RGS7, RGS11, Gβ5 and R9AP in the Nyx$^{mob}$ mice, which lack expression of nyctalopin (Pardue et al., 2001). Immunohistochemistry on fixed transverse retinal sections followed by confocal microscopy showed that the proteins mGluR6, GPR179, RGS7, RGS11, Gβ5 and R9AP all localized properly in the absence of nyctalopin (Fig. 22). These data indicate that nyctalopin plays a specific role in recruiting the TRPM1 channel to the DBC dendritic tips but is not important for the localization of mGluR6, GPR179, RGS7, RGS11, Gβ5 or R9AP.

RGS7 and RGS11 deletion does not effect localization of TRPM1, GPR179 or mGluR6
Using the RGS$^{7/11}$ mouse model, which lacks expression of RGS7 and RGS11 in the DBCs, I examined the expression pattern of mGluR6, TRPM1, GPR179, R9AP and Gβ5. Immunohistochemical staining for mGluR6, TRPM1, GPR179, R9AP and Gβ5 was performed on fixed transverse retinal sections from RGS$^{7/11}$ mice then visualized using confocal microscopy (Fig. 22). I found that expression mGluR6, TRPM1 and GPR179 at the DBC dendritic tips was normal, and as expected Gβ5 expression was absent (Fig. 22 and Cao et al. (2012)). Surprisingly, R9AP expression was absent in the RGS$^{7/11}$ DBCs, suggesting that the formation of the complete RGS complex may be critical for complex localization.

**Localization of the RGS proteins to the DBC dendritic tips is lost in the Gpr179$^{nob5}$ mice**

To determine if the absence of GPR179 affected the localization of the RGS proteins, immunohistochemical staining for RGS7, RGS11, Gβ5 and R9AP was performed in Gpr179$^{nob5}$ fixed retinal sections and the proteins were visualized by confocal microscopy. These data revealed that RGS7 and RGS11 are absent from the DBCs of the Gpr179$^{nob5}$ retina (Fig. 21) and (Orlandi et al., 2012). In addition to RGS7 and RGS11 not localizing to the DBC dendritic tips of the Gpr179$^{nob5}$ retina, the RGS membrane anchor R9AP and Gβ5 also do not localize to the DBC dendritic tips (Fig. 21). These data suggest that GPR179 either acts as an RGS protein anchor in addition to R9AP, or GPR179 is required to recruit another unidentified RGS anchor to the DBC dendritic tips. These data also suggest that the disruption in DBC signaling in the Gpr179$^{nob5}$ retina may be the result of the RGS complex not localizing correctly.
Figure 21: Signlaplex protein expression in mouse models of DBC dysfunction I. Immunohistochemical labeling of signlaplex proteins mGluR6, TRPM1, GPR179, RGS11, RGS7, nytalopin and R9AP in fixed retinal sections from WT and cCSNB mouse models Grm6\(^{-/-}\), Gpr179\(^{nobs}\) and Trpm1\(^{-/-}\). The image of TRPM1 staining in the Grm6\(^{-/-}\) mice is adapted from (Xu et al., 2012). Open box indicates that this particular experiment has not been done. Scale bar is 5 µm.
Figure 22: Signlaplex protein expression in mouse models of DBC dysfunction II. Immunohistochemical labeling of signlaplex proteins mGluR6, TRPM1, GPR179, RGS11, RGS7, nyctalopin and R9AP in fixed retinal sections of WT and cCSNB mouse models RGS7^{−/−}/11^{−/−} and Nyx^{nob}. Data for R9AP^{−/−} mice adapted from (Cao et al., 2009). Open box indicates that this particular experiment has not been done. Scale bar is 5 µm.
Screening of GPR179 interacting partners using the Membrane Yeast-Two Hybrid

To determine the role of GPR179 in the DBC signalplex I screened for proteins that may interact with GPR179. My first attempt at identifying interacting partners relied on the membrane yeast two-hybrid (MYTH) system (Iyer et al., 2005). The MYTH system requires cloning your genes of interest into two different yeast expression vectors, bait and prey (Fig 23AB). The bait and prey vectors each contain a yeast membrane signal sequence, multiple cloning site and split ubiquitin. In addition, they contain genes involved in the de novo synthesis of leucine or tryptophan, which allows for selection of yeast carrying both plasmids on –leu/-trp selective media. The bait vector expresses your membrane protein of interest fused to the carboxy terminus of a split ubiquitin and the artificial transcription factor LexA-VP16 (Fig. 23C). The prey vector expresses a second TM protein of interest fused to the amino terminus of a split ubiquitin (Fig. 23C). The bait and prey vectors are co-transfected into yeast and plated on –Leu/-Trp selective media to select for yeast containing the bait and prey vectors. The yeast containing both plasmids are then plated on –leu/-trp/-his/-ade selective media. If the proteins interact, the split ubiquitins can dimerize, which recruits the cell's ubiquitin specific proteases to cleave the bait protein and release the transcription factor. The transcription factor will enter the nucleus and promote transcription of genes responsible for de novo synthesis of histidine and adenine in the genetically engineered NMY32 yeast strain allowing growth of yeast that contain interacting bait and prey proteins (Fig. 23C).
Figure 23: Schematic of MYTH assay. (A) Bait vector contains CYCI promoter, Cub split ubiquitin and LexA VP16 transcription factor. The bait vector also contains a gene critical for de novo leucine synthesis. (B) The prey vector contains the ADH1 promoter and NubG split ubiquitin. The prey vector also contains a gene critical for de novo synthesis of tryptophan. (C) Bait and prey vectors containing genes encoding membrane proteins are co-expressed in yeast. The bait and prey vectors also contain genes critical for de novo amino acid synthesis that allows positive selection. (D) If the bait and prey proteins interact the split ubiquitin molecule dimerizes and proteases cleave the bait protein, releasing the synthetic transcription factor. The transcription factor controls expression of genes needed for de novo synthesis of histidine and adenine in transgenic NMY32 yeast strain, allowing for positive selection of protein interaction. Modified from (Iyer et al., 2005).
To screen for interactions, Gpr179 was cloned into bait and prey vectors. The size of the Gpr179 ORF (~7.1 kb) and the size of the bait vector (~7.7 kb) made cloning difficult. Therefore, I cloned truncated genes into bait vectors that would help identify regions critical for interactions with other signalplex proteins. The clone Gpr179 TM1 contains 211-1,938 bp of the ORF and encodes a protein that contains the 7 TM domains and a truncated 17 amino acid carboxy terminus (Fig. 24A). The clone Gpr179 TM2 contains 82-2,782 bp of the ORF minus the signal sequence and encodes a protein that contains the entire amino terminus, 7 TM domain and truncated carboxy terminus (Fig. 24B). The clone Gpr179 Cterm contains 1,885-7,101 bp of the ORF and encodes the 1,739 amino acid carboxy terminus (Fig. 24C). The clone Gpr179 contains the entire ORF and encodes the full-length protein (Fig. 24D). Our lab was already in possession of prey vectors containing the genes Grm6, Trpm1, Ga, and Gβ5 which I used to screen for interactions with GPR179.

To test for correct expression and stability of the different Gpr179 bait constructs, I cotransfected them with ALG5 or FUR4 positive and negative control vectors. ALG5, localizes to the endoplasmic reticulum (ER) and FUR4 localizes to the plasma membrane (PM). The positive control vector contains the WT amino terminus of the split ubiquitin, NubI, and will reconstitute with any bait protein in the same cellular compartment allowing yeast to grow on selective media. The negative control prey vector contains a mutated version of the split ubiquitin amino terminus, NubG, and will only reconstitute with Cub if the bait and prey proteins interact. Co-transfection of Gpr179 TM1 bait vector with the positive control prey vectors yielded positive growth on selective media (Fig. 24A), indicating that the bait protein was being translated in yeast and expressed in the ER and at the PM. Co-transfection of Gpr179 TM1 bait vector with the negative control prey vectors also yielded growth on interaction selective media (Fig. 22A), indicating that the bait protein was likely self-activating. This likely occurs because
the protein is being translated but is not able to be processed properly in the yeast, resulting in proteolysis. The proteolysis liberates the transcription factor allowing the yeast to grow on interaction selective media. The other reason for growth on the selective media is that GPR179 TM1 interacts with ALG5 and FUR4. GPR179 TM1 was deemed unsuitable for screening with signalplex proteins.

Co-transfection of the Gpr179 TM2 bait vector with the positive control prey vectors yielded growth on interaction selective media (Fig. 24B), indicating that the bait protein was being translated in yeast and expressed in the ER and at the PM. Co-transfection of Gpr179 TM2 bait vector with the negative control prey vector that localizes to the PM yielded growth on interaction selective media (Fig. 24B). However, co-transfection of Gpr179 TM2 bait and the negative control prey that localizes to the ER did not yield growth on the selective media. This result was puzzling, but due to the growth with one of the negative controls I determined the truncated protein was not suitable for screening for interactions.

Co-transfection of the full length Gpr179 Cterm bait vector with the positive control prey vectors yielded positive growth on interaction selective media (Fig. 24C), indicating that the bait protein was being translated in yeast and expressed in the ER and at the PM. Co-transfection of Gpr179 Cterm bait with both ER and PM negative control prey did not yield growth on interaction selective media (Fig. 24C), indicating that GPR179 Cterm is suitable for screening in the MYTH system.

Co-transfection of the Gpr179 bait vector with the positive control prey vectors yielded positive growth on selective media (Fig. 22D), indicating that the bait protein was being translated in yeast and expressed in the ER and at the PM. Co-transfection of Gpr179 bait vector with the negative control prey vectors did not yield growth on interaction selective media (Fig. 24D), indicating that the full-length protein is suitable for
screening in the MYTH system. Therefore, I also cloned the full length Gpr179 ORF into a prey vector to test for interaction with the mGluR6 and GPR179 bait proteins.
Figure 24: Screening Gpr179 bait vectors for use in MYTH system. (A) The Gpr179 TM1 bait vector encodes a truncated GPR179 protein that does not contain the large cytoplasmic domain. The protein was self-activating when expressed in the MYTH system. (B) The Gpr179 TM2 bait vector encodes a truncated GPR179 protein that contains a small portion of the cytoplasmic domain. The protein was self-activating when expressed in the MYTH system. (C) The Gpr179 Cterm bait vector encodes the cytoplasmic domain of the GPR179 protein and is suitable for screening in the MYTH system. (D) The Gpr179 bait vector encodes the entire GPR179 protein and is suitable for screening in the MYTH system.
The GPR179 bait protein was co-expressed with TRPM1, Nyctalopin, Gβ5, Gαo or mGluR6 prey proteins (Fig. 25 rows 1-5) and plated on interaction selective media to test for interactions. The bait GPR179 did not test positive for interactions with TRPM1, Nyctalopin, Gβ5, Gαo or mGluR6 prey proteins. GPR179 was tested for interaction with mGluR6 again by co-expressing the GPR179 prey protein with the mGluR6 bait protein. The yeast did not grow on interaction selective media (Fig. 25 row 6) indicating that the proteins do not interact.

The GPR179 bait protein was tested for self-interaction with the GPR179 prey protein. The screen resulted in positive growth on the interaction selective media indicating that the proteins interact (Fig. 25 row 7). This indicates that GPR179 may be capable of forming homodimers or homooligomers in the yeast system. To test whether this interaction may be occurring through the cytoplasmic protein domain or the TM and extracellular domain, I tested the GPR179 Cterm protein for interaction with the GPR179 prey protein (Fig. 25 row 8). Yeast co-transfected with the Gpr179 Cterm bait and the Gpr179 prey vector did not grow on interaction selective media, indicating that the interaction that allows GPR179 to form homodimers in yeast does not take place on the carboxy terminus of the protein. This means the interaction among GPR179 proteins likely occurs between the 7 TM domains or the extracellular domain.
Figure 25: Screening for interactions with the MYTH system. The GPR179 bait protein was tested for interaction with TRPM1, Nyctalopin, Gβ5, Gαo and mGluR6 bait proteins (Rows 1-5). The mGluR6 prey protein was tested for interaction with GPR179 prey protein (Row 6). Self-interaction was tested with GPR179 bait and prey proteins (Row 7). The self-interaction between GPR179 proteins is not mediated through the cytoplasmic domain of the protein (Row 8).
Duolink Analyses

The second method I used to screen for GPR179 interacting partners was the Duolink Proximity Ligation Assay (PLA). The Duolink PLA assay is an antibody ligation assay that produces a positive fluorescent signal only when proteins localize within a critical radius of ~40 nm of one another. The assay is performed in vitro or in vivo by probing for proteins of interest with primary antibodies produced in different species (Fig. 26A). The primary antibodies are probed for with (+) and (-) PLA probes that consist of secondary antibodies conjugated to an oligonucleotide. The (+) PLA probe oligonucleotides will circularize with the (-) PLA oligonucleotides in the presence of a ligation mixture if the probes are located within 40 nm of one another. A DNA amplification solution is added to initiate rolling circle amplification of the circularized DNA. The newly synthesized DNA is bound by fluorescent probes, which produce a fluorescent signal that can be visualized by confocal microscopy.

To evaluate the system, I tested whether Grm1, which encodes the protein mGluR1, a Class C GPCR that has been shown to form homodimers in vitro (Doumazane et al., 2011), will interact in this system. Myc and FLAG tagged Grm1 vectors were co-transfected in COS-7 cells and 24 h post transfection the PLA assay was performed using mouse anti-myc and rabbit anti-FLAG antibodies. Confocal microscopy revealed that the cells co-expressing mGluR1-myc and mGluR1-FLAG produced positive PLA signals, indicating formation of mGluR1 homodimers (Fig. 26B). As a negative control, the assay was performed but application of the primary antibodies was withheld. The negative control shows very little PLA fluorescent signals as expected (Fig. 26C).
Figure 26: *In vitro* Duolink PLA assay. (A) Schematic of the Duolink PLA assay. (B) As a positive control, cells were co-transfected with vectors containing Grm1-myc and Grm1-FLAG and the Duolink assay was performed. A representative confocal microscopy image shows positive PLA fluorescence is present in cells co-transfected with Grm1-myc and Grm1-FLAG. (C) As a negative control the assay was performed minus the primary antibodies.
To test for interaction between GPR179 and mGluR6, I co-transfected \textit{Gpr179-myc} and \textit{Grm6-FLAG} expression vectors into COS-7 cells. The Duolink PLA assay was performed and confocal microscopy revealed cells were co-expressing GPR179-myc and mGluR6-FLAG (Fig. 27A,B,C) and that there is a positive PLA fluorescent signal (Fig. 27D). These data indicated that GPR179 and mGluR6 localize within close proximity to one another and likely interact in this system.

To test for protein interactions between GPR179 and TRPM1, I co-transfected \textit{Gpr179-myc} and \textit{Trpm1-FLAG} into Cos-7 cells. After performing the PLA assay confocal microscopy revealed cells were co-expressing GPR179-myc and TRPM1-FLAG (Fig. 27E,F,G) and that a positive PLA fluorescent signal is present (Fig. 27H). These data indicate that GPR179 and TRPM1 localize within close proximity to one another and likely interact in this system.
Figure 27: GPR179 interacts with mGluR6 and TRPM1 in vitro. (A-C) GPR179 and TRPM1 are co-expressed in Cos-7 cells and co-localize. (D) PLA fluorescence is present in cells that co-express GPR179 and TRPM1, indicating the proteins interact. (E-g) GPR179 and mGluR6 are co-expressed in Cos-7 cells and co-localize. (H) PLA fluorescence is present in cells that co-express GPR179 and mGluR6, indicating the proteins interact.
To test if GPR179 and TRPM1 form complexes in vivo, I used the Duolink PLA assay on fixed transverse retinal sections. I used WT retinal sections and antibodies against GPR179 and TRPM1. The PLA assay was performed in conjunction with fluorescent immunostaining for GPR179 and TRPM1 followed by analysis using confocal microscopy. WT retinas labeled with GPR179 and TRPM1 antibodies showed the proteins were localized to the DBC dendritic tips (Fig 28A,B) and there is positive PLA fluorescence in the OPL (Fig. 28C). The PLA fluorescent signal co-localizes with the GPR179 and TRPM1 staining (Fig. 28D), indicating that the signal is specific to regions where both proteins are expressed.

As a negative control, the assay was performed on transverse retinal sections from Nyx\textsuperscript{nob} mice. In the Nyx\textsuperscript{nob} retinas TRPM1 does not localize to the DBC dendritic tips (Fig. 28E) but GPR179 is expressed normally (Fig. 28F), as expected. However, there were no consistent PLA signals present in the OPL that co-localize with GPR179 (Fig. 28G), indicating that a positive PLA signal is dependent on GPR179 and TRPM1 expression at the DBC dendritic tips.

A notable feature of the positive signal from Fig. 28C is that they are not present at every DBC dendritic tip where GPR179 and TRPM1 are expressed. This suggests that the antibody epitopes for GPR179 and TRPM1 may reside close to the critical distance for the assay, which would lower the efficiency of a positive reaction. The protein dense environment where GPR179 and TRPM1 reside could also decrease the Duolink assay efficiency, accounting for puncta expressing both proteins but lacking a positive signal.
Figure 28: TRPM1 and GPR179 localize within the Duolink critical radius. (A) WT retina slices labeled with antibodies against GPR179 (blue) and TRPM1 (green) and PLA assay was performed. Red puncta indicate positive interactions. (B) PLA assay was performed in nyx<sup>bob</sup> retinas (negative control) which lack TRPM1 expression at the DBC dendritic tips. (C) A 4.5 µm line was drawn through a cone pedicle in (A) and fluorescence was plotted across the line. GPR179, TRPM1 and the PLA fluorescence overlap, indicating the PLA fluorescent signal is specific to puncta expressing GPR179 and TRPM1.
Discussion

The immunohistochemistry data shows GPR179 is critical for the localization of RGS7, RGS11, Gβ5 and R9AP (Fig. 21 and Orlandi et al. (2012)). We also showed that GPR179 physically interacts with the RGS7/Gβ5 and RGS11/Gβ5 protein complexes (Orlandi et al., 2012). These data indicate that GPR179 may act as a scaffolding protein for both RGS7 and RGS11 in the DBCs and that the interaction is dependent on the expression of Gβ5. We know that the RGS proteins are critical to setting the light response in the DBCs (Cao et al., 2012). One aspect of my data that has not been studied further is the absence of the membrane anchor R9AP in the Gpr179 knocked out DBCs. It is unknown whether R9AP interacts directly with GPR179. Further, it appears that RGS7/Gβ5 or RGS11/Gβ5 binding with R9AP is required for R9AP localization at the DBC dendritic tips because R9AP is absent in RGS7−/−/RGS11−/− retinas (Fig. 22). It will also be important to determine whether R7BP, another RGS anchor expressed in the DBCs, is affected by GPR179 expression. It seems likely that GPR179 may act as a hub to recruit a large number of RGS proteins to the DBC dendritic tips so that a large number of GAP proteins are localized to the signalplex to set the speed of the BC light response. The large cytoplasmic tail on GPR179 is an ideal domain to act as a hub. Large, unstructured protein domains are known to act as protein scaffolds (Dyson and Wright, 2005; Coletta et al., 2010).

It was previously reported that RGS7, RGS11, Gβ5 and TRPM1 expression were decreased or absent in the Grm6−/− retina (Morgans et al., 2007; Cao et al., 2009; Xu et al., 2012). Immunolabeling GPR179 in the Grm6−/− retina reveals that GPR179 is properly localized to the dendritic tips of the DBCs. The dependence of TRPM1 localization on mGluR6 is controversial. Cao et al. (2011) reported TRPM1 was absent from the OPL in Grm6 knocked out retinas, a Grm6 spontaneous mutant, whereas Xu et al. (2012) found it was present but greatly decreased. Other proteins absent from the DBC
dendritic tips in *Grm6*−/− retinas include Gβ5, RGS7, RGS11 and R9AP (Fig. 21) and (Morgans et al., 2007; Xu et al., 2012). These data indicate that Gβ5, RGS7, RGS11 and R9AP localization is dependent on mGluR6 expression, whereas GPR179 is not (Fig. 21). When taken together with the data from the *Gpr179*^{nob5} retinas, which also lose localization of Gβ5, RGS7, RGS11 and R9AP but maintain mGluR6 localization, the mechanism by which the proteins do not localize in the *Grm6*−/− retinas is unclear. The creation of a dominant negative mGluR6 mouse in which it traffics to the DBC dendritic tips would help sort out whether the loss of proteins is because they are physically dependent on mGluR6 for trafficking or if the signalplex is downregulated by another mechanism.

The MYTH assay to test for protein interactions between GPR179 and other DBC signalplex proteins did not produce positive data that GPR179 interacts with other signalplex proteins. This could be due to the fact that yeast and BCs are such different systems that proteins may not behave the same way in each system. In the DBCs there are likely additional proteins that traffic and add posttranslational modifications to DBC signalplex proteins. This facilitates organization and trafficking of signalplex proteins at the dendritic tips in an ordered complex. In contrast, the MYTH system requires two exogenous mammalian fusion proteins to be expressed in a foreign system. The yeast may not be able to add the correct post-translational modifications to the exogenous proteins. They also lack expression of the rest of the signalplex proteins, which may facilitate protein localization.

The *in vitro* Duolink assay suggested that GPR179 forms complexes with mGluR6 and TRPM1. The downside to the assay is that the positive signal could be due to the overexpression of two membrane proteins, which could cause false positive signals because the cell is being overloaded with proteins, which may force proteins to localize within the Duolink distance. I think these experiments should be performed
under more stringent conditions by driving gene expression using a weak gene promoter to reduce false positives due to high protein concentration.

The *in vivo* Duolink assay suggests that GPR179 and TRPM1 are a part of the same protein complex in the DBCs. Therefore, I performed the assay on retinal sections to determine if GPR179 is in the same complex as mGluR6 and nyctalopin. I did not get positive interaction data for these experiments, but I think the reason for this is not because GPR179 does not localize in close proximity with these proteins. Instead, I think the negative results reflect the shortcomings of the assay. If the protein epitopes are on opposite sides of the membrane, as they are with mGluR6 and GPR179, then the assay efficiency is likely decreased dramatically. If the antibody epitopes are on distal regions of the proteins they may be too far apart for the PLA probes to ligate. Finally, the dense protein matrix at the DBC signalplex may inhibit the assay, accounting for the reason the assay was more robust in the *in vitro* system.

To further examine the positive interaction data between GPR179/TRPM1 and GPR179/mGluR6 Dr. Nazarul Hasan, a postdoc in the lab, performed immunoprecipitation (IP) experiments in parallel. Using antibodies against GPR179, the GPR179 protein complex was immunoprecipitated from retinal lysates and the precipitate was examined by mass spectrometry. These experiments showed that TRPM1 interacts with GPR179 (Ray et al., 2013 and Appendix III). Performing the IP with antibodies against TRPM1 identified GPR179 in the proteins that were precipitated. To validate the mass spectrometry identification that GPR179 and TRPM1 were in the same protein complex Co-IP experiments were performed in mammalian cell lines. The Co-IP experiments showed positive interaction between GPR179 and TRPM1 (Ray et al., 2013 and Appendix III). IP experiments from retinal lysates also suggested that TRPM1 and GPR179 directly interact (Ray et al., 2013 and Appendix III).
The self-interaction between GPR179 bait and prey vectors should be further studied. It is well accepted that many GPCRs are known to form homo and heterodimers and even higher order oligomers (Bouvier, 2001; Milligan, 2006; Filizola, 2010). The ability of GPR179 to form dimers or oligomers would enable it to be the focal point of the signalplex protein structure, especially if it is found to interact with more DBC signaling proteins.

The interaction between GPR179 and TRPM1 likely enhances the sensitivity of the glutamate signaling cascade by bringing the RGS machinery in close proximity to the TRPM1 channel. This enables the TRPM1 channel to quickly respond to changes in glutamate release from the photoreceptors because the RGS proteins continuously hydrolyze Go,GTP to Go,GDP, which presumably inhibits G protein closure of the TRPM1 channel. Our collaborator Neal Peachey observed a small ERG b-wave response in the Gpr179<sup>nob5</sup> mouse that was not present in other cCSNB mouse models (Ray et al., 2013 and Appendix III). When the ERG flash duration was increased, the b-wave response increased in the Gpr179<sup>nob5</sup> mouse but the Trpm1<sup>−/−</sup> did not have a b-wave. These observations were the catalyst for performing single cell recording experiments that identified a role for GPR179 beyond anchoring RGS proteins.

The immunohistochemistry revealed that mGluR6 and TRPM1 localized correctly in the Gpr179<sup>nob5</sup> DBCs and the ERG suggested these mice had a small b-wave. Therefore, we asked whether mGluR6 could gate the TRPM1 channel in the Gpr179<sup>nob5</sup> retina. To answer that question we used single cell recordings from rod BCs and found that CPPG, an mGluR6 antagonist, could illicit a response in the Gpr179<sup>nob5</sup> rod BCs that was significantly decreased from WT but significantly greater than the Trpm1<sup>−/−</sup> rod BC response (Ray et al., 2013 and Appendix III). We followed that experiment up by directly testing the gating of the TRPM1 channel in the Gpr179<sup>nob5</sup> rod BCs using single cell recordings and the drug Capsaicin, a TRPM1 channel modulator, to gate the channel
open. We found that in the Gpr179$^{nob5}$ rod BCs there was a significantly decreased capsaicin response compared to WT. These data suggested that the TRPM1 channel is not as sensitive to gating in the absence of GPR179.

We hypothesized that the interaction between GPR179 and TRPM1 enhanced the TRPM1 sensitivity to gating by changing the state of the channel in the membrane through physical interaction. To determine if GPR179 was required for the high sensitivity of the TRPM1 channel to capsaicin we performed the capsaicin experiments in the RGS7$^{-/-}$/RGS11$^{-/-}$ rod BCs, which do not express the RGS protein complex but retain all other signalplex components (Fig. 22). We found that the RGS7$^{-/-}$/RGS11$^{-/-}$ rod BCs had a capsaicin response similar to WT and significantly greater than the response in the Gpr179$^{nob5}$ rods BCs (Ray et al., 2013 and Appendix III). These data suggested that GPR179 is required to enhance the sensitivity of the TRPM1 channel to gating.

Channel variance can be measured when the retinal sections are bathed in L-AP4 and indicate the state of the TRPM1 channel when the glutamate signaling cascade is maximally active. High variance indicates the channels are in a state of constant opening and closure, whereas low variance indicates the channels are either maximally open or maximally closed. In WT retinal sections variance should be low under L-AP4 due to G protein closure of the TRPM1 channel. We observed significantly lower channel variance in Gpr179$^{nob5}$ rod BCs compared to WT. The channel variance in the Gpr179$^{nob5}$ rod BCs was similar to the variance in Trpm1$^{-/-}$ rod BCs. These data suggest that in WT rod BCs the TRPM1 channel is constantly opening and closing in response to free Gβγ, presumably because Gβγ gating of the TRPM1 channel is a transient process. In the Gpr179$^{nob5}$ rod BCs the variance is decreased similar to the RGS7$^{-/-}$/RGS11$^{-/-}$ rod BCs (Ray et al., 2013 and Appendix III). We attribute these results to the mislocalized or absent RGS proteins in the Gpr179$^{nob5}$ and RGS7$^{-/-}$/RGS11$^{-/-}$ rod BCs that allows a build up of active G proteins shifting the equilibrium of TRPM1 channels to more closed.
We were able to show that GPR179 plays a structural role in the DBCs by recruiting the RGS proteins to the signalplex. We also found that GPR179 directly interacts with TRPM1 and that this interaction is critical to the gating sensitivity of the TRPM1 channel. The disruption in glutamate signaling in the Gpr179
nob5 rod BCs appears to be the compound result of a loss of RGS proteins and decreased TRPM1 channel sensitivity. A recent publication also reported that GPR179 physically interacts with mGluR6 in the DBCs (Orlandi et al., 2013). Taken together, these data suggest that GPR179 is a hub for DBC signalplex components. Although GPR179 is not critical for the localization of mGluR6 and TRPM1 to the DBC dendritic tips, it may play an important role in recruiting these proteins into a larger oligomeric structure.

There is still disagreement about whether Gαo or Gβγ is responsible for gating the TRPM1 channel (Koike et al., 2010a; Koike et al., 2010b; Shen et al., 2012) and there is almost nothing known about the detailed mechanism of gating. Whole cell patch clamp experiments in rod BCs suggest that introduction of Gβγ into the rod BCs via the patch pipette closes the TRPM1 channel, whereas Gαo does not (Shen et al., 2012). These data suggest that the rod BCs respond to glutamate by mGluR6 binding glutamate and activating the heterotrimeric G proteins through GαoGDP to GαoGTP exchange, which subsequently causes GαoGTP to release from the Gβγ complex. The Gβγ dimer binds and directly closes the TRPM1 channel or closes the TRPM channel through an effector protein.

Methods

Antibodies

The following primary antibodies and their concentrations were used: sheep anti-GPR179 (peptide KVQEETPGEDLDRPVLQKR), 1:2,000 (Peachey et al., 2012b); mouse monoclonal anti-ctbp2/Ribeye (BD Bioscience), 1:1,000; guinea pig anti-mGluR6 1:1,000 (Koike et al., 2010b); sheep anti-TRPM1 1:1,000 (Cao et al., 2011); rabbit anti-
GFP (MBL), 1:800 and Rhodamine Peanut Aglutinin (PNA) conjugate 566 (Vector Labs), 1:1,000; goat anti-Gβ5 (peptide MATDGLHENETLASLKC), 1:1,000 (Morgans et al., 2007); goat anti-R9AP 1:1,000 (Hu and Wensel, 2002); mouse anti-DIG HRP, 1:1,000 (Perkin Elmer); mouse anti-GFP, 1:1,000 (Molecular Probes); sheep anti-RGS11 (peptide CSPALQSTPREPAATSSP EGADGE), 1:1,000 (Martemyanov et al., 2005); sheep anti-RGS7 1:1,000 (Morgans et al., 2007); mouse anti-RIBEYE 1:1,000 (BD Transduction Labs); rabbit anti-PKC 1:1,000 (Sigma Aldrich).

Secondary antibodies (Invitrogen, 1:1,000) appropriate to each primary antibody included: donkey anti-sheep Alexa-488, donkey anti-rabbit Alexa-680, donkey anti-rabbit Alexa-546, donkey anti-mouse Alexa-647 and donkey anti-guinea pig Cy3 (Millipore, 1:1,000). In lieu of an antibody specific to nyctalopin, we used Tg(Gabrr1-YFP/nyx)Rgg1 transgenic mice that express a yellow fluorescent protein (YFP) tagged nyctalopin (Gregg et al., 2007). They are labeled as WT in the figures.

**Immunohistochemistry**

Mice were euthanized with CO₂ followed by cervical dislocation. Their eyes were enucleated and the lens was removed. Eyecups were washed in phosphate buffered saline (PBS) then fixed for 30 min in a 4% formaldehyde PBS solution (pH 7.4). Eyecups were washed 3 times with PBS then cryoprotected in increasing concentrations of sucrose in PBS (10%, 15% for 1 h each and 30% overnight). Eyecups were embedded in 2:1 OCT/ 20% sucrose PBS solution frozen in a liquid nitrogen cooled bath of isopentane. Eyecups were sectioned (18 µm) using a Leica 1850 cryostat. Sections were mounted on glass slides (SuperFrost) and allowed to dry for 10 mintues at room temperature before being stored at -80°C. Sections were air dried at 37°C for 15 min and washed with PBS and PBS containing 0.05% Triton X-100 (PBX) for 5 min each, then blocked in PBX plus 5% normal donkey serum blocking solution for 1 h. Sections were incubated overnight at room temperature in the presence of the primary antibody diluted
in blocking solution, then washed 3 times for 10 min each with PBX followed by incubation in secondary antibody in blocking solution for 1 h at room temperature. Sections were washed 2 X 10 min in PBX and 1 X PBS. Slides were cover-slipped using Immu-Mount (Thermo Scientific) and slides were imaged using an Olympus FV1000 confocal microscope. Images were universally adjusted for brightness using Photoshop.

**In-Fusion HD Cloning**

PCR fragments were amplified using primers designed to have 14-18 bp 5’ overhangs that share homology with the cut site on a linearized vector. PCR fragments were treated with Cloning Enhancer (Clontech) and incubated at 37°C for 15 min then at 80°C for 15 min on a thermal cycler. Linearized vector, treated PCR fragment and water was added to 5X In-Fusion HD Enzyme Premix according to the manufacturers protocol (Clontech). The reaction was mixed by pipetting and incubated for 15 min at 50°C on a thermal cycler. The reaction mix was placed on ice until transformation or stored at -20°C.

**Plasmid Isolation**

Plasmid purification was performed using the Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturers protocol. Single colonies from transformed bacteria were selected from agarose plates using a sterile toothpicks and used to inoculate 2 mL of Circlegrow media supplemented with 100 µg/mL kanamycin. The culture was grown overnight at 37°C while shaking at 225 RPM. The bacterial cells were transferred to a 2 mL centrifuge tube and pelleted by centrifugation at max speed for 5 minutes to pellet cells. The supernatant was discarded and the cells were resuspended by vortexing in 250 µl of Cell Resuspension Solution. 250 µl of Cell Lysis Solution and 10 µl Alkaline Protease Solution was added, and mixed by inverting tubes several times, then incubated at room temperature for 5 min. 350 µl of Neutralization Solution then was added and mixed by inverting several times. Tubes
were centrifuged at 12,000 x g for 10 min to pellet cellular debris and the supernatant was decanted into a spin column. The spin column was placed into a collection tube and centrifuged for 1 min at 12,000 x g. The flow through was discarded and the plasmid bound to the column was washed 2 times by adding 500 µl of Column Wash Solution followed by centrifugation for 1 min at 12,000 x g. The spin column was transferred to a clean microcentrifuge tube and 100 µl of sterile water was added to the column to elute plasmid DNA. The water was incubated on the column for 1 min at room temperature then centrifuged for 1 min at 12,000 x g to collect DNA. DNA was stored at -20°C.

**Cell culture, transfection and immunocytochemistry**

COS-7 (African green monkey kidney) cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. 25 h prior to transfection, cells were seeded on 1 cm² chamber slides (Lab-Tek). Expression plasmids were transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 24 h after transfection, cells were washed in PBS and fixed in ice-cold methanol for 5 min then ice-cold methanol/acetone (50:50) for 5 min. Cells were washed in PBS and blocked for 1 h in blocking solution (PBS containing 0.01% Triton X-100 and 5% normal donkey serum). Antibodies were diluted in blocking solution and incubated on cells overnight at 4°C, then washed with PBS. Fluorescent secondary antibodies were diluted 1:1,000 and added to slides. Cells were incubated for 1 h with secondary antibodies at room temperature then washed with PBS. Slides were mounted with coverslips using Vectashield mounting medium with DAPI (Vector Laboratories). Slides were imaged with an FV-1000 confocal microscope.

**Duolink**
Duolink assay was performed as described in the Duolink II Fluorescence user manual (OLink Bioscience). Retina sections were prepared for immunohistochemistry as previously described. Sections were air dried at 37°C for 15 min and washed with PBS and PBS containing 0.05% Triton X-100 (PBX) for 5 min each and then blocked in PBX containing 5% normal donkey serum for 1 h. Primary antibodies were diluted in blocking serum and incubated on sections overnight at room temperature. Slides were washed 3 X 10 min in PBX. In the meantime, PLA probes specific to the primary antibodies were mixed 1:5 in blocking solution and incubated at room temperature for 20 min. After the slides were washed, the solution containing the PLA probes was applied and incubated at 37°C in a humidity chamber for 1 h. Slides were washed 2 X 5 min in PBX. In the meantime, Ligation stock was diluted 1:5 in PCR grade water and mixed. Just before adding to slides, Ligase was added to the Ligation mixture at a 1:40 dilution, mixed gently. Slides were incubated at 37°C in a humidity chamber for 100 min. Slides were washed 2 X 10 min in PBX and 1 X 10 min in PBS. For fluorescent immunostaining, fluorescent secondary antibodies were diluted in blocking solution and incubated for 1 h at room temperature. Slides were washed 2 X 10 min in PBX and 1 X 10 min in PBS. Slides were mounted with glass coverslips using Duolink Mounting Medium containing DAPI then visualized on a confocal microscope.

**Membrane yeast-two hybrid**

Yeast were grown overnight in 10 ml YPD media (Clontech) to A_{600} 0.5 to 1.0. Yeast were pelleted for 5 min at 1500 x g. The pellet was washed with ddH_{2}O and centrifuged again for 5 min at 1500 x g. The pellet was re-suspended in (100 µl or 200 l per tube) of TE buffer (10mM Tris-HCl, 0.1mM EDTA) pH 7.5 containing 0.1M lithium acetate (LiAC). Yeast solution was transferred to a microfuge tube (200ul per tube) and 1 ug of bait and prey plasmid DNA was added in addition to 5 µl (50 µg) carrier DNA (salmon sperm, denatured for 5 min at 99°C put on ice, denatured again then place on
ice). To the tube, 300 µl 40% polyethylene glycol (250 µl 50%) in TE containing 0.1M LiAC was added and vortexed to mix well. Solution was incubated for 30 min at 30°C, then DMSO added to 10%v/v. Cells were heat shocked for 10 min at 42°C then centrifuged for 15 sec at 10,000 x g. The supernatant was removed and the cells were resuspended in 100 µl of ddH₂O and plated on selective plates (Minimal SD agar, -His/-Ade selective DO supplement, Clontech). Plates were incubated at 30°C for 2-3 days. After 2-3 days, yeast colonies were selected with a sterile pipette tip and re-suspended in 20 µl of ddH₂O. Yeast were spotted on interaction selective plates (Minimal SD agar, -His/-Ade/-Leu/-Trp selective DO supplement, Clontech) and incubated at 30°C for 2-3 days.
CHAPTER IV: LRIT3 IS REQUIRED FOR NORMAL DBC FUNCTION

Aim 3: Determine the mechanism by which LRIT3 contributes to glutamate signaling in the DBCs

Rationale: Zeitz et al. (2013b) reported that LRIT3, a gene of previously unknown function, was critical to the glutamate signaling cascade in the DBCs. Two human patients with cCSNB of a previously unknown cause were found to have mutations in the LRIT3 (Zeitz et al., 2013b). The mechanism by which LRIT3 contributes to the glutamate signaling cascade remains unknown. To determine the role LRIT3 plays in the glutamate signaling cascade of the DBCs, we developed a Lrit3−/− mouse model. An ERG will be performed on the Lrit3−/− mouse to confirm a disruption in LRIT3 causes a no b-wave phenotype. Using the Lrit3−/− mouse model immunohistochemistry will be performed to determine the effect deletion of LRIT3 has on signalplex proteins.

Hypothesis: LRIT3 is critical to DBC signalplex maintenance and Lrit3−/− mice will have a no b-wave ERG.

Introduction

Despite many of the molecules critical for glutamate signaling in the DBCs having been identified (Masu et al., 1995; Pardue et al., 1998; Bech-Hansen et al., 2000; Pusch et al., 2000; Gregg et al., 2007; Audo et al., 2009; Cao et al., 2009; Shen et al., 2009; van Genderen et al., 2009; Koike et al., 2010b; Okawa et al., 2010; Audo et al., 2012; Cao et al., 2012; Dhingra et al., 2012; Orlandi et al., 2012; Peachey et al., 2012b; Shen et al., 2012), there are likely additional proteins that are required to develop and maintain a functional synapse between the photoreceptors and ON BCs. At the presynaptic and
postsynaptic membrane, proteins are arranged into a dense network consisting of scaffolding proteins, receptors, ion channels and signaling machinery. These protein networks are likely organized by scaffolding proteins that ensure the pre- and postsynaptic protein architecture are aligned to facilitate efficient signal transmission (Waites et al., 2005; McAllister, 2007; Jin and Garner, 2008). The protein network on the postsynaptic side, termed the postsynaptic density (PSD), is highly complex and consists of ~1,000 different proteins from a broad array of functional classes (Yamauchi, 2002; Yoshimura et al., 2004; Collins et al., 2005). Given that there are 10 proteins known to be critical to the mGluR6 to TRPM1 glutamate signaling cascade, additional proteins are expected to be required for structural support and organization. The absence of some of these proteins would be expected to cause cCSNB.

One such protein is LRIT3, a leucine-rich repeat, immunoglobulin like and transmembrane domain 3 protein that when absent causes cCSNB (Zeitz et al., 2013b). LRIT3 is the second leucine-rich-repeat (LRR) protein identified as being critical to the glutamate signaling cascade in the DBCs, the other being nyctalopin (Pardue et al., 1998; Bech-Hansen et al., 2000; Pusch et al., 2000; Pardue et al., 2001; Gregg et al., 2007; Cao et al., 2011; Pearring et al., 2011). Nyctalopin contains an extracellular LRR domain and is connected to the plasma membrane by a single pass transmembrane domain or a GPI anchor, depending on the species (O’Connor et al., 2005; Bojang and Gregg, 2012). Nyctalopin is required for the recruitment of TRPM1 to the DBC dendritic tips by an unknown mechanism (Gregg et al., 2007). The function of the LRR domain in nyctalopin remains to be discovered, but the importance of it can be highlighted by the fact that in humans twenty one of the thirty five cCSNB causing mutations in NYX, the gene that encodes nyctalopin, reside in regions encoding the LRR domain (Matsushima et al., 2005).
The LRR domain is one of the most common protein domains in mammals and is highly conserved across species (Hynes and Zhao, 2000; Bjorklund et al., 2006). There are ~330 LRR-containing proteins in the human proteome, many of these being expressed in neuronal systems (Ko, 2012). The LRR domain consists of 20-29 amino acid motif that contains a conserved LxxLxLxxN/CxL amino acid sequence that is typically repeated in a tandem array from 2-52 times (Kobe and Deisenhofer, 1994; Matsushima et al., 2005). The first crystal structure of a LRR protein, ribonuclease inhibitor, revealed the tandem array of repeats forms a characteristic horseshoe shaped structure (Kobe and Deisenhofer, 1993) that all known LRR proteins adopt (Matsushima et al., 2005). The concave side of this structure is formed by continuous β-sheets and the convex side often consists of α-helices, but the composition can vary (Bella et al., 2008). LRR proteins may interact with ligands or other LRR proteins through the concave and convex surface of the horseshoe shaped structure (Matsushima et al., 2005). The LRR domain is capable of interacting with a wide range of ligands by varying the length and number of tandem repeats, which affects its curvature (Buchanan and Gay, 1996; Bella et al., 2008).

The majority of LRR proteins are expressed in the nervous system, often giving very restrictive expression patterns among neurons of the same functional type (Lauren et al., 2003; Beaubien and Cloutier, 2009; Homma et al., 2009; Hong et al., 2009). LRR proteins have been shown to play critical roles in the nervous system. TrkA, TrkB and TrkC are critical for axon guidance during development; NGL1, NGL2 and NGL3 are critical for synaptic contact formation; LGI1 is critical for stabilization of mature neurons at synapses; and NGL-3 is critical for recruitment of AMPA receptors at glutamatergic synapses (Linhoff et al., 2009; Woo et al., 2009; Siddiqui et al., 2010; de Wit et al., 2011). Mutations in LRR containing proteins lead to several neuronal diseases such as schizophrenia, epilepsy, autism, Tourette’s syndrome and Alzheimer’s disease (Fig. 29).
The mechanism of how LRR proteins recruit and maintain signal complex proteins has not been determined. Further, the function and ligands of many of these LRR proteins remain unknown (de Wit et al., 2011).
Figure 29: Overview of LRR proteins in neural circuits. LRR proteins are grouped by their known roles in neural circuits including axon guidance (A), target selection (B), synapse formation (C), myelination (D), limiting plasticity (E) and nervous system disorders (F). Different protein domains are overviewed in the diagram key (bottom left). Domain abbreviations: LRRNT and LRRCT, LRR N- and C-terminal flanking domains; Ig, immunoglobulin-like; CT3, cysteine knot; TyrK, tyrosine kinase; EPTP, epitempin; Laminin G, laminin globular; TIR, Toll/interleukin-1 receptor; EGF, epidermal growth factor-like; FN3, fibronectin type III; GPI-anchor, glycoprophatidylinositol; PDZ-IS, postsynaptic density protein. Diagram adapted from de Wit et al. (2011)
Human patients with mutations in \textit{Lrit3} have cCSNB but are otherwise healthy, suggesting that \textit{Lrit3} may have a limited expression pattern. Because the human patients are healthy we do not need to make a conditional knockout mouse, which can be more costly and time consuming. To develop an \textit{Lrit3}^{-/-} mouse, Zinc Finger Nuclease (ZFN) technology will be utilized. ZFNs are synthetic DNA binding proteins that are fused with a non specific endonuclease (Chandrasegaran and Smith, 1999). The ZFN proteins consist of a chain of two finger modules with specificity for a DNA hexamer. The modules can be paired together to form specificity up to 24 bp. The DNA binding zinc finger modules are fused with the DNA cleavage domain of the restriction endonuclease FokI. The FokI restriction endonuclease must dimerize to cleave DNA (Bitinaite et al., 1998). To produce target specificity, two ZFNs must be designed on opposite strands with a 5-7 bp separation for DNA cleavage (Carroll, 2011). When the two ZFNs bind to the target site, the FokI dimerizes and cleaves the DNA. The dependency of the restriction endonuclease to dimerize allows ZFNs to target highly specific regions within the genome and reduces off target effects. The double stranded DNA breaks created by the ZFNs induce a DNA repair mechanism, most commonly non-homologous end joining (NHEJ), to repair the DNA double stranded break. The result of NHEJ repair can be the insertion or deletion of one to several DNA base pairs. By careful placement of target sites in an exon, some of the events will cause a frameshift resulting in a null allele.

To make a ZFN knockout mouse, ZFN mRNA is injected into a fertilized mouse single cell embryo. The mRNA is translated into the functional ZFN proteins by the embryo’s cell machinery. The ZFNs then bind and cleave the targeted DNA causing double stranded breaks, followed by DNA repair. The injected embryos are transplanted into a foster mother for development. Once the pups are born they are screened for mutations at the ZFN target site by sequencing genomic DNA.
Results

*Lrit3*⁻/⁻ mouse model is created using ZFN technology

To begin the design process for generating a *Lrit3* knockout mouse we cloned *Lrit3* cDNA from the retina, to ensure the retinal isoform matches that present in the NCBI database. To clone *Lrit3* from the retina, I designed In-Fusion Cloning (Clontech) primers that annealed to the predicted *Lrit3* start and stop sites and used PCR to amplify the ORF from mouse retinal cDNA. I predicted the *Lrit3* ORF in the retina was larger than the NCBI database annotated ORF based on next-generation transcriptome sequence analysis from retina (Brooks et al., 2011). The amplified PCR fragment was cloned into a pcDNA3.1 mammalian expression vector and sequenced using both internal and pcDNA3.1 vector primers. The sequencing results revealed the retinal isoform of *Lrit3* is 363 bp larger than the NCBI database version. The additional base pairs reside on the 5' end of exon 4 (Fig. 30A).

I used the SMART online resource (http://smart.embl.de/) (Letunic et al., 2012) to predict the domain structure of LRIT3 (Fig. 30B). The predicted protein structure was used to determine which protein domains may be most critical function and therefore be targeted by ZFNs to make the *Lrit3*⁻/⁻ mouse. The NCBI database isoform of *Lrit3* is 560 aa in length and produces a protein predicted to have six extracellular LRR domains, an immunoglobulin (Ig) like domain, a fibronectin type 3 domain followed by a transmembrane (TM) domain and a 76 aa cytoplasmic carboxy terminus (Fig. 30B). The retention of part of intron 3 in the retinal isoform changes the prediction of the Ig like domain to an Ig C-2 Type domain and inserts regions of low complexity following the Ig domain (Fig. 30B). Regions of low complexity may provide flexibility to the extracellular domain allowing the LRR domain to bind ligands more easily.

Based on the predicted protein structure, the most critical functional domain, therefore the most desirable domain to disrupt would be the single TM domain.
Designing ZFNs to target DNA that encodes for the TM domain would likely disrupt protein function if a missense or nonsense mutation was introduced. Targeting the ZFNs to the LRR domains would also likely knockout protein function if a missense mutation was introduced into the region because the LRR domain is likely critical to binding an extracellular ligand. Based on the predicted protein structure it was decided that ZFNs should most ideally be designed to target the LRR or TM domain to effectively eliminate protein function.
Figure 30: Predicted LRIT3 protein domains. (A) The NCBI database *Lrit3* gene is 363 bp shorter than the isoform cloned from mouse retina. The increase in size is the result of an alternative splice acceptor site in intron 3. (B) The increased size in the retinal isoform changes the IG like domain to a IG C-2 domain and inserts a region of low complexity into the protein.
To produce a Lrit3 knockout mouse (Fig. 31), we contracted a team at Sigma-Aldrich to produce ZFNs. They designed and tested several ZFNs in mammalian cell lines to identify those most likely to target regions of the gene that would result in a knockout. The ZFN pair provided to us targeted the first nucleotides that encode the second LRR domain (Fig. 31). This DNA sequence encodes an amino acid sequence that is highly conserved across species (Fig 32B). At this location, missense mutations have a high probability of causing a deleterious allele by changing the secondary structure of the conserved LRR domain. The ZFN mRNAs were sent to the Fox Chase Cancer Center for creation of the knockout mouse.
Figure 31: Workflow of the generation of a ZFN KO mouse model. ZFN mRNA pairs targeted against exon 2 of *Lrit3* were injected into fertilized single cell embryos. In the embryos the mRNA is translated and the ZFNs can make double stranded breaks in the target sequence. The fertilized embryos are transferred to a foster mother for development. Modified image from Sigma-Aldrich
Genotyping ZFN induced mutations in *Lrit3*

To genotype the mice, tail biopsies were obtained and genomic DNA was isolated. Primers were designed that flanked the ZFN target site and PCR products from each mouse were sequenced. Two sets of primers were designed; primer set 1 annealed ~200 bp upstream and ~200 bp downstream of the ZFN target site, primer set 2 annealed ~400 bp upstream and ~400 bp downstream of the target site (Fig. 32A). The second primer set was used to account ensure large deletions could be detected. PCR products from mice with deletions resulted in low quality sequence in regions where mutations resided. To get sequence for the individual alleles the PCR fragments were cloned into a pCR-Blunt cloning vector (Invitrogen). Several clones from each mouse were screened for mutations using Sanger Sequencing to ensure both clones of both alleles were identified.

A total of 363 embryos were injected and implanted into 9 Swiss Webster mothers. 44 offspring were obtained, 15 of which had mutations on at least one allele (Fig. 32C). The mutations ranged from a single to a 40 bp deletion. 4 of the mice were compound heterozygotes and were predicted to be *Lrit3<sup>−/−</sup>* due to the mutations being located in a gene region that encodes a highly conserved peptide. Mouse G48 contained a 4 bp deletion that will result in a frameshift and a 15 bp deletion that will cause a loss of 5 highly conserved amino acids. Mouse G49 contained a complex DNA inversion that disrupts a highly conserved amino acid encoding region and a single base pair deletion that will result in a frameshift. Mouse G50 contains a 3 bp deletion that deletes a conserved tyrosine before the second LRR domain and a single bp deletion that results in a frameshift. Mouse J7 contains a 15 bp deletion that will cause a loss of highly conserved amino acids and a single bp deletion that will result in a frameshift.

To generate a knockout line we chose to breed mouse G47 because he carried a 40 bp deletion on one allele, which deletes critical amino acids and produces a
frameshift. The 40 bp deletion also allows easy genotyping of the offspring by agarose gel electrophoresis.
Figure 32: ZFN mutations disrupt a highly conserved protein-coding region. (A) gene map of *Lrit3* including primer annealing sites used to capture ZFN targeted region (*
). (B) LRIT3 amino acid alignment using the Profile Alignment (PRALINE) application (Simossis and Heringa, 2005). The ZFNs flank and cut the DNA sequence ACCT which partially encodes the Tyr/Lys amino acids (black line) that reside in a peptide sequence that is highly conserved across species. (C) Sequences from *Lrit3* ZFN mutant offspring. Mutations range from single bp deletions to 40 bp deletions. Sequence was aligned using the Clustal V sequence alignment (Higgins, 1994). Mouse G49b had a large inversion on one allele and is not shown.
ERG analysis reveals ZFN Lrit3 compound heterozygotes have a no b-wave ERG

From the sequence alignment I predicted the two founder mice G48 and G49 who had a mutation on both alleles would be null for the LRIT3 protein. These were tested for visual function using the ERG. We performed a dark adapted ERG with a -0.4 log cd*s*m⁻² flash stimulus. This stimulus intensity elicits a response from both rod and cone ON BCs (Stockman and Sharpe, 2006). Both mutants lacked b-waves in the ERGs, whereas littermate controls had normal ERGs (Fig. 33). These data demonstrate that similar to human (Zeitz et al., 2013b), LRIT3 is required for normal function of the DBCs in mice and that the Lrit3⁻/⁻ mouse is a new model of cCSNB.
Figure 33: Predicted $Lrit3^{/-}$ mice have a no b-wave ERG phenotype. ERG from G51 littermate WT (blue) and G48 $Lrit3^{/-}$ (red) mice. Waveforms reflect response from dark adapted mice subjected to a 200 msec -0.4 log cd*s*m$^{-2}$ strobe flash. WT mice have a normal ERG a- and b-wave. The $Lrit3^{/-}$ mice lack the ERG b-wave, indicating a disruption in DBC function.
TRPM1 does not localize to the DBC dendritic tips in the \textit{Lrit3}\textsuperscript{−/−} retina

Data from human patients with cCSNB indicated LRIT3 was critical to DBC signaling and that it was expressed at the DBC dendritic tips (Zeitz et al., 2013b). However, the mechanism by which the absence of LRIT3 disrupts glutamate signaling in the DBCs remains unknown. To determine if LRIT3 plays a critical role in localizing other proteins to the DBC dendritic tips, immunostaining for DBC components was performed on WT and \textit{Lrit3}\textsuperscript{−/−} retinas.

Due to the similarities between LRIT3 and nyctalopin, we first investigated the expression of TRPM1 in the \textit{Lrit3}\textsuperscript{−/−} retina. Fixed transverse retinal sections from \textit{Lrit3}\textsuperscript{−/−} and WT mice were labeled with antibodies against TRPM1 and mGluR6 and the proteins were visualized by confocal microscopy. As expected, TRPM1 immunostaining labeled the DBC bodies and gave punctate labeling in the OPL of WT mice. In the \textit{Lrit3}\textsuperscript{−/−} retina, TRPM1 was not localized to the DBC dendritic tips, although the bipolar cell bodies were positive for TRPM1 protein expression (Fig. 34). These results are similar to the TRPM1 immunostaining pattern in the \textit{Nyx}\textsuperscript{nob} mice (Fig. 19, 34 and Pearring et al. (2011).
Figure 34: TRPM1 is not localized correctly in Lrit3⁻/⁻ DBCs. Fixed transverse retinal sections were labeled with antibodies against TRPM1 (green) and mGluR6 (red) and visualized with confocal microscopy. As expected, TRPM1 is expressed in the cell bodies and at the dendritic tips of the DBCs in WT retina and it co-localizes with mGluR6. In the Lrit3⁻/⁻ and Nyx⁺/⁻ retinas, TRPM1 is expressed in the cell bodies but does not localize to the DBC dendritic tips and does not colocalize with mGluR6 in the OPL. Scale bar represents 5 µm.
Other known signalplex proteins are present in the Lrit3<sup>−/−</sup> retina

To determine if the absence of LRIT3 impacted additional signalplex proteins I labeled Lrit3<sup>−/−</sup> and WT transverse retinal sections with antibodies against GPR179, mGluR6, Gβ5, RGS7 and RGS11 and visualized the staining with confocal microscopy. These studies showed that GPR179, mGluR6, Gβ5, RGS7 and RGS11 all localized properly in the absence of LRIT3 (Fig. 35). These results mirror the expression pattern of these proteins in the Nyx<sup>−/−</sup> mouse model (Fig. 21 and (Cao et al., 2011; Pearring et al., 2011)). These data suggest that the defect in glutamate signaling in the Lrit3<sup>−/−</sup> mouse is caused by the inability of TRPM1 to be localized at the DBC dendritic tips.
Figure 35: GPR179, mGluR6, G\(\beta\)5, RGS7, RGS11 and R9AP localization is normal in \(Lrit3^{-/-}\). DBC signalplex proteins were labeled with antibodies in WT and \(Lrit3^{-/-}\) retinal slices and visualized using confocal microscopy. The proteins GPR179, mGluR6, G\(\beta\)5, RGS7, RGS11 and R9AP are localized correctly in the \(Lrit3^{-/-}\) retina.
Discussion

My experiments show that LRIT3 is required for normal DBC function in mice, consistent with the report that mutations in human Lrit3 cause cCSNB (Zeitz et al., 2013b). LRIT3 is an extracellular LRR protein with an Ig C-2 domain, fibronectin type 3 domain, and a single TM domain (Fig 30B). The molecular function of LRIT3 in the DBCs is unclear, however, I show that its expression is required for localization of the TRPM1 cation channel to the DBCs dendritic tips (Fig. 34). Like other LRR proteins (de Wit et al., 2009; Linhoff et al., 2009; Siddiqui et al., 2010), LRIT3 appears to be critical for the recruitment of synaptic components and synaptic maintenance.

Based on the immunohistochemistry data showing that TRPM1 is not localized in the Lrit3−/− DBCs, we anticipated the rod BCs would not respond to the mGluR6 antagonist, CPPG, or the TRPM1 modulator, capsaicin. To test whether the rod BCs respond to CPPG in the Lrit3−/− retina, transverse retinal sections were prepared for single cell recordings. Retinal slices were bathed in a solution containing L-AP4, which maximally activates the glutamate signaling cascade and closes the TRPM1 channel. Application of 0.6 mM CPPG for 200 msec elicited a response in the Lrit3−/− retina that was significantly decreased compared to WT. This response was not significantly different than the response from Trpm1−/− rod BCs but was significantly smaller than the response generated in the Gpr179nob5 rod BCs (Fig. 36A,B). These results support our data that the TRPM1 channel is not localized to the DBC dendritic tips in the Gpr179nob5 retinas.

To further test whether TRPM1 is not localized to the DBC dendritic tips in the rod BCs of the Lrit3−/− retina we measured the rod BC response to exogenous capsaicin application. Capsaicin is a modulator of the TRPM1 channel. If the channel is localized to the plasma membrane of the rod BC dendritic tips in the Lrit3−/− retina it should have a capsaicin response greater than the Trpm1−/− capsaicin response. We found that the
capsaicin response in rod BCs from \textit{Lrit3}\textsuperscript{-/-} retina were no different than the capsaicin response in the \textit{Trpm1}\textsuperscript{-/-} rod BCs, and as expected both responses are significantly decreased compared to WT (Fig. 36C,D). The capsaicin response in the \textit{Lrit3}\textsuperscript{-/-} rod BCs was not significantly different from the response in \textit{Gpr179}\textsuperscript{nob5} rod BCs (Fig. 36D). These data further support that in the \textit{Lrit3}\textsuperscript{-/-} retina the absence of TRPM1 channel localization is the cause of the cell’s inability to respond to light.
Figure 36: Lrit3−/− rod BC response to CPPG and capsaicin. Whole cell recordings from WT, Gpr179nobs, Trpm1−/− and Lrit3−/− rod BCs in the presence of L-AP4. (A) Representative traces of WT and Lrit3−/− rod BCs in response to CPPG. (B) Scatter diagrams of peak amplitude responses. One way ANOVA (**): Trpm1−/− is significantly decreased in amplitude compared to Gpr179nobs. (*) Lrit3−/− is significantly decreased in amplitude compared to Gpr179nobs. LRIT3 does not significantly differ from Trpm1−/−. (C) Representative traces of WT and Lrit3−/− rod BCs in response to capsaicin. (D) Scatter diagrams of peak amplitude responses. One way ANOVA (**): Gpr179nobs and Trpm1−/− are significantly different. Lrit3−/− does not significantly differ from either Gpr179nobs or Trpm1−/−. Experiments performed by Kathryn Heath.
Like LRIT3, nyctalopin is critical for the localization of TRPM1 at the DBC dendritic tips. It will be important to determine the mechanism by which LRIT3 is responsible for the localization of TRPM1, whether the function of LRIT3 is redundant with nyctalopin, and if LRIT3 serves a purpose beyond localization of the TRPM1 channel. Determining the mechanism by which LRIT3 localizes the TRPM1 channel to the DBC dendritic tips will be important because the general mechanism by which LRR proteins recruit other proteins to the synapse is largely unknown.

Leucine-rich repeat transmembrane protein 1 (LRRTM1) is responsible for the assembly of excitatory synapses. LRRTM1 plays a role in recruiting the AMPA receptor to the plasma membrane and is responsible for long-term potentiation in pyramidal neurons (Soler-Llavina et al., 2013). In the BCs, we know that the number of TRPM1 channels at the DBC dendritic tips rate limits the response (Peachey et al., 2012a). It will be important to determine if LRIT3 plays a role in regulating the number of TRPM1 channels at the DBC dendritic tips, possibly shaping DBC plasticity.

LRR proteins are known to have roles in synapse maintenance (de Wit et al., 2011), therefore, it will be important to perform electron microscopy (EM) on the Lrit3−/− retinas to ensure they are formed properly and maintained through adulthood. The Lrit3−/− line should also be crossed with the Nyx^{nob} mouse and EM performed. Deleting both LRIT3 and nyctalopin from the DBC dendritic tips may cause the synapse to be less stable, especially if both proteins have non-redundant roles in synapse maintenance. These experiments would help us understand if LRIT3 has a function beyond recruiting the TRPM1 channel to the DBC dendritic tips.

To study LRIT3 function further an antibody must be made or a transgenic mouse expressing a tagged LRIT3 protein produced to do immunohistochemistry and biochemistry. With the emergence of new genome editing techniques this can be efficiently accomplished and provide a reliable method of detecting LRIT3 in vivo. The
tag would ideally be placed on the carboxy terminus. The carboxy terminus is a short intracellular domain likely only needed for anchoring the protein to the membrane and addition of a protein tag should not interfere with protein function.

There are several protein domains in LRIT3 in which a functional role needs to be identified, including the LRR domain, Ig C-2 domain and Fibronectin type 3 domain (Fig. 30). The extracellular LRR domain is likely critical for ligand binding in the EC matrix. The Ig C-2 domain of LRIT3 likely is a protein interaction domain. Ig domains of synaptic proteins have been demonstrated to be critical for stabilization of synaptic protein complexes (Rapti et al., 2011). Fibronectin type 3 domains have been shown to play critical roles in neural synaptic plasticity (Strekalova et al., 2002; Dityatev et al., 2010), calcium channel potentiation and integrin binding to the EC matrix (Dityatev et al., 2010). Genome editing techniques can be used to create dominant negative LRIT3 transgenic mice that contain mutations in the different protein domains. Ideally, these mutations would disrupt a protein domain but allow the protein to localize properly. These experiments would help differentiate which domains are critical for TRPM1 and LRIT3 localization.

Methods

DNA isolation

For mouse genotyping, tail samples were collected from mice and placed in a 0.7 mL microcentrifuge tube. To the tube, 200 µL of DirectPCR (Viagen Biotech) was added along with 2 µL proteinase K (20 mg/ml). Sample was incubated for at least 4 h at 55°C in a dry bath with brief vortexing every h. After the tail sample was adequately digested, the sample was heated to 85°C for 30 min to inactivate the proteinase K. Samples were then ready for PCR.

General PCR
PCR was performed using either Phire II or Phusion taq polymerase (Thermo Scientific). Reactions were made to 20 µl consisting of 0.5 µM of each forward and reverse primers, 200 µM of dNTPs, 4 µl of 5X reaction buffer, and either 0.4 µl of Phire II or Phusion DNA Polymerase, template DNA and water varied by application. Reactions were run on a Bio Rad C1000 Touch Thermal Cycler.

**RNA Isolation from Retina**

Mice were euthanized by CO₂ inhalation and cervical dislocation. Eyes were extracted and enucleated and the retinas were removed and stored at -80°C in RNAlater (Ambion). RNA was isolated according to the manufacturers protocol for the PureLink® RNA Mini Kit (Ambion). Briefly, retinal tissue was removed from RNAlater and homogenized in 10-20 volumes of Tri Reagent (Ambion) by pipetting. The homogenized mixture was incubated for 5 min at room temperature then 200 µl chloroform per ml of homogenate was added and the mixture vortexed, and incubated for an additional 5 min at room temperature; followed by centrifugation at 12,000 x g for 10 min at 4°C. The aqueous phase volume was transferred to a new 1.5 ml centrifuge tube and 200 µl of 100% ethanol was added to the sample and vortexed for 15 sec. The sample was placed in a filter cartridge (Ambion) and centrifuged at 12,000 x g for 30 sec. Flow through was discarded and 500 µl wash solution was applied to the filter cartridge and centrifuged at 12,000 x g for 30 sec. The wash step was repeated and filter cartridge was transferred to a new microcentrifuge tube and 100 µl of Elution buffer was applied to elute the RNA. The RNA was collected by centrifugation for 30 sec at 12,000 x g, and stored at -80°C.

**Zero Blunt pCR Cloning**

pCR-Blunt cloning was performed according the manufacturers instructions (Invitrogen MAN0007763). In brief, ligation reactions were set up containing 1 µl pCR-Blunt vector, 1 µl of blunt-end PCR product, 2 µl 5X ExpressLink T4 DNA Ligase Buffer,
5 µl sterile water and 1 µl ExpressLink T4 DNA Ligase. The ligation reaction was gently mixed and incubated at room temperature for 10 minutes. In the meantime, One Shot Top10 cells (Invitrogen) were thawed on ice. 2.5 µl of the ligation reaction was added to one vial of One Shot Top10 cells and mixed by swirling. Cells were incubated on ice for 15 min then heat shocked for 30 sec at 42°C and returned to ice for at least 1 minute. 250 µl of SOC medium was added to the transformed cells and they were incubated at 37°C while shaking at 225 rpm for 1 h. Cells were then plated on Circlegrow agarose plates (MP Biomedicals) supplemented with kanamycin 100 µg/mL.

**In-Fusion HD Cloning**

PCR fragments were amplified using primers designed to have 14-18 bp 5’ overhangs that share homology with the cut site on a linearized vector. PCR fragments were treated with Cloning Enhancer (Clontech) and incubated at 37°C for 15 min then at 80°C for 15 min on a PCR thermal cycler. Linearized vector, treated PCR fragment and water was added to 5X In-Fusion HD Enzyme Premix according to manufacturers suggestion (Clontech). The reaction was mixed by pipetting and incubated for 15 min at 50°C on a thermal cycler. The reaction mix was placed on ice or stored at -20°C until transformation.

**Plasmid Isolation**

Plasmid purification was performed using the Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturers protocol. Single colonies from transformed bacteria were selected from agarose plates using a sterile toothpicks and used to inoculate 2 ml of Circlegrow media supplemented with 100 µg/ml kanamycin. The culture was grown overnight at 37°C while shaking at 225 RPM. The bacterial cells were transferred to a 2 ml centrifuge tube and pelleted by centrifugation at max speed for 5 minutes to pellet cells. Supernatant was discarded and cells were resuspended by vortexing in 250 µl of Cell Resuspension Solution. 250 µl of Cell Lysis
Solution and 10 µl Alkaline Protease Solution was added, and mixed by inverting tubes several times then incubated at room temperature for 5 min. 350 µl of Neutralization Solution then was added to the tube and mixed by inverting several times. Tubes were centrifuged at 12,000 x g for 10 min to pellet cellular debris and the supernatant was decanted into a spin column. The spin column was placed into a collection tube and centrifuged for 1 min at 12,000 x g. The flow through was discarded and the plasmid bound to the column was washed 2 times by adding 500 µl of Column Wash Solution to the column and centrifugation for 1 min at 12,000 x g. The spin column was transferred to a clean microcentrifuge tube and 100 µl of sterile water was added to the column to elute plasmid DNA. The water was incubated on the column for 1 min at room temperature then centrifuged for 1 min at 12,000 x g to collect DNA. DNA was stored at -20°C.

**Antibodies**

The following primary antibodies and their concentrations were used: sheep anti-GPR179 (peptide KVQEETPGEDLDRPVLQKR), 1:2,000 (Peachey et al., 2012b); guinea pig anti-mGluR6 1:1,000 (Koike et al., 2010b); sheep anti-TRPM1 1:1,000 (Cao et al., 2011); goat anti-Gβ5 (peptide MATDGLHENETLASLKC), 1:1,000 (Morgans et al., 2007); goat anti-R9AP 1:1,000 (Hu and Wensel, 2002); sheep anti-RGS11 (peptide CSPALQSTPREPAATSSPEGADGE), 1:1,000 (Martemyanov et al., 2005); sheep anti-RGS7 1:1,000 (Morgans et al., 2007).

Secondary antibodies (Invitrogen, 1:1,000) appropriate to each primary antibody included: donkey anti-sheep Alexa-488, donkey anti-rabbit Alexa-680, donkey anti-rabbit Alexa-546, donkey anti-mouse Alexa-647 and donkey anti-guinea pig Cy3 (Millipore, 1:1,000).

**Immunohistochemistry**
Mice were euthanized with CO$_2$ followed by cervical dislocation. Their eyes were enucleated and the lens was removed. Eyecups were washed in phosphate buffered saline (PBS) then fixed for 30 min in a 4% formaldehyde PBS solution (pH 7.4). Eyecups were washed 3 times with PBS then cryoprotected in increasing concentrations of sucrose in PBS (10%, 15% for 1 h each and 30% overnight). Eyecups were embedded in 2:1 OCT/ 20% sucrose PBS solution frozen in a liquid nitrogen cooled bath of isopentane. Eyecups were sectioned (18 µm) using a Leica 1850 cryostat. Sections were mounted on glass slides (SuperFrost) and allowed to dry for 10 minutes at room temperature before being stored at -80°C. Sections were air dried at 37°C for 15 min and washed with PBS and PBS containing 0.05% Triton X-100 (PBX) for 5 min each, then blocked in PBX plus 5% normal donkey serum blocking solution for 1 h. Sections were incubated overnight at room temperature in the presence of the primary antibody diluted in blocking solution, then washed 3 times for 10 min each with PBX followed by incubation in secondary antibody in blocking solution for 1 h at room temperature. Sections were washed 2 X 10 min in PBX and 1 X PBS. Slides were cover-slipped using Immuno-Mount (Thermo Scientific) and slides were imaged using an Olympus FV1000 confocal microscope. Images were universally adjusted for brightness using Photoshop.
CHAPTER V: SUMMARY

I showed in Aim I that a mutation in Gpr179 was the cause of the no b-wave phenotype in the Gpr179\textsuperscript{nob5} mouse. The Gpr179\textsuperscript{nob5} mouse contains a \~6.5 kb insertion in intron 1 of the gene Gpr179 that is thought to disrupt mRNA processing between exon 1/2. Analysis of gene expression using qRT-PCR revealed exon 1/2 expression was greatly decreased in the Gpr179\textsuperscript{nob5} mouse. Morpholino disruption of GPR179 protein translation in zebrafish resulted in a greatly diminished ERG b-wave compared to scrambled control. Human patients with cCSNB were screened for mutations in Gpr179 and two patients were identified with inactivating mutations on each allele. These data indicate that Gpr179\textsuperscript{nob5} is a new model of cCSNB.

I determined Gpr179 is expressed in the INL of the retina using \textit{in situ} hybridization. Immunohistochemistry followed by confocal microscopy revealed the protein is localized to the tips of the DBCs in the OPL and GPR179 puncta juxtapose RIBEYE staining and colocalize with mGluR6. Finally, we generated a Gpr179\textsuperscript{-/-} mouse model to confirm its role in DBC function. The line is currently being generated at the University of Louisville.

In Aim II I examined the interdependency of DBC signalplex components on the expression of other cascade components using immunohistochemistry and confocal microscopy. I found that the localization of RGS7, RGS11, Gβ5 and R9AP to the DBC dendritic tips was dependent on expression of GPR179 (Fig. 21). Through collaboration we showed that GPR179 interacts with RGS7/Gβ5, RGS11/Gβ5 (Appendix II and Orlandi et al., (2012)), further supporting our hypothesis that GPR179 acts to
recruit and localize the RGS proteins to the DBC signalplex. I showed that GPR179 forms complexes with mGluR6 and TRPM1 using the Duolink PLA Assay. Similar conclusions were made by Orlandi et al. (2013). In Ray et al. (2013) (Appendix III) we showed that GPR179 and TRPM1 IP together from retinal lysates. Taken together, it appears GPR179 acts as a hub and recruits the RGS proteins to the DBC signalplex and brings them within close proximity of mGluR6 and TRPM1. Localizing all of the signalplex components into a macromolecular complex enhances the efficiency and fidelity of signaling between the mGluR6 receptor and the TRPM1 channel.

The finding that R9AP does not localize in the RGS7−/−/RGS11−/− DBCs is an interesting finding and suggests that the RGS complex consisting of R9AP, Gβ5 and RGS7 or 11 must form in an intracellular compartment before the complex can localize at the DBC dendritic tips. The RGS complex cannot form and does not localize properly if Gβ5 (Chen et al., 2003) or RGS7/11 are absent (Fig. 22). It was previously reported that R9AP localization in the DBCs was decreased in a hypomorphic RGS7−/−/RGS11−/− mouse (Zhang et al., 2010) but it was later determined that the mouse still retained RGS7 function.

The small ERG b-wave response in the Gpr179nob5 mice suggested that mGluR6 was capable of signaling to TRPM1 in the absence of GPR179 (Ray et al. 2013 and Appendix III). Using whole cell patch clamp we confirmed that mGluR6 could signal to TRPM1, albeit with reduced sensitivity in both the Gpr179nob5 and RGS7−/−/RGS11−/− rod BCs (Ray et al., 2013 and Appendix III). This reduced response is the result of the absence of RGS proteins in both models. The capsaicin response in the rod BCs displays another role for GPR179 in the DBCs. In the Gpr179nob5 rod BCs the capsaicin response is significantly decreased compared to WT, whereas in the RGS7−/−/RGS11−/− rod BCs the capsaicin response is no different than WT. These data indicate that
TRPM1 has reduced sensitivity to capsaicin because the TRPM1 interaction with GPR179 enhances the sensitivity of the channel to gating.

In Aim III we created a new mouse model of cCSNB based on a report of a newly identified gene being involved in DBC function in humans (Zeitz et al., 2013b). The new mouse model was created using ZFNs, which allowed the quickest and most efficient generation of a knockout animal. Using ZFN technology, we created several compound \textit{Lrit3} mutants that had a no b-wave ERG. We selected a \textit{Lrit3} mutant with a 40 bp deletion to generate the knockout line.

The gene \textit{Lrit3} encodes a single pass transmembrane protein with an extracellular LRR domain, similar to another LRR containing protein critical to DBC function, nyctalopin. We found that LRIT3 may serve a similar role as nyctalopin in the DBCs because both are required for TRPM1 localization at the DBC dendritic tips. We confirmed that TRPM1 is absent from the DBC dendritic tips in the \textit{Lrit3}\textsuperscript{-/-} mouse using whole cell recordings in response capsaicin puffs. These experiments indicate the disruption in the LRIT3 mutant is due to the absence of the TRPM1 channel. The mechanism by which LRIT3 controls TRPM1 localization to the DBC dendritic tips will be an important further line of investigation. Because the amount of TRPM1 at the DBC dendritic tips is known to set the light response amplitude (Peachey et al., 2012a), it will be important to determine if LRIT3 can actively change the amount of TRPM1 at the DBC dendritic tips in response to changes in light levels.

**Model of DBC signaling**

Using the knowledge gained from this project, we have developed a more thorough understanding of the rod BC signalplex protein arrangement and mechanism of signaling. When I started the project we had a very limited understanding of how the mGluR6 receptor signaled to the TRPM1 cation channel in the DBCs (Fig 5). Over the last 4 years we identified 2 new proteins, GPR179 and LRIT3, involved in DBC signaling.
(Peachey et al., 2012b) and characterized the interdependency of the DBC signalplex proteins (Orlandi et al., 2012). We used physiology in models of DBC dysfunction to understand the role of individual DBC signalplex proteins (Appendix III and Ray et al., (2013)). These data were used to assemble a more accurate model of the DBC signalplex and how it may function (Fig. 37).

We propose that GPR179 is the focal point of the signalplex protein scaffold. GPR179 interacts with TRPM1, mGluR6 and is responsible for recruiting the RGS machinery in close proximity to TRPM1 and mGluR6. The LRR proteins nyctalopin and LRIT3 play a critical role in recruiting TRPM1 to the DBC dendritic tips and they likely interact with extracellular matrix proteins to position TRPM1 at the synapse. The glutamate receptor mGluR6 is physically associated with this complex and likely interacts with one or more proteins through a transmembrane domain. The G proteins are tethered to the membrane through posttranslational modifications.

In the dark, photoreceptors are continuously releasing glutamate into the synaptic cleft where it becomes bound by mGluR6. This induces a conformational change in the receptor that allows it to bind GαoGDP and activate it to GαoGTP by GTP exchange. The newly formed GαoGTP is released from the receptor and the heterotrimeric G protein dissociates into GαoGTP and the Gβγ dimer. The Gβγ complex binds and gates the TRPM1 channel closed in a transient manner. The RGS proteins through their constitutive GTPase activity immediately inactivate the GαoGTP subunit. The GαoGDP subunit reforms the heterotrimeric complex with Gβγ. For gating of the TRPM1 channel to occur, activation of Gαo must outpace the constitutive RGS GTPase activity. Once the equilibrium shifts in favor of the RGS proteins through a decrease in glutamate release by the photoreceptors, the TRPM1 channel opens, causing the cell to depolarize.
Whether the cell is depolarized or hyperpolarized depends on the equilibrium of open to closed TRPM1 channels. When the glutamate signaling cascade is maximally activated by the presence of glutamate in the synaptic cleft, the equilibrium favors closed TRPM1 channels because G protein activation outpaces the constitutive RGS activity. In the absence of glutamate in the synaptic cleft, G protein activation ceases and the RGS proteins quickly deactivate any remaining active G proteins. The equilibrium shifts to mostly inactive G proteins and the TRPM1 channel opens, producing the light response.
Figure 37: Model of DBC signaling. The ratio of free G\(\beta\gamma\) to G\(\alpha\)G\(\beta\gamma\) determines the gating of the TRPM1 channel. In the dark glutamate in the synaptic cleft activates the mGluR6 receptor, which activates G proteins. Free G\(\beta\gamma\) transiently binds and gates the TRPM1 channel closed. In order for TRPM1 channels to be gated closed and keep the cell hyperpolarized G protein activation must outpace the constitutive RGS deactivation of G proteins. When glutamate is decreased in the synaptic cleft, G protein activation by mGluR6 is halted and the RGS proteins quickly deactivate any remaining active G proteins. G protein suppression of the TRPM1 channel is relieved causing the channel to open and the cell to depolarize. GPR179 serves to anchor the RGS complex in close proximity with the TRPM1 channel for increased gating sensitivity. GPR179 interaction with TRPM1 enhances the sensitivity of the channel to gating. LRIT3 and nytalopin serve to localize the TRPM1 channel at the synapse.
Future directions

This study has answered many questions regarding glutamate signaling in the DBCs. Just as important, it creates new questions that, upon answering, will give rise to a greater understanding of DBC function. It will be important to explore the role of the extracellular EGF domain of GPR179. Instead of GPR179 binding a ligand, this domain may be critical for interaction with the extracellular matrix or other proteins on the pre or postsynaptic side of the synapse. The large intracellular carboxy terminus of GPR179 is a large and contains an amino acid sequence of low complexity that likely gives it a disordered secondary structure. The current literature suggests that large scaffolding proteins often contain a disordered structure in solution, but upon binding their interacting partners they create a highly ordered protein scaffold (Dyson and Wright, 2005; Xie et al., 2007; Coletta et al., 2010; Ferreon et al., 2013). It will be important to identify the carboxy terminus-binding partners to gain a deeper understanding of signalplex architecture.

The GABA\textsubscript{B1} and GABA\textsubscript{B2} GPCRs form a heterodimer and are both required to carry out signaling by a trans-activating mechanism that involves one receptor binding the ligand and the other receptor activating the G proteins (Margeta-Mitrovic et al., 2000; Pagano et al., 2001; Rondard et al., 2008; Monnier et al., 2011). It will be interesting to determine if GPR179 and mGluR6 are capable of functioning in this manner. While it seems unlikely that GPR179 binds glutamate due to its lack of extracellular glutamate binding domain, it is possible that it can still play a role in the activation of G proteins because it is predicted to couple to Ga\textsubscript{i/o} G proteins.

The gating mechanism of G\beta\gamma on the TRPM1 channel will be important to determine. At this time it is unknown where the G\beta\gamma binding site on TRPM1 is located and what its stoichiometry is with TRPM1. Biophysical techniques such as fluorescent spectroscopy may be able to characterize this interaction.
The extracellular LRR domains of LRIT3 and nyctalopin are likely critical to protein function, yet nothing is known about their function in the DBCs. Understanding what these proteins interact with in the extracellular matrix will provide a greater understanding about how they coordinate the localization of proteins on both sides of the synapse. Crossing the Lrit3<sup>−/−</sup> mouse with various mouse models of cCSNB and transgenic models can help pinpoint the function of LRIT3. It will be important to cross the LRIT3 mouse with the Tg(Gabrr1-YFP/nyx)<sup>Rgg1</sup> mouse, which expresses a YFP tagged nyctalopin protein (Gregg et al., 2007), to determine if nyctalopin is localized to the DBC dendritic tips in the Lrit3<sup>−/−</sup> mouse.
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APPENDIX I: GPR179 Is Required for Depolarizing Bipolar Cell Function and Is Mutated in Autosomal-Recessive Complete Congenital Stationary Night Blindness

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Complete congenital stationary night blindness (cCSNB) is a clinically and genetically heterogeneous group of retinal disorders characterized by nonprogressive impairment of night vision, absence of the electroretinogram (ERG) b-wave, and variable degrees of involvement of other visual functions. We report here that mutations in GPR179, encoding an orphan G protein receptor, underlie a form of autosomal-recessive cCSNB. The Gpr179 Nob5/nob5 mouse model was initially discovered by the absence of the ERG b-wave, a component that reflects depolarizing bipolar cell (DBC) function. We performed genetic mapping, followed by next-generation sequencing of the critical region and detected a large transposon-like DNA insertion in Gpr179. The involvement of GPR179 in DBC function was confirmed in zebrafish and humans. Functional knockdown of GPR179 in zebrafish led to a marked reduction in the amplitude of the ERG b-wave. Candidate gene analysis of GPR179 in DNA extracted from patients with cCSNB identified GPR179-inactivating mutations in two patients. We developed an antibody against mouse GPR179, which robustly labeled DBC dendritic
terminals in wild-type mice. This labeling colocalized with the expression of GRM6 and was absent in Gpr179nob5/nob5 mutant mice. Our results demonstrate that GPR179 plays a critical role in DBC signal transduction and expands our understanding of the mechanisms that mediate normal rod vision.

Congenital stationary night blindness (CSNB) is a severe disability that impairs night vision. Complete CSNB (cCSNB) is a genetically heterogeneous form of the disorder that is caused by mutations in genes that are required for signal transduction through retinal depolarizing bipolar cells (DBCs).\(^1\)–\(^8\) The function of photoreceptors and DBCs can be assessed noninvasively with the electroretinogram (ERG), and their light-induced activities are reflected in the a-wave and b-wave, respectively.\(^9\) Individuals with cCSNB and animal models of the disorder have an ERG waveform that lacks the b-wave because of a failure to transmit the photoreceptor signal through the DBCs. Depolarization of the DBCs is initiated by a metabotropic glutamate receptor-mediated (GRM6)\(^10\) modulation of a transient receptor potential melastatin 1 cation channel (TRPM1).\(^11\)–\(^13\) This G protein signal transduction cascade utilizes G\(\alpha_\text{o},\)\(^14\) G\(\beta_\text{5,16}\) and depends on the auxiliary protein nyctalopin.\(^16,17\) Mutations GRM6 (MIM604096), TRPM1 (MIM 613216), or NYX (MIM 300278), which encodes nyctalopin, all can cause cCSNB in humans.\(^1\)–\(^8\) Mice with mutations in Grm6, Trpm1, Gna0, and Gnb5 or Nyx also have a no b-wave (nob) ERG phenotype.\(^10\)–\(^16,18\)–\(^20\) In this report, we define a critical role for GPR179, a previously uncharacterized orphan G protein receptor, in the DBC signal transduction cascade and in human cCSNB. Specifically, mutations in GPR179 in humans are responsible for a form of cCSNB. Consistent with this result, nob5 mice have a mutation in Gpr179 (Gpr179\(^\text{nob5/nob5}\)) and a nob ERG phenotype. Finally, zebrafish, whose Gpr179 expression is knocked down via morpholino injection, have a reduced ERG b-wave amplitude.
The *nob5* mouse arose as a spontaneous mutation in a colony of C3H mice and was identified via ERG when this line was crossed to a line of C3H mice lacking the rd1 mutation (C3H-fb/fb). To identify the causative mutation, we crossed affected *nob5* mice to wild-type (WT) C57BL/6J mice and the resulting F1 mice were intercrossed to generate a segregating mapping cross. We identified F2 progeny homozygous for the *nob5* locus by ERG and used them to map the phenotype by using a genome-wide screen with 103 simple sequence length polymorphic markers distributed throughout the genome. Initial mapping localized the gene to chromosome 11. Subsequently, > 600 additional informative meiosis refined the map location of the *nob5* locus to between D11Mit54 and D11Mit67. This 1.3 Mb region contains over 90 genes, none of which were known to be involved in DBC signal transduction or had been identified in molecular analyses of enriched pools of DBCs. To identify the mutation underlying the *nob5* phenotype we used genome capture and high-throughput sequencing. Comparison of the sequence encompassing the critical region in *nob5/nob5* and WT C3H mice revealed the presence of an insertion in intron 1 of Gpr179 (Figure 1A). The next-generation sequence data provided only 10 bp of sequence on either side of the insertion, but these data suggested the insertion was a transposable element. To examine this directly, we used PCR to amplify the insertion and its flanking intronic DNA. This revealed the presence of the predicted 1.3 kb fragment in WT mice and a 7.8kb fragment in homozygous affected littermates, indicating the insertion is ~6.5 kb (Figure 1A). Both bands were seen in heterozygotes. Henceforth, the mutant *nob5* allele will be referred to as *Gpr179*$_{nob5}$. Sequence analyses of the ends of the insertion indicated it was an endogenous retroviral element of the ERV2 class (Figure 1B). To evaluate the impact of this insertion on Gpr179 expression, we used a quantitative intronspanning Taqman RT-PCR assay to determine Gpr179 mRNA levels of WT and Gpr179*_{nob5}/nob5* retinas (Figure 1C). The expression of mRNA representing Gpr179 in the Gpr179*$_{nob5/nob5}$
retina was decreased more than 800-fold compared to the expression of mRNA in the WT retina. These data indicate that the $Gpr179^{nob5/nob5}$ phenotype is caused by a large insertion mutation in intron 1 of Gpr179; this insertion dramatically reduces gene expression and probably represents a null allele.
Figure 2: Transposable Element Disrupts Gpr179 Expression in nob5 Mouse (A) PCR fragments from Gpr179\textsuperscript{nob5/nob5} (lane 1), Gpr179\textsuperscript{nob5/+} (lane 2), and WT C3H (lane 3). The insertion is ~6.5 kb. (B) Schematic exon map of Gpr179 indicating location of nob5 insertion mutation. The arrows indicate location of PCR primers used in (A). (C) Quantitative PCR of Gpr179 cDNA generated from mRNA isolated from retinas of WT and Gpr179\textsuperscript{nob5/nob5} mice. Expression of Gpr179 was normalized to that of 18S RNA and is relative to the Gpr179 expression in WT. The error bars indicate mean ± standard deviation for three mice. Gpr179 expression in Gpr179\textsuperscript{nob5/nob5} retina is significantly reduced (p < 0.0005). All animal studies were approved by the local institutional animal care and use committees and conformed to all regulatory standards.
A series of dark-adapted ERGs obtained from representative WT, Gpr179nob5/p, and Gpr179nob5/nob5 mice are shown in (Figure 2A). Throughout the stimulus range examined, WT ERGs are dominated by a positive polarity b-wave, which increases in amplitude with increasing flash luminance and reflects the light-induced activity of DBCs.25 At higher flash luminance, the b-wave was preceded by a negative polarity a-wave, reflecting the light-induced closure of cation channels along rod photoreceptor outer segments.26 ERG responses in heterozygous Gpr179nob5/+ mice resembled the responses of WT mice, consistent with autosomal-recessive inheritance. In contrast, whereas large a-waves are obtained from homozygous Gpr179nob5/nob5 mice, these responses lack the b-wave component, revealing slow PIII, an ERG component generated by the radial Müller glial cells.27 Summary plots for the major components of the dark-adapted ERGs are shown in Figure 2B. ERG a-wave amplitudes were comparable across the three genotypes and the b-waves of Gpr179nob5/+ and WT mice were indistinguishable. The b-wave component is absent in Gpr179nob5/nob5 mice, and therefore, these data are not plotted. This ERG phenotype, in which the b-wave is absent while the a-wave is preserved, indicates that rod phototransduction is unaffected in Gpr179nob5/nob5 mice, whereas synaptic transmission between photoreceptors and DBCs, or DBC activity itself, is grossly abnormal.28 Light-adapted ERGs obtained from representative WT, Gpr179nob5/+ , and Gpr179nob5/nob5 mice are shown in Figure 2C. In WT mice, the cone ERG was dominated by the positive polarity b-wave and higher frequency oscillatory potentials, which reflect activity through the DBC pathway.29 In contrast, cone ERGs of Gpr179nob5/nob5 mice are electronegative. Summary plots for cone ERGs recorded from all three genotypes are shown in Figure 2D. When cone ERG amplitude is measured from the negative trough to the following positive peak, the Gpr179nob5/nob5 response is reduced in amplitude, whereas those from WT and Gpr179nob5/+ heterozygotes are comparable. The Gpr179nob5/nob5 ERG phenotype is
essentially indistinguishable from those of mouse mutants for other proteins involved in DBC signal transduction, GRM6, TRPM1, and NYX, protein required for correct localization of TRPM1 channels in DBC dendrites.
Figure 3: ERG Phenotype of Gpr179nobs/nobs Mice ERGs were recorded from mice anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) after overnight dark adaptation via a published procedure.\(^6\) (A) Dark-adapted ERG series obtained from representative WT (left), Gpr179nobs/+ (middle), and Gpr179nobs/nobs (right) littermates at 6 months of age. The scale bars indicates 100 ms and 500 mV. Values to the left of each row of waveforms indicate flash luminance in log cd s/m\(^2\). (B) Luminance- response functions for the major components of the dark-adapted ERG. The b-wave component is absent in Gpr179nobs/nobs mice and therefore these data are not plotted. (C) Cone-mediated ERG series obtained from WT (left), Gpr179nobs/+ (middle), and Gpr179nobs/nobs (right) littermates at 6 months of age. Scale bar indicates 100 ms and 100 mV. (D) Luminance- response functions for the cone ERG b-wave. Values to the left of each row of waveforms indicate flash luminance in log cd s/m\(^2\).
GPR179 encodes a predicted orphan G protein-linked receptor that has not been previously characterized in any cell or tissue. The nob5 phenotype predicts that the GPR179 gene product is required for DBC function and therefore should be present in DBCs. Because DBCs receive input from photoreceptors via ribbon synapses in the outer plexiform layer (OPL), we initially characterized gross retinal morphology and OPL ultrastructure of the Gpr179\textsuperscript{nob5/nob5} retina. However, all cellular and synaptic layers appeared normal in the Gpr179\textsuperscript{nob5/nob5} retina (Figure 3A). Furthermore, Gpr179\textsuperscript{nob5/nob5} ribbon synapses are indistinguishable from WT (Figure 3B). A normal retinal morphology is typical of all other mouse models of cCSNB.\textsuperscript{10,12,13,18,30}

To examine the cellular localization of GPR179 in the mouse retina, we developed a polyclonal sheep antibody to a peptide (KVQETPGEDLDRPVLQKR) located within the amino terminal extracellular domain. Retinal cryosections from Gpr179\textsuperscript{nob5/×} and Gpr179\textsuperscript{nob5/nob5} littermates were reacted with our antibody to GPR179 and an antibody against PKCα to label rod DBCs. In Gpr179\textsuperscript{nob5/+} retina, PKCα labeled the entire rod DBC (Figures 3C and 3E), whereas the GPR179 antibody produced a punctuate-labeling pattern (Figures 3D and 3E), which corresponds to the location of the OPL. In the Gpr179\textsuperscript{nob5/nob5} retina, labeling for PKCα (Figures 3F and 3H) is comparable to Gpr179\textsuperscript{nob5/nob5} and labeling for GPR179 is absent (Figures 3G and 3H), consistent with decreased Gpr179 mRNA expression in Gpr179\textsuperscript{nob5/nob5} retinas (Figure 1C). The punctuate-labeling pattern of GPR179 in the OPL is typical of proteins that are localized to the dendritic tips of DBCs, including GRM6, TRPM1 and NYX.\textsuperscript{12,16} To confirm this localization, we double-labeled retinal sections with antibodies to GPR179 (Figure 3I) and GRM6 (Figure 3J) The punctate labeling for GPR179 colocalizes with GRM6 (Figure 3L), the glutamate receptor known to mediate signaling in DBCs. These data show that GPR179 is expressed on the dendritic terminals of DBCs. GPR179 is not expressed elsewhere in the retina (data not shown). The combined ERG, genetic and
immunohistochemical data argue strongly that the \textit{Gpr179}^{nob5/nob5} mouse phenotype is caused by the insertion and results in a functionally null allele of GPR179.
Figure 4: Anatomical Phenotype of Gpr179\textsuperscript{nob5/nob5} Retina (A) Retinal cross-sections obtained from Gpr179\textsuperscript{nob5/+} and Gpr179\textsuperscript{nob5/nob5} mouse retinas fixed with 2.5% glutaraldehyde/2% paraformaldehyde and prepared according to published procedures.\textsuperscript{35} (B) Electron micrographs of ribbon structures in Gpr179\textsuperscript{nob5/+} and Gpr179\textsuperscript{nob5/nob5} mouse retinas prepared according to published procedures. (C–K) Confocal immunohistochemistry of retinas fixed with 4% paraformaldehyde for 15 min and prepared according to published procedures.\textsuperscript{16} The scale bar indicates 5 µm. (C–E) Gpr179\textsuperscript{nob5/+} retina labeled with antibodies against (C) PKCα, (D) GPR179, and (E) merge of (C) and (D). (F–H) Gpr179\textsuperscript{nob5/nob5} retina labeled with antibodies against (F) PKCα, (G) GPR179, and (H) merge of (F) and (G). (I–K) Gpr179\textsuperscript{nob5/+} retina labeled with antibodies for (I) GPR179, (J) GRM6, and (K) merge of I–J. The following antibodies were used: GPR179; affinity purified polyclonal sheep anti-GPR179 peptide (KVQETPGE+DLDRPVLQKR), 1:1,000; mouse monoclonal anti-ctbp2/Ribeye, 1:1,000 (BD Biosciences); guinea pig anti-GRM6, 1:1000 (see Koike et al.\textsuperscript{12}); rabbit anti PKCα (1:1,000, Sigma). The following secondary antibodies were used: Alexa-488 donkey anti-sheep, Alexa-555 goat anti-rabbit, and Alexa-633 goat anti-guinea pig (all at 1:1,000; Invitrogen).
To directly determine whether reduced GPR179 expression could recapitulate the reduced b-wave phenotype, we used morpholino knockdown in zebrafish, which have a single copy of gpr179 in their genome. We injected 1 cell stage zebrafish embryos with morpholinos (MOs) targeted against the Gpr179 translation start site (MO-Gpr179 50-GCCCCATACCTTTAGCAACTGCTTCT-30), and recorded ERGs at 4-6 days post fertilization. As comparisons, MOs against either the nyx translation start site (MO-Nyx 50-GATGAAACACATCACTGGCTTC-30) or control (C-MO 50-CCTCTTACCTCAGTTACAATTATA-30) were injected. Embryos injected with MO Gpr179 had a significantly reduced ERG b-wave amplitude, similar to embryos injected with MO-Nyx (positive control) (Figures 4A and 4B). The b-wave was unaffected following injection of the control MO (Figure 4B). The b-wave/a-wave ratio decreased from 3.79 ± 0.46 (n = 4) in control to 0.75 ± 0.11 (n = 7) in Gpr179-MO (p = 0.005). These results indicate that gpr179 expression is required for normal DBC function in zebrafish.
Figure 4: GPR179 Knockdown in Zebrafish Decreases ERG b-Wave Injected MOs (Gene Tools) were designed against the gpr179 translation site (MO 50-GCCCATACTTTTAGCAACTGCTTCTTCTTCT-30), which occurs as a single copy in the zebrafish genome, or the nyx translation site (MO 50-GATGAAACACATCACTGGCTTC-30) or were a standard control (MO 50-CCTCTTACCTCAGTTACAATTATA-30). In each case, 30 ng of MO was injected into the chorions of one cell-stage zebrafish embryos. ERGs were recorded from larvae at 4–6 days post fertilization with a 1 s stimulus after 30 min of dark adaption, as previously described.36 (A) ERGs from embryos injected with control (left column) or MOgpr179 (right column) MOs and tested at four flash intensities. Flash intensity at 0 log is 9.3 W/m². Neutral density (ND) filters reduced the intensity by the indicated amount in log units. The b-wave amplitudes obtained from MO-gpr179 injected embryos were strongly reduced at all intensities. The b-wave/a-wave ratio decreased from 3.79 ± 0.46 (n = 4) in control to 0.75 ± 0.11 (n = 7) in MO-gpr179 (p = 0.005), showing that a specific knockdown of gpr179 reduces DBC function. (B) The magnitude of b-wave reduction for MO-gpr179 and MO-nyx were comparable. Injections of MO-control did not affect the ERG (data not shown).
Mutations in *NYX*, *GRM6* or *TRPM1* have been identified in patients with cCSNB.\(^1\)\(^–\)\(^8\) However, a small number of individuals with cCSNB did not bear mutations in any of these genes.\(^8\) To evaluate the potential involvement of GPR179 in cCSNB patients lacking DBC function, we sequenced the 11 exons and flanking splice sites of the human gene in 44 patients (see Table 1 for primers). All human studies were undertaken with the approval of the appropriate institutional review board. We identified two probands with inactivating mutations in the GPR179 gene. Proband 1 had no family history of night blindness or consanguinity and was 10 years old at the time of diagnosis. He presented with 20/70 best corrected visual acuities, mild myopic refractive error, congenital nystagmus, a history of early onset nightblindness, a normal retinal appearance and full Goldmann visual fields. ERGs obtained under ISCEV standard conditions from proband 1 are shown in Figure 5A. Under dark-adapted conditions (upper traces), the ERG b-wave recorded to a low luminance stimulus was markedly reduced in amplitude, whereas the ERG obtained to a high flash luminance had a robust a-wave without the subsequent b-wave seen in controls (middle traces). Under light-adapted conditions (lower pair of traces), the ERG waveform showed a square a-wave but retained a late positive ERG component. ERGs of proband 2 showed a similar selective absence of the dark-adapted b-wave and a square light-adapted ERG a-wave (data not shown). These ERG abnormalities have been uniquely associated with human cases of DBC dysfunction\(^\text{32–34}\) and readily cCSNB from other retinal disorders with a reduced b-wave, such as incomplete CSNB (MIM 300071 and 610427).\(^33\)

Proband 1 was a compound heterozygote for two frameshift mutations in GPR179, c.187delC and c.984delC (NM_001004334.2) resulting in predicted protein truncations p.Leu63Serfs*12 and p.Ser329Leufs*4, respectively (Figure 5B). The premature chain termination is expected to result in functional null alleles. The probands’ unaffected parents were each heterozygous for one of the mutations (Figure 5B).
Proband 2 was 20 years old at the time of diagnosis. She is of Norwegian descent and presented with rotatory nystagmus, a very unusual blond fundus, and congenital nightblindness and was able to see 20/30 with a -12.00 D prescription. Although not known to be related, proband 2 also carried the c.984delC frameshift mutation identified in proband 1, suggesting that this might be a founder mutation. Proband 2 carried a second mutation, c.659A>G, that would result in missense mutation, p.Tyr220Cys. (Figure 5C). The three variants in probands 1 and 2 were not present in 210 healthy control chromosomes. Two out of three Alamut analyses, which predict mutation impact on function, classified the p.Tyr220Cys as potentially pathogenic. Moreover, by introducing a new cysteine into the GRP179 protein, the mutation is likely to impact its structure. The functional importance of Tyr220 is supported by its conservation across species ranging from human to Tetraodon (Figure 5D). DNA of family members of proband 2 was not available for segregation analysis.
Figure 5: GPR179 Mutations Are Present in Two Probands with Autosomal-Recessive cCSNB (A) ERGs obtained from a control subject and proband 1 for a standard series of stimulus conditions that allow rod- and cone-mediated responses to be evaluated. For proband 1 (patient 06-130), the two records indicate ERGs obtained from the two eyes. Under dark-adapted conditions, ERGs obtained from the proband had markedly reduced b-waves. Under light-adapted conditions (30 cd/m²), the cone ERG had a squared a-wave (arrow). Values indicate flash luminance in log cd s/m². (B and C) GPR179 (accession number NM_001004334.2) exons were sequenced from DNA samples isolated from 44 patients with cCSNB. Chromatograms containing the mutant sequences found in probands 1 (B) and 2 (C). In addition to the chromatogram, each subsection shows the mutation, the predicted impact on the amino acid sequence, and the segregation pattern. The pedigree for proband 1 shows that he inherited one mutant GPR179 allele from each parent (c.187delC and c.984delC), who had normal vision (data not shown). The parents of proband 2 were not available for analyses. (D) Comparison of the region of GPR179 containing the amino acid substitution (p.Tyr220Cys) identified in proband 2 across phyla. With the exception of Drosophila melanogaster, Tyr220 is conserved for every species for which data were available. In general, this region of the protein is highly conserved (the shade of blue indicates the amount of conservation; dark blue indicates the most conserved). The study followed the tenets of the Declaration of Helsinki and was approved by the ethics committee of the Academic Medical Centre, Amsterdam. All participants provided signed informed consent for participation in the study.
The combined data indicate that GPR179 is required for DBC signal transduction and that mutations that disrupt the function of GPR179 cause a recessive form of cCSNB. Although Gpr179nob5/nob5 mice lack expression of GPR179, we have not detected any anatomical defect in the retina, and the mice have no apparent health problem. Similarly, the two human patients we have identified with cCSNB and mutations in GPR179 have no other known health problems. Given the ERG phenotype in mice, zebrafish, and humans, as well as the colocalization of GPR179 with GRM6, we postulate that GPR179 plays a critical role in DBC signal transduction, possibly by forming heterodimers with GRM6. Future studies will be required to define the specific role of GPR179 in this process. The availability of the $Gpr179^{nob5/nob5}$ mouse model will be an important tool with which to address this question.
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APPENDIX II: GPR158/179 regulate G protein signaling by controlling localization and activity of the RGS7 complexes.

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Abstract

The extent and temporal characteristics of the GPCR signaling is shaped by the Regulator of G protein Signaling (RGS) proteins that promote G protein deactivation. With hundreds of the GPCRs and dozens of RGS proteins, compartmentalization plays key role in establishing signaling specificity. However, the molecular details and mechanisms of this process are poorly understood. Here we report that the R7 group of RGS regulators is controlled by the interaction with previously uncharacterized orphan
GPCRs: GPR158 and GPR179. We show that GPR158/179 recruit RGS complexes to the plasma membrane and augment their ability to regulate GPCR signaling. The loss of GPR179 in a mouse model of night blindness prevents targeting of RGS to the postsynaptic compartment of bipolar neurons in the retina, illuminating the role of GPR179 in night vision. We propose that the interaction of RGS proteins with orphan GPCRs serves as a mechanism that aids establishing signaling selectivity in G protein pathways.

**Introduction**

Signal transduction via heterotrimeric G proteins is fundamental for mediating a wide range of the cellular responses to changes in the extracellular environment (Offermanns, 2003). In these pathways, the signaling is initiated upon binding of ligand to a G protein coupled receptor (GPCR) that catalyzes the GDP/GTP exchange on the G protein, which leads to their dissociation into active Ga-GTP and Gbg subunits. Control of the kinetics and extent of the signaling in the G protein pathways is realized through the action of the RGS proteins that inactivate the signaling by promoting the GTP hydrolysis on G protein a subunits (Hollinger and Hepler, 2002; Ross and Wilkie, 2000). In mammalian nervous systems, the R7 family of RGS proteins (RGS6, RGS7, RGS9 and RGS11) play key roles in synaptic transmission, light perception and sensitivity to addictive drugs by regulating several GPCR pathways (Anderson et al., 2009; Slepak, 2009). The function of the R7 RGS proteins depends on the formation of the macromolecular complexes with other proteins that dictate their catalytic activity and compartmentalization and allows achieving signaling specificity. Two homologous membrane-anchoring subunits have been previously shown to form complexes with R7 RGS proteins: RGS9 anchor protein (R9AP) and R7 Binding Protein (R7BP) (Jayaraman et al., 2009). Knockout of R9AP or R7BP in mice has been shown to dramatically affect the localization and expression of RGS9 and RGS11 (Anderson et al., 2007a; Cao et al.,
2009; Keresztes et al., 2004). However, the protein levels of RGS6 and RGS7 were not affected upon the elimination of R7BP and only minor changes in the membrane recruitment of these proteins was observed in neurons lacking R7BP (Anderson et al., 2007a; Cao et al., 2008; Panicker et al., 2010). These observations suggest the presence of other, yet unidentified membrane anchor(s) for R7 RGS proteins. However, homology searches of genomic sequences revealed no proteins with sufficient similarity to R7BP/R9AP.

In this report we used an unbiased proteomic approach to identify additional membrane anchors for RGS7 in the nervous system. We demonstrate that the previously uncharacterized orphan GPCRs, GPR158 and GPR179, control localization and activity of RGS7/Gb5 complexes both in reconstituted cells and in vivo. These findings for the first time describe the role of orphan GPCRs GPR158 and GPR179 in the regulation of G protein signaling.

Results and Discussion

Identification of GPR158 as a binding partner of RGS7 in the brain

We conducted an unbiased screen aimed at identifying novel binding partners of RGS7. RGS7 was immunoprecipitated from the total brain lysates followed by the mass-spectrometric sequencing of pulled down proteins. Gb5 knockout mice, which show dramatically reduced expression of RGS7 (Chen et al., 2003), were used as a negative control to exclude non-specific interactions. We found only 2 proteins with confidence similar to RGS7 (Fig. 1A). The first protein was Gb5, a well-known binding partner of RGS7, validating our identification strategy. The second protein was identified as an orphan G protein coupled receptor 158, or GPR158 (Fig. 1B; Supplemental Table 1). Tandem mass-spectrometry analysis of the identified peptides revealed high confidence of sequence assignment (Supplemental Fig. S1).
Based on amino acid sequence similarity, GPR158 belongs to the class C GPCR family (Bjarnadottir et al., 2005). Our bioinformatics analysis indicates that the GPR158 (accession NP_065803.2) is conserved across multiple species and contains several conserved residues in the intracellular face of the TM3 and TM6 (Fig. 1 B). However, GPR158 lacks the extracellular venus-flytrap module that plays an essential role in ligand binding and receptor activation in all known class C receptors (Bjarnadottir et al., 2005; Jingami et al., 2003). Instead, GPR158 features two other conserved elements that are not found in typical class C receptors: a calcium binding EGF-like domain (aa 314-359) and leucine repeat region (aa 108-136). The expression of GPR158 is detected in many tissues, but the protein is particularly prominent in the nervous system (Fig. 1C).

**GPR158 specifically interacts with RGS7/Gb5 complex and competes with R7BP**

To validate the interaction of RGS7 with GPR158 we first verified their co-immunoprecipitation from the brain lysates by Western blotting. As illustrated in Fig. 1D precipitation of RGS7 pulls down GPR158 from wild type but not from Gb5 knockout tissues confirming the specificity of the interaction. Conversely, antibodies against GPR158 but not non-immune IgG effectively co-precipitate RGS7 from the brain (Fig. 1D). We next examined interaction between GPR158 and RGS7 in transfected HEK293. Immunoprecipitation of GPR158 by the engineered affinity myc-tag resulted in efficient pull-down of RGS7 together with Gb5 when the proteins were co-expressed (Fig. 1E). Similarly, reciprocal immunoprecipitation of HA-tagged-RGS7 led to co-precipitation of GPR158 when both proteins were present in the cells (Fig. 1E).

Since RGS7 shares high degree of similarity with other R7 RGS proteins we tested if GPR158 interacts with RGS6/Gb5, RGS9-2/Gb5 and RGS11/Gb5 complexes. We found that GPR158 could only co-precipitate with RGS6 but not with RGS9-2 or
RGS11 (Fig. 1F). Although interaction with RGS6 was specific, it was apparently less efficient relative to RGS7 binding.

RGS7/Gb5 dimer has been previously shown to bind membrane anchor R7BP (Drenan et al., 2005; Martemyanov et al., 2005). Therefore, we next explored whether RGS7/Gb5 can simultaneously bind to both GPR158 and R7BP. Co-immunoprecipitation studies following the reconstitution in HEK293 cells show that the interaction of RGS7 with GPR158 and R7BP is mutually exclusive (Fig. 1G). R7BP pulls down only RGS7 but not GPR158. Conversely, GPR158 pulls down RGS7 but not R7BP. This mutually exclusive nature of R7BP and GPR158 binding to RGS7/Gb5 was further confirmed in the competition experiments (Fig. 1H). The interaction of RGS7 with GPR158 was progressively reduced upon increase in R7BP expression. Similarly, the binding of RGS7 to R7BP decreased when more GPR158 was supplied to the cells.
Figure 1. GPR158 is a novel binding partner of RGS7. A, Summary of the mass-spectrometric analysis of proteins present in the eluates from the preparative immunoprecipitation reaction (IP) using RGS7 antibodies. Positive identification criteria were set to 95% confidence. Yellow field shows hits above this threshold while red- below. In addition, only proteins with the number of identified unique peptides similar to the target protein (RGS7) were considered (green field). B, Bioinformatic analysis of GPR158 organization. Predicted transmembrane domains and conserved features are depicted as cylinders. Cytoplasmic surface of GPR158 features conservation of key residues important for the G protein activation in class C GPCRs, including Lys 502 and Arg 505 at the cytoplasmic end of the TM3 and Glu 609 in the third intracellular loop. C, Regional specificity of GPR158 expression as evidenced by Western blotting analysis of tissue lysates. D, RGS7 and GPR158 co-immunoprecipitate from native brain lysates. Whole brain tissue was extracted and used for the immunoprecipitation experiments using either RGS7 or GPR158 specific antibodies. E, RGS7 and GPR158 form complex upon co-expression in HEK293 cells. Cells were transfected with the indicated
constructs and proteins were immunoprecipitated using antibodies directed against affinity tags. F, GPR158 binds to RGS6 but not to RGS9 or RGS11. GPR158 was co-transfected into HEK293 cells with indicated RGS constructs, and co-precipitating RGS proteins were revealed by Western blotting. G, GPR158 does not co-immunoprecipitate with R7BP in the presence of RGS7/Gb5 complex in transfected HEK293 cells. H, GPR158 and R7BP compete for binding to RGS7. Transfection of increasing amounts of R7BP reduced co-immunoprecipitation of GPR158 with RGS7 and conversely, increasing concentrations of GPR158 reduced binding of RGS7 to R7BP.
GPR158 targets RGS7/Gb5 complex to the plasma membrane via the interaction with the DEP domain

Since GPR158 is a membrane protein we next asked whether it can change the localization of RGS7 complex in the cells. We found that when expressed in HEK293 cells, GPR158 is efficiently targeted to the plasma membrane (Fig. 2A). In contrast, as previously noted (Drenan et al., 2006; Zhang et al., 2001), localization of RGS7/Gb5 was mostly cytoplasmic. However, co-expression of RGS7/Gb5 with GPR158 resulted in its efficient translocation to the plasma membrane (Fig. 2A). We further confirmed recruitment of RGS7/Gb5 to the plasma membrane biochemically (Fig. 2B). Consistent with the immunocytochemistry data, most of RGS7 was found in the cytosolic fraction upon sedimentation analysis but moved to the membrane pellet when co-expressed with GPR158 (Fig. 2B). These results suggest that GPR158 serves as a membrane anchor for RGS7/Gb5 complex.
Figure 2. GPR158 recruits RGS7 to the plasma membrane in a DEP domain-dependent manner. A, Analysis of GPR158 and RGS7 localization in transfected HEK293 cells by immunocytochemistry followed by confocal microscopy. RGS7/Gb5 and GPR158 were expressed alone or together and their localization was examined. B, RGS7 fractionates with the plasma membrane fraction in the presence of GPR158. Following transfection cells were disrupted and fractionated by sedimentation into membrane and cytosol portions. Band densities were quantified from 3 independently conducted experiments. **, p<0.01, t-test. C, full-length RGS7, but not RGS7 mutant with the deleted DEP domain (DEPless RGS7) binds to GPR158. HEK293 cells were co-transfected with the indicated constructs and interaction of proteins was studied by the immunoprecipitation. D, DEPless RGS7 does not co-segregate with GPR158 in the membrane fraction upon sedimentation analysis. E, DEPless RGS7 fails is not recruited to the plasma membrane of the transfected HEK293 cells that express GPR158. Cells were co-transfected with indicated constructs and protein localization was studied by immunostaining.
Competition between GPR158 and R7BP for binding to RGS7 suggests that their interactions are mediated by the same or overlapping determinants. Since binding to R7BP requires the presence of the DEP domain of RGS7 (Anderson et al., 2009) we hypothesized that this domain also mediates the interaction of RGS7 with GPR158. Indeed, a truncated mutant of RGS7 lacking the DEP domain (DEPless), while preserving the interaction with Gβ5 did not co-immunoprecipitate with GPR158 upon co-transfection in HEK293 cells (Fig. 2C). Consequently, DEPless-RGS7 failed to be recruited to the plasma membrane by GPR158 as evidenced by either biochemical fractionation (Fig. 2D) or immunocytochemistry (Fig. 2E).

**GPR158 augments GAP activity of RGS7 towards Gao**

The identification of the novel interaction of RGS7/Gb5 complex with GPR158 raises the question about its physiological significance. Members of the R7 RGS family are efficient GAPs for the Go class of the proteins, downstream from multiple GPCRs including m-opioid receptor (Anderson et al., 2009). We therefore used a cell based BRET assay to monitor the effects of RGS7/Gb5 on Gao activated by the m-opioid receptor (Fig. 3A; Hollins et al., 2009). We primarily focused on analyzing the deactivation kinetics of G protein signaling that reflect the catalytic activity of RGS proteins. Consistent with the previous reports, we found that RGS7/Gb5 complex accelerated Gao deactivation kinetics (Fig. 3B). Co-transfection of GPR158 resulted in further acceleration of signaling termination. Notably, GPR158 did not influence the kinetics of μ-opioid signaling when supplied without RGS7/Gb5 complex, indicating that it acts via increasing the activity of RGS7 (Fig. 3C). Indeed, the catalytic activity of RGS7/Gb5 as measured by the k_GAP parameter was increased by GPR158 by ~2 fold (from 0.081±0.016 s⁻¹ to 0.152±0.022 s⁻¹). We found no effect of GPR158 on either the activation kinetics of Gao that reflects MOR activity in the cells (Fig. 3D) or the expression of reporter constructs (Supplemental Figure S2). Furthermore, no
significant effect of GPR158 on the expression of RGS7 was detected under the conditions and concentrations of components used for the BRET assays (Fig. 3E). This suggests that GPR158 exerts direct stimulatory effect the activity of RGS7/Gb5 complex.
Figure 3. GPR158 potentiates the ability of RGS7/Gb5 to deactivate Gao signaling. 

A, Schematic representation of the BRET-based assay to monitor G protein signaling cycle. Activation of the m-opioid receptor (MOR) causes the G protein heterotrimer to dissociate into Ga and Gbg subunits. Released Gbg subunits tagged with Venus fluorescent protein interact with luciferase (Rluc) –tagged reporter GRK to produce BRET signal. Upon termination of MOR activation by antagonist naloxone, Gao subunit hydrolyses GTP and re-associates with Gbg subunits quenching the BRET signal. B, Time-course of the normalized BRET responses recorded in a representative experiment. Individual data points show BRET values averaged from 6 replicates. Application of MOR agonist (morphine) and antagonist (naloxone) is indicated by the upper bars. Deactivation phase of the response was fitted with the single exponent (solid line). C, Quantification of the deactivation time constant following the addition of naltrexone. Exponential fits of the data shown in panel B were used to derive time constant t. Asterisks indicate statistical significance of the differences (**, p<0.01 and ***, p<0.001; Mann-Whitney Rank Sum Test; n=18) as compared to control experiment with no regulators added (black bar). D, Quantification of the activation time constant derived from the exponential fitting of the onset kinetics (fits not shown). Note no significant differences between experimental conditions. E, Analysis of the GPR158 effect on expression level of RGS7. The HEK293 cells were transfected with all constructs used for the BRET experiments using the same ratios and conditions. Proteins of interest were detected by Western blotting using specific antibodies.
GPR158-like protein, GPR179 targets RGS/Gb5 complexes to the dendritic tips of ON-bipolar cells

Regulation of RGS7/Gb5 complex localization and activity by GPR158 prompted us to ask whether the interaction with the GPCR-like proteins is a general mechanism for controlling RGS7 function in neurons and whether proteins similar to GPR158 might also be engaged in this process. Our analysis shows that GPR158 shares substantial sequence similarity with another orphan receptor GPR179 (Fig 4A). We found that GPR179 (accession NP_001004334.2), just like GPR158, also forms specific complexes with RGS7 (Fig. 4B). However, unlike GPR158, it could interact with all members of the R7 RGS subfamily (Fig. 4C). Interestingly, GPR179 exhibited much more restricted expression and was detected only in the retina (Fig. 4D). Two recent studies showed that mutations in GPR179 gene cause congenital stationary night blindness (CSNB) in humans indicating GPR179 is required for normal synaptic transmission between photoreceptors and ON-bipolar cells in the retina (Audo et al., 2012; Peachey et al., 2012). Furthermore, a mouse mutant nob5 lacks an ERG b-wave and is a model for this form of cCSNB (Peachey et al., 2012). Earlier studies showed that RGS7/Gb5 and RGS11/Gb5 complexes co-localize with the essential components of the signaling cascade at the dendritic tips of the ON-bipolar cells and play an important role in normal transmission at this synapse (Chen et al., 2010; Mojumder et al., 2009; Zhang et al., 2010). We therefore asked whether GPR179 could influence localization and function of RGS proteins in these neurons. Indeed, our examination revealed that RGS7 and RGS11 co-localizes with GPR179 at the dendritic tips of the ON-bipolar cells (Fig. 4E). Remarkably, loss of GPR179 in the nob5 mice resulted in loss of the punctate staining for both RGS7 and RGS11 (Fig. 4 G,H), although their protein levels were unchanged (Fig. 4F). These data suggest that GPR179 is essential for the postsynaptic targeting of the RGS/Gb5 complexes in retinal ON-bipolar neurons.
Figure 4. GPR179 is a parologue of GPR158 required for subcellular targeting of RGS7/Gb5 complex in vivo. A, GPR158 shares considerable sequence homology and conservation among species with GPR179 as revealed by phylogenetic analysis. B, GPR179 forms complexes with RGS7 in transfected cells. Forward and reverse immunoprecipitation experiments were carried using indicated antibodies following co-transfection of GPR158 with RGS7 in HEK293 cells. C, Interaction of GPR179 with members of the R7 RGS subfamily. RGS/Gb5 complexes were co-expressed with GPR179 in HEK293 cells and the interactions between proteins were studied by the co-immunoprecipitation assays. D, Expression profile of GPR179 across nervous tissues as determined by the Western blotting of total tissue lysates. Ponceau S staining for total protein indicates equal sample loading. E, GPR179 co-localizes with RGS7 and RGS11 at the dendritic tips of the ON-bipolar cells in the outer plexiform layer of the retina. Retina cross-sections were immunolabeled for GPR179 (green) and RGS7 (red). Note characteristic punctate pattern of staining that indicates synaptic localization of both proteins. F, Loss of GPR179 in nob5 retinas does not affect the expression of RGS7 and RGS11. Western blot analysis of RGS7 and RGS11 expression in total retina lysates from wild type mice (WT) or mice lacking GPR179 (nob5). G and H, Elimination of GPR179 prevents targeting of RGS7 and RGS11 but not TRPM1 to the dendritic tips. Retina cross-sections were double immunostained for RGS7 (red) and GPR179 in panel G or RGS7/RGS11 (red) and TRPM1 (green) in panel H. Cell nuclei are labeled with DAPI (blue).
GPR158/179 and G protein signaling

The results of our study reveal the existence of a new family of membrane anchors and activity modulators for the R7 RGS proteins that belong to the group of orphan GPCRs and contain two members GPR158 and GPR179. Two other membrane anchors for the R7 RGS family have been described previously (Jayaraman et al., 2009). R9AP was found to form complexes with RGS9 and RGS11 but not with RGS6 or RGS7 and was demonstrated to play key role in controlling posttranslational stability, subcellular targeting and activity of its RGS partners (Anderson et al., 2009; Jayaraman et al., 2009). Subsequently, R9AP homologue, R7BP, was identified as universal partner for all members of the R7 RGS subfamily (Drenan et al., 2005; Martemyanov et al., 2005). Similarly to R9AP, R7BP was demonstrated to play role in stabilization, localization and activity regulation for some, but not all R7 RGS proteins in some, but not all neurons (Anderson et al., 2009; Jayaraman et al., 2009). For example, R7BP was shown to be important for achieving the high expression level of RGS9-2 in the striatum, but did not affect the expression of RGS7 in the same region (Anderson et al., 2007a). Likewise, while R7BP is important for the recruitment of RGS7/Gb5 complex to the nucleus (Panicker et al., 2010), it was not required for the delivery of the same complex to the dendritic tips of the ON-bipolar neurons in the retina (Cao et al., 2008) and only mildly affected RGS7/Gb5 recruitment to the plasma membrane in the brain (Panicker et al., 2010). These observations suggested the existence of additional targeting mechanisms for the R7 RGS complexes, particularly for the RGS7/Gb5. The identification of GPR158 and GPR179 as RGS membrane anchors, suggests a new mechanism for achieving subcellular targeting of this important class of GPCR regulatory proteins.

Our findings provide a new example for the interaction between R7 RGS proteins with the GPCRs. Previous studies found that RGS9 can form complexes with m-opioid
Garzon et al., 2005) and D2 dopamine receptors (Kovoor et al., 2005). Furthermore, RGS7/Gb5 complexes have been shown to bind to the third intracellular loop of M3 muscarinic receptor, via a direct protein-protein interaction involving the DEP domain of the molecule (Sandiford et al., 2010). As in case of the GPR158, this binding was mutually exclusive with R7BP recruitment, and R7BP prevented the interaction. Similarly, the DEP domain of the primordial yeast RGS protein Sst2 mediates its recruitment to the pheromone sensing GPCR Ste2 (Ballon et al., 2006). These observations indicate that in addition to specialized membrane anchoring subunits, the DEP domains of R7RGS proteins are recruited to elements found in some GPCRs. Yet, no common motifs are detected across any DEP domain interacting proteins reinforcing an idea that these modules potentially recognize a diverse set of targets.

The interaction with GPR158 results in translocation of RGS7/Gb5 complexes to the plasma membrane compartments and augmentation of their catalytic activity. In the case retinal ON-bipolar cells, GPR179 is required for the localization of both RGS7 and RGS11. Since these RGS proteins in complex with the Gb5 are essential for the synaptic transmission at the ON-bipolar synapse (Chen et al., 2003), their mistargeting in mice with GPR179 deletion or human patients with mutations in GPR179 gene (Audo et al., 2012; Peachey et al., 2012) may account for their no b-wave phenotype and night blindness, respectively. What remains unexplored however is whether GPR158 and GPR179 only serve as RGS anchor proteins, or whether they can act as bona fide GPCRs. Both are distant members of the class C GPCRs (Bjarnadottir et al., 2005) and our bioinformatics analysis shows that the amino acids that are critical for the ability of the class C receptors to activate G proteins (Binet et al., 2007) also are conserved in GPR158 and GPR179. This suggests a possibility that these orphan receptors can, in principle, activate G proteins. However, the lack of typical class C ligand binding domain
in GPR158/GPR179 suggests that if they can in fact activate G proteins the mechanism must be substantially different.

**Materials and Methods**

**Mice, antibodies and genetic constructs**

Generation of sheep anti-RGS6, sheep anti-RGS9-2 and sheep antiRGS11 antibodies was described (Cao et al., 2008; Martemyanov et al., 2005). Rabbit anti-ß5, rabbit anti-RGS7 (7RC1) and rabbit anti-R7BP (TRS) were generous gifts from Dr. William Simonds (National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD). Mouse anti-HA (Millipore), rabbit anti-myc (Genscript), rabbit anti-GPR179 (Sigma) and rabbit anti-GPR158 (Sigma) were purchased. The *GPR179<sup>nob5/nob5</sup>* mice are described elsewhere (Peachey et al., 2012).

Cloning of full-length RGS7, DEPless-RGS7, RGS6, RGS9-2, RGS11, ß5, R7BP and N-terminal HA-tagged RGS7 in pcDNA3.1/V5-His-TOPO was described (Anderson et al., 2007b; Martemyanov et al., 2003; Martemyanov et al., 2005; Panicker et al., 2010; Porter et al., 2010). Human myc-tagged-GPR158 and myc-tagged-GPR179 were purchased from OriGene. BRET sensor constructs Venus155-239-Gb1, Venus1-155-Gg2 and masGRKct-Rluc8 were kindly provided by Dr. Nevin A. Lambert (Medical College of Georgia).

**Cell culture, Transfection, Immunoprecipitation and Western blotting**

HEK293T cells were cultured at 37°C and 5% CO2 in DMEM supplemented with 10% FBS, MEM non-essential amino acids, 1 mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin. Cells were transfected using Lipofectamine LTX (Invitrogen), harvested 24 h later, lysed in ice-cold IP buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Triton X-100 and complete protease inhibitor cocktail) by sonication. For immunoprecipitation, lysates were cleared by centrifugation at 14,000
rpm for 15 min, and the supernatants were incubated with 20 µl of Dynabeads (Invitrogen) and 2 µg of antibodies on a rocker at 4°C for 1 h. After three washes with IP buffer, proteins were eluted with 50 µl of 4X SDS sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting using HRP-conjugated secondary antibodies and an ECL West Pico (Thermo Scientific) detection system.

**Preparative immunoprecipitation and mass spectrometry**

Whole brains were removed from mice, homogenized in IP buffer (1xPBS, 0.3 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Roche)) with a series of needles with increasing gauge and lysed for 30 min at +4 °C. Samples were centrifuged (30,000 g, 20 min) to remove debris, and supernatants were incubated for 45 min with 5 mg of anti-RGS7 antibody covalently coupled to Protein G beads as previously described (Martemyanov et al., 2005). Protein complexes were eluted with 5% ammonium hydroxide and vacuum-dried.

Proteins were dissolved in 0.5 M triethylammonium bicarbonate (pH 8.5) containing 0.1% SDS, reduced with 5 mM tris-(2-carboxyethyl) phosphine for 1 h at 60 °C and alkylated with 10 mM methyl methanethiosulfonate for 10 min at room temperature. Proteins were digested with 20 µg of modified porcine trypsin (Promega) in the presence of 3mM CaCl$_2$ at 37 °C for 16 h. Peptide mixtures were vacuum-dried, reconstituted in 0.2% formic acid (Pierce, Rockford, IL) and applied to a MCX cartridge (Waters, Milford, MA) pre-equilibrated with methanol/water (1:1, v/v). The cartridge was washed with 0.1% formic acid in 5% methanol, followed by a 100% methanol wash. Peptides were eluted from the MCX resin in 1 ml of 1.5% NH$_4$OH in methanol and vacuum-dried. The resulting peptide mixtures were desalted, resolved by liquid chromatography and analyzed by LTQ-Orbitrap XL mass spectrometry as described (Cao et al., 2011).

**Membrane/cytosol fractionation**
HEK293T transfected cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 2.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, complete protease inhibitor cocktail) by 10 passages through a 27-gauge needle and then centrifuged at 400 x g for 5 min at 4°C to remove nuclei and intact cells. Supernatants were ultracentrifuged at 150,000 x g for 20 min at 4°C. The cytosolic soluble fraction was collected from the supernatant while the pellet containing the membrane fraction was resuspended in an equal amount of lysis buffer. Samples were then sonicated and 4X SDS sample buffer added for Western blot analysis.

Immunocytochemistry

HEK293T transfected cells or retina cross-sections were fixed for 15 min with 4% paraformaldehyde, permeabilized for 5 min with 0.2% Triton X-100/PBS, blocked with 10% donkey serum for 1 h, and incubated with primary antibody in 2% donkey serum for 1 h. After three washes, sections were incubated with AlexaFluor488 or 546-conjugated secondary antibodies for 1 h. Cells were stained 5 min with DAPI before mounting in Fluoromount (Sigma). Images were taken by the LSM 780 Zeiss confocal microscope.

Monitoring G protein cycle in live cells by fast kinetic BRET assay

Agonist-dependent cellular measurements of bioluminescence resonance energy transfer (BRET) between masGRKct-Rluc8 and Gb1g2-Venus were performed to visualize the action of G protein signaling in living cells as previously described with slight modification (Hollins et al., 2009; Lambert et al., 2010). HEK293T/17 were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), MEM non-essential amino acids, 1 mM sodium pyruvate, and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) at 37°C in a humidified incubator containing 5% CO2. For transfection, cells were seeded into 6-cm dishes at a density of 4×10^6 cells/dish. After 4 h, expression constructs (total 5 µg/dish) were transfected into the cells using Lipofectamine LTX (8 µL/dish) and PLUS (5 µL/dish)
reagents. MOR, Gao, Venus155-239-Gb1, Venus1-155-Gg2, masGRKct-Rluc8, RGS7, Gb5, and GPR158 constructs were transfected using equal DNA amounts. Empty vector was used to balance the amount of transfected DNA. The cells were used for experiments at 16-24 h after transfection. BRET measurements were made using a micro plate reader (POLARstar Omega; BMG Labtech) equipped with two emission photomultiplier tubes, allowing to detect two emissions simultaneously with resolution of 50 milliseconds for every data point. All measurements were performed at room temperature. The BRET signal is determined by calculating the ration of the light emitted by the Gb1g2-Venus (535 nm) over the light emitted by the masGRKct-Rluc8 (475 nm). The average baseline value recorded prior to agonist stimulation was subtracted from BRET signal values, and the resulting difference (R) was normalized against the maximal value (Rmax) recorded upon agonist stimulation.

**Statistical analyses**

We used Student’s t-test to analyze densitometry data from biochemical fractionation experiments. For the analysis of the non-parametric data reporting differences in the exponential rate constant of the G protein deactivation kinetics observed in BRET experiments we used Mann-Whitney rank-sum test. The confidence values below p<0.05 were considered to be statistically significant.

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**Abbreviations List**

RGS: Regulators of G protein Signaling
GPCR: G Protein Coupled Receptor

GAP: GTPase Activating Protein

DEP: Disheveled, EGL-10, Pleckstrin homology domain

R7BP: R7 family Binding Protein

R9AP: RGS9 Anchor Protein

References


Appendix III: GPR179 is required for high sensitivity of the mGluR6 signaling cascade in depolarizing bipolar cells

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ABSTRACT

The two visual parallel pathways are initiated at the first retinal synapse by signaling between the photoreceptors and two types of bipolar cells. For normal function, ON or depolarizing bipolar cells (DBCs) require the G protein-coupled receptor, mGluR6, its G protein-coupled cascade and the transient receptor potential melastatin 1 (TRPM1) cation channel. Another seven transmembrane protein, GPR179, also is required for DBC function and recruits the regulators of G-protein signaling (RGS) proteins, RGS7 and RGS11, to the dendritic tips of the DBCs. Here we show that GPR179 and
RGS7/RGS11 play different roles in modulation of the mGluR6 mediated gating of TRPM1. We show that expression of several mGluR6 cascade components including, mGluR6, TRPM1 and nytalopin are independent of GPR179 expression and GPR179 interacts with TRPM1. In Gpr179<sup>nob5</sup> mice a small dark-adapted electroretinogram b-wave is present that can be enhanced with long flashes, indicating some rod DBC function. Patch clamp recordings demonstrate that Gpr179<sup>nob5</sup> and RGS7<sup>-/-</sup>/RGS11<sup>-/-</sup> rod DBCs retain mGluR6-mediated gating of TRPM1 when strong stimuli are used. In contrast, the two mutants differ in that direct gating of TRPM1 by capsaicin in RGS7<sup>-/-</sup>/RGS11<sup>-/-</sup> and WT rod DBCs is similar, but severely compromised in Gpr179<sup>nob5</sup> rod DBCs. Noise and standing current analyses indicate that TRPM1 channels in Gpr179<sup>nob5</sup> rod DBCs have a lower open probability than in WT, presumably from G protein suppression. We propose that the GPR179 not only localizes RGS7 and RGS11 to the dendritic tips of DBCs, but alters the “state” of the TRPM1 channel by direct interactions.

**INTRODUCTION**

Visual processing in the mammalian retina is initiated through two parallel vertical pathways that are established at the synapse between photoreceptors and bipolar cells (BCs). In the dark, photoreceptors constantly release glutamate and in response to a light increment they hyperpolarize, decreasing glutamate release. Two general classes of BCs form synapses with cone photoreceptor terminals and are defined by their response to a light increment. Hyperpolarizing BCs (HBCs) signal via ionotropic glutamate receptors of the AMPA/Kainate type (Kaneko and Saito, 1983; Saito and Kaneko, 1983; Slaughter and Miller, 1983) and DBCs signal via the metabotropic glutamate receptor 6 (mGluR6) (Masu et al., 1995). A single class of DBC, the rod DBC is postsynaptic to rod photoreceptors (Boycott et al., 1969). As a result defects at this synapse lead to complete congenital stationary night blindness (cCSNB). This defect is
detected by the absence of the b-wave in the electroretinogram (ERG) in both humans and animal models (Miyake et al., 1986; McCall and Gregg, 2008).

The rod light response is initiated when a decrease in glutamate occupancy of mGluR6 signals the opening of the TRPM1 channel (Audo et al., 2009; Li et al., 2009; Morgans et al., 2009; Shen et al., 2009; Koike et al., 2010b; Morgans et al., 2010; Peachey et al., 2012a). This cascade also includes proteins of known function: the heterotrimeric G proteins Gαo and Gβ3 (Dhingra et al., 2000; Dhingra et al., 2002; Koike et al., 2010b; Dhingra et al., 2012), and the regulators of G proteins Gβ5, RGS7 and RGS11 (Morgans et al., 2007; Jeffrey et al., 2010; Cao et al., 2012). Other known components with unresolved function include nyctalopin, GPR179 and LRIT3 (Gregg et al., 2003; Audo et al., 2012; Peachey et al., 2012b; Zeitz et al., 2013).

GPR179 is a seven transmembrane receptor that interacts with RGS7 and RGS11 and is critical for proper localization of these proteins to the DBC dendritic tips (Orlandi et al., 2012). GPR179 expression is required for normal mGluR6 signaling (Peachey et al., 2012b) and mutations in Gpr179 in mouse and human patients cause a no b-wave ERG phenotype (Audo et al., 2012; Peachey et al., 2012b). To gain further insight into the role of GPR179 in the rod DBC light response we studied Gpr179nob5 and RGS7+/−/RGS11+/− mice.

We show that expression of both mGluR6 and TRPM1 are independent of GPR179 expression and that GPR179 and TRPM1 proteins interact. Functional analysis using ERG and whole cell patch clamp recording of rod DBCs confirm that TRPM1 is present in both Gpr179nob5 and RGS7+/−/RGS11+/− rod DBCs, although the sensitivity of the cascade is significantly lower in each compared to WT. However, the two mutants differ in that direct gating of TRPM1 by capsaicin is similar in WT and RGS7+/−/RGS11+/− rod DBCs and severely compromised in Gpr179nob5 rod DBCs. Taken together, our results suggest that the GPR179/RGS7/RGS11 complex sets the sensitivity of the
mGluR6 signaling cascade and that the GPR179/TRPM1 interaction sets the sensitivity of gating of the TRPM1 channel to light stimuli.

Materials and Methods

Animals

All procedures were performed in accordance with the Society for Neuroscience policies on the use of animals in research and each local Institutional Animal Care Use Committees. Descriptions of all mice used have been published previously (Masu et al., 1995; Pardue et al., 1998; Pearring et al., 2011; Cao et al., 2012; Peachey et al., 2012b) and every line was either generated on a C57BL/6J background or backcrossed onto this background for at least 6 generations. All mice were housed in local AALAC approved facilities under a 12 h/12 h light/dark cycle. Trpm1<sup>−/−</sup> (Trpm1<sup>tm1Lex</sup>) mice can be obtained from the European Mouse Mutant Archive (emmanet.org) and were originally generated by Lexicon Genetics.

Antibodies

In experiments to examine the pattern of protein expression in the outer plexiform layer (OPL), the following primary antibodies (and their concentrations) were used: sheep anti-GPR179 (peptide KVQETPGEDLDRPVLQKR), 1:2,000 (Peachey et al., 2012b); mouse monoclonal anti-ctbp2/Ribeye (BD Bioscience), 1:1,000; guinea pig anti-mGluR6 1:1,000 (Koike et al., 2010b); sheep anti-TRPM1 1:1,000 (Cao et al., 2011); rabbit anti-GFP (MBL), 1:800 and Rhodamine Peanut Aglutinin (PNA) conjugate 566 (Vector Labs), 1:1,000. Secondary antibodies (Invitrogen, 1:1,000) appropriate to each primary antibody included: donkey anti-sheep Alexa-488, donkey anti-rabbit Alexa-680, donkey anti-rabbit Alexa-546, donkey anti-mouse Alexa-647 and donkey anti-guinea pig Cy3 (Millipore, 1:1,000). In lieu of an antibody specific to nyctalopin, we used Tg(Gabrr1-YFP/nyx)<sup>Rgg1</sup> transgenic mice that express a yellow fluorescent protein (YFP) tagged nyctalopin (Gregg et al., 2007). They are labeled WT in the figures.
**Immunohistochemistry**

Mice were anesthetized, their eyes were enucleated and the lens was removed. Eyecups were washed in phosphate buffered saline (PBS) then fixed for 30 minutes in 4% paraformaldehyde PBS solution (pH 7.4). Eyecups were washed 3 times in PBS then cryoprotected in increasing concentrations of sucrose in PBS (10%, 15% for 1 h each and 20% overnight). Eyecups were embedded in 2:1 OCT/20% sucrose PBS solution frozen in a liquid nitrogen cooled bath of isopentane. Eyecups were sectioned (18 µm) using a Leica 1850 cryostat, mounted on glass slides and stored at -70°C. Sections were warmed to 37°C and washed with PBS and PBS containing 0.05% Triton X-100 (PBX) for 5 min each, then blocked in PBX 5% normal donkey serum blocking solution for 1 h. Sections were incubated overnight at room temperature in the presence of the primary antibody diluted in blocking solution, then washed 3 times for 10 min each with PBX followed by incubation in secondary antibody in PBX for 1 h at room temperature. Sections were washed for 10 min in PBX twice and in PBS once and cover-slipped using Immu-Mount (Thermo Scientific). Slides were imaged using an Olympus FV1000 confocal microscope. Images were universally adjusted for brightness using Photoshop.

**Mass Spectrometry**

Retinas were isolated from WT mice and homogenized in lysis buffer (1% Nonidet P-40, 2 mM EDTA, 20 mM HEPES pH 7.4, supplemented with protease inhibitors cocktail) by rotating at 4°C for 45 min. Samples were centrifuged at 17,000 g for 20 min to remove the debris and supernatant was precleaned with Dynabeads (Invitrogen) for 1 h at 4°C. Samples were incubated with TRPM1 or GPR179 antibody overnight at 4°C. Lysates and antibody complexes were incubated with Dynabeads for 1.5 h at 4°C. Protein complexes were eluted with Nupage LDS sample buffer (Invitrogen) and electrophoresed on Nupage gel (Invitrogen) until the highest molecular weight
standard (260 kDa) had moved ~5 mm into the gel. Electrophoresed gel pieces were cut from the top of the gel and an in-gel tryptic digestion was performed as described previously (Rood et al., 2010).

The resulting peptide mixture was resolved by liquid chromatography (LC) using an EASY n-LC (Thermo Scientific) UHPLC system with buffer A = 2% v/v acetonitrile / 0.1% v/v formic acid and buffer B = 80% v/v acetonitrile / 0.1% v/v formic acid as mobile phases. The mass spectrometry data from LC elutes was collected using an Orbitrap Elite – ETD mass spectrometer (Thermo Scientific). A decision tree was used to determine whether CID or ETD activation was used. Proteome Discoverer v1.3.0.330 was used to analyze the data collected by the mass spectrometer. Scaffold v3.6.5 was used to calculate the false discovery rate using the Peptide and Protein Prophet algorithms.

**Cell culture, transfection and immunoblotting:**

HEK293T (Human Embryonic Kidney) cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. One day prior to transfection, cells were seeded on 60 mm culture dishes. *Gpr179* and *Trpm1* expression plasmids were transfected into HEK293T cells using JetPrime reagent (Polyplus-transfection) or Lipofactamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24-48 h of transfection, cells were harvested in NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40, pH 8.0, supplemented with protease inhibitors cocktail (Sigma-Aldrich) by rotating for 45 min at 4°C and centrifuged at 17,000 g for 15 min at 4°C. Protein content was quantified by Bradford reagent (BioRad). Protein lysates were analyzed on 4-12% Nupage gels (Invitrogen) or 6% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted using HRP-conjugated secondary antibodies and ECL West Pico detection system (Thermo Scientific).
Co-immunoprecipitation

Dissected mouse retinas were homogenized in lysis buffer (1% Nonidet P-40, 2 mM EDTA, 20 mM HEPES pH 7.4, supplemented with protease inhibitors cocktail) by rotating at 4°C for 45 min. Homogenates were cleared by centrifugation at 17,000 g for 20 min at 4°C. For co-immunoprecipitation, retinal lysates or transfected HEK cells lysates were precleared by incubating with 12 µl Dynabeads protein G (Invitrogen) at 4°C for 1 h. Precleared lysates were incubated with 2-5 µg of anti-GPR179 or anti-Flag antibodies overnight at 4°C on an orbital rocker. 45 µl of Dynabeads protein G were added and incubated for 1-2 h at 4°C. Dynabeads were collected and washed 4 times with Tris-buffered saline containing 0.3% Tween-20. Protein complexes were eluted with 40 µl of 4X LDS loading buffer by incubation at 70°C for 10 min, separated by SDS-PAGE and analyzed by immunoblotting.

Electroretinography

Mice were tested in two recording protocols in which either a strobe flash or variable duration stimuli were used to evoke ERG responses. For both, animals were dark-adapted overnight and anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). Eyedrops were used to dilate the pupil (1% tropicamide, 2.5% phenylephrine HCl) and to anesthetize the corneal surface (1% proparacaine HCl). ERGs were recorded using a stainless steel (strobe flash) or gold (variable duration) electrodes contacting the corneal surface wetted with 1% methylcellulose. Platinum needle electrodes in the cheek and tail serve as reference and ground, respectively. Strobe flash ERGs were recorded using an LKC UTAS E-3000 signal averaging system to stimuli that ranged in luminance from -3.6 to 1.4 log cd sec/m². ERGs were also recorded using a Diagnosys Espion system with a -1.2 log cd/m² stimulus that ranged in duration from 10 – 1000 msec.

Retinal Slice Preparation and Whole Cell Patch Clamp Recording
Mice were anesthetized with an intraperitoneal injection of Ringer’s solution containing ketamine/xylazine (127/12 mg/kg, respectively) and sacrificed by cervical dislocation. The eyes were enucleated and the retinas removed and placed in fresh Ames solution (Sigma-Aldrich). The retina was adhered to nitrocellulose paper (Millipore) and then sliced perpendicular to the retinal layers using a tissue slicer. The slices were then placed in a recording chamber. Recording electrodes were pulled from borosilicate glass (FHC, Inc., Bowdoin, ME) on a P-97 Flaming/Brown Micropipette Puller (Sutter Instruments Co., Novato, CA). Electrode resistance measured between 6-9 MΩ. Glass electrodes were filled with intracellular solution that contained Cs-gluconate solution (20 mM CsCl, 107 mM CsOH, 107 mM D-Gluconic Acid, 10 mM NaHEPES, 10 mM BAPTA, 4 mM ATP, and 1 mM GTP). The intracellular solution contained 1% sulforhodamine to visualize the cell and classify its morphology (Ghosh et al., 2004). Rod DBC somas were targeted for whole cell patch clamp recording. A 2-4 GΩ seal was created on the cell body and cells with an input resistance ~ 1 GΩ and access resistance < 25 MΩ were used for recording. The recording chamber was maintained at 34-35°C.

To block inhibitory inputs, Ames solution was supplemented with: 1µM strychnine, 100 µM picrotoxin and 50 µM 6-tetrahydropyridin-4-yl methylphosphinic acid (TPMPA). Four µM L-AP4 was added to the bath solution to saturate mGluR6 receptors. The mGluR6 receptor antagonist α-cyclopropyl-4-phosphonophenylglycine (CPPG) was dissolved in Ames medium to a working concentration of 0.6 mM or 3 mM. CPPG was applied by pressure application using a Picospritzer II (Parker Instrumentation, Cleveland, OH) onto the rod DBC dendritic tips in the outer plexiform layer (OPL). In separate experiments, capsaicin (10 µM), a TRPM1 agonist, was puffed onto the rod DBC dendrites to gate the opening of the TRPM1 channel. Reagents were purchased from Sigma-Aldrich, except for L-AP4, CPPG, and capsaicin, which were purchased from Tocris Bioscience.
Voltage Clamp Protocols

Rod DBC responses were recorded via a Multiclamp 700A amplifier with a Digidata 1440A digitizer (MDS Analytical Technologies, Union City, CA) and filtered at 2.4 kHz with a four-pole Bessel low pass filter, sampled at 10 kHz. Clampex 10.2 software (MDS Analytical Technologies, Sunnyvale, CA) was used to generate command outputs and acquire and analyze analog whole cell current. Rod DBCs were voltage clamped at +50mV (Nawy, 2004; Shen et al., 2009). CPPG was puffed at rod DBC dendrites for either 200 msec or 1 sec. For capsaicin experiments 200 msec and 1 sec puffs were applied. Three to five responses were recorded from each cell and then averaged. Variance and standing current were measured across the first 1.5 sec of the recording for each rod DBC. Offline analyses of data were performed using Clampfit 10.2. Prism 5.04 software (Graphpad Software, Inc., La Jolla, CA) was used to perform two-way repeated measures ANOVAs, two-way ANOVAs, one-way ANOVAs, or t-tests as suited for the necessary comparison. Statistical significance = \( P < 0.05 \).

RESULTS

The localization of mGluR6, TRPM1 and NYX to the DBC dendritic tips is independent of GPR179 expression

Since GPR179 is critical to localization of the RGS7 and RGS11 (Orlandi et al., 2012), we examined if it plays a similar role for other proteins in the DBC cascade. We compared expression and localization of mGluR6, the receptor that initiates signaling and TRPM1 the channel that is ultimately gated by the cascade in the outer retina of WT and \( Gpr179^{nob5} \) mice. We also examined the expression pattern of nyctalopin (EYFP-Nyx), a protein critical to expression/localization of TRPM1 (Pearring et al., 2011). Fig. 1 shows representative transverse retinal section reacted with antibodies to label these three proteins in WT (A) and \( Gpr179^{nob5} \) retina. The expression pattern is indistinguishable between WT and \( Gpr179^{nob5} \). The merged images in triple labeled
retinal sections show that TRPM1, mGluR6 and nyctalopin expression overlaps on the dendritic tips of the DBCs.
Figure 1. mGluR6, TRPM1 and NYX expression is independent of GPR179 expression. Representative confocal images of cross sections of the OPL and INL of WT (A) and Gpr179<sup>nob5</sup> (B) retinas reacted with antibodies to TRPM1 (green), mGluR6 (blue) and EYFP-Nyc (red). The merged images (bottom) show that in both WT and in Gpr179<sup>nob5</sup> retinas expression patterns are similar and that TRPM1, mGluR6 and nyctalopin expression co-localize at the dendritic tips of DBCs. Scale bars, 5 µm.
To determine if GPR179 expression depends on expression of mGluR6, Nyctalopin and TRPM1, we reacted retinal sections from WT and knockout or mutant mice: *Grm6<sup>−/−</sup>*(Grm6 encodes mGluR6), *Nyx<sup>rob</sup> and *Trpm1<sup>−/−</sup> with an antibody to GPR179 (Peachey et al., 2012b). We co-labeled these sections with the lectin, PNA, to mark the cone pedicles in the OPL. Fig. 2 shows that the pattern of GPR179 expression is indistinguishable in representative sections from all four mouse strains. As expected, GPR179 expression is absent in the negative control, *Gpr179<sup>rob</sup> retina* (Peachey et al., 2012b).
Figure 2. GPR179 expression at the dendritic tips of the DBCs is independent of mGluR6, TRPM1 and nyctalopin expression. Representative confocal images of cross sections of the OPL and INL of a series of WT and mutant mouse retinas reacted with an antibody to GPR179 (green) and the cone pedicle marker, PNA (red). (A) In WT retinas expression of GPR179 colocalizes with PNA (yellow puncta in merged image). (B-D) GPR179 expression also is localized at the dendritic tips of DBCs even in the absence of expression of: mGluR6 (B), nyctalopin (C) and TRPM1 (D). As shown here (E) and previously (Peachey et al., 2012b) GPR179 expression is absent from Gpr179nob5 mouse retina. Green puncta in the merged images represent GPR179 expression at the dendritic tips of rod DBCs. Scale bars, 5 µm.
Independence of localization of GPR179, mGluR6, TRPM1, and nyctalopin

Although not evident in Figs 1 and 2, we observed what might be subtle differences in the level of GPR179 expression across these mutants. We used western blotting from retinal lysates of each to examine and to quantify expression levels of GPR179 relative to WT (Fig. 3A,B). As expected, the negative control, Gpr179
nob5
mutant retina showed no expression. In Grm6
−/−
and Trpm1
−/−
mutant retina, expression levels of GPR179 were reduced, by ~50% and ~20%, respectively. In addition, we examined and quantified TRPM1 expression levels in Gpr179
nob5
and Grm6
−/−
retinas (Fig. 3C,D). Again, the negative control shows no expression in the TPRM1
−/−
. In Gpr179
nob5
retinas, TRPM1 expression is decreased by ~30%, but is similar to WT in Grm6
−/−
retina. Others have reported that TRPM1 is mislocalized in Grm6 mutant retina (Morgans et al., 2009; van Genderen et al., 2009; Cao et al., 2011; Pearring et al., 2011; Peachey et al., 2012a; Xu et al., 2012) and it has been shown that TRPM1 expression is decreased ~30% in the Grm6
nob3
mutant (Cao et al., 2011). The discrepancy between our results and previously published results could be due to animal age or variation in the Grm6 mutant (Maddox et al., 2008; McCall and Gregg, 2008). In summary, our data show that GPR179 is localized correctly in the OPL independent of mGluR6, TRPM1, and nyctalopin and that the localization of these proteins is independent of GPR179, although expression levels may change.

GPR179 and TRPM1 are part of the same protein complex

We have previously shown a physical interaction between TRPM1 and nyctalopin (Cao et al., 2011; Pearring et al., 2011) and between GPR179 and RGS7 and RGS11 (Orlandi et al., 2012). Using an affinity purification by immunoprecipitation (IP) approach, we examined GPR179 interactions with TRPM1. Using antibodies to GPR179, we immunoprecipitated protein complexes from WT mouse retinal lysates. The identity of co-immunoprecipitated proteins was characterized using mass spectroscopy. IP with
antibodies to GPR179 (a positive control) yielded 121 peptides identified as GPR179 and giving a coverage of 55%. Two peptides were identified as TRPM1. IP with antibodies to TRPM1 yielded 48 peptides matching TRPM1 (57% coverage). Twelve peptides were identified as GPR179 (9% coverage). As a negative control for the specificity of the assay, we used an identical IP approach with a non-specific IgG. None of the peptides in the resulting data set matched either GPR179 or TRPM1. Additional negative controls included IPs from GPR179<sup>mob5</sup> retinas with GPR179 antibodies and IPs from Trpm1<sup>−/−</sup> retinas with TRPM1 antibodies. In these latter two controls, neither TRPM1 nor GPR179 were identified in the resulting data sets. Combined these data indicate our IPs were specific and that GPR179 and TRPM1 are part of the same protein complex.

To test the validity of the mass spectrometry results, and to determine whether GPR179 and TRPM1 interact directly, we used IP from HEK293T cells co-transfected with Gpr179 and FLAG-Trpm1 expression vectors (Pearring et al., 2011) and from WT mouse retinas (Fig. 3 E,F). Western blot analyses of singly (Fig. 3E, lanes 1,2) and doubly (Fig. 3E, Lane 3) transfected HEK293T cells show that both proteins were expressed as expected. IP with the FLAG antibody (Fig. 3E, lanes 4-6) shows on the singly transfected cells (Fig. 3E, lanes 4,5) that it precipitates FLAG-TRPM1 only, as expected. IP from the double transfected cells shows that the FLAG antibody co-IPs GPR179 and FLAG-TRPM1 (Fig. 3E, lane 6), indicating that the two proteins interact. To control for the possibility that this interaction occurs during the lysis procedure, we transfected GPR179 and FLAG-TRPM1 expressing into different HEK293T cell cultures, lysed the cells and mixed the lysates. IP with FLAG antibodies and subsequent western blotting for GPR179 showed that GPR179 is absent from the immunoprecipitates (data not shown), supporting the conclusion that GPR179 and TRPM1 when expressed in HEK293T cells interact directly.
When we repeated the IP experiments using retinal lysates (Fig. 3F), both GPR179 and TRPM1 were present in WT as expected (Fig. 3F, lane 1), and GPR179 was absent in Gpr179\textsuperscript{nob5} retinal lysate (Fig. 3F, lane 2). IP from WT retinal lysates with an antibody to GPR179 and subsequent western blotting of the precipitates shows that TRPM1 is co-immunoprecipitated, along with GPR179 (Fig. 3F, lanes 3,5). As controls, we repeated the IP experiments using lysates from Gpr179\textsuperscript{nob5} (Fig. 3F, lane 4) and Trpm1\textsuperscript{−/−} (Fig. 3F, lanes 6) retinas. Both GRP179 and TRPM1 were absent in precipitates from the Gpr179\textsuperscript{nob5} retinas (Fig. 3F, lane 4) and only GPR179 was present in precipitates from Trpm1\textsuperscript{−/−} retinas (Fig. 3F, lane 6). Combined the mass spectrometry and IP studies support the conclusion that GPR179 and TRPM1 are part of the same synaptic complex and interact directly.
Figure 3. GPR179 interacts with TRPM1. Western blots of total retinal lysates probed with antibodies to (A) GPR179 and (C) TRPM1. Each blot was reprobed with antibodies to β-actin to determine total protein and for use as an internal standard. Band intensities were analyzed and quantified with NIH ImageJ software and normalized to the β-actin expression level in the same sample. The histograms (B, D) plot the mean expression from four experiments on independent samples. (B) GPR179 expression was lower in Grm6<sup>−/−</sup> and Trpm1<sup>−/−</sup> retinas compared to WT. (D) TRPM1 expression was similar to WT in Grm6<sup>−/−</sup> and in GRP179<sup>nox5</sup> retinas. Errors bars represent standard error *P<0.05, **P<0.001. (E) Western blot of HEK293T cells transfected with plasmids expressing GPR179 (lane 1), FLAG-TRPM1 (lane 2) or both (lane 3) and probed with GPR179 and FLAG antibodies. The presence of a specific expression construct is indicated by “+” above the lane on the blot. Lysates from HEK293T samples (lanes 1-3) were immunoprecipitated with antibodies to GPR179 and the precipitates analyzed by western blotting, using antibodies to GPR179 (lanes 4-6, top row) or TRPM1 (lanes 4-6, bottom row). These data show that TRPM1 is co-immunoprecipitated with GPR179 (lane 6). (F) Western blot of retinal lysates from WT (lane 1) and GPR179<sup>nox5</sup> (lane 2) retinas, probed for presence of GPR179 (top row) or TRPM1 (bottom row). Western blots of proteins co-immunoprecipitated with antibodies to GPR179 from WT (lanes 3 and 5), GPR179<sup>nox5</sup> (lane 4) and Trpm1<sup>−/−</sup> (lane 6) probed for GPR179 (top row) or TRPM1 (bottom row). Immunoprecipitation with GPR179 antibody from retinal lysates of Gpr179<sup>nox5</sup> and Trpm1<sup>−/−</sup> mice served as controls for nonspecific binding. These data were representative of at least three independent experiments using independent samples. Data show that GPR179 and TRPM1 co-immunoprecipitate (lanes 3 and 5).
**Gpr179<sup>nob5</sup> mice have ERG b-waves absent in other cCSNB mouse models**

Previously we compared the ERG responses of WT and Gpr179<sup>nob5</sup> and showed a significant reduction in the Gpr179<sup>nob5</sup> b-wave and interpreted this result as a no b-wave phenotype (Peachey et al., 2012b). When ERG responses from Gpr179<sup>nob5</sup> and Trpm1<sup>−/−</sup> mice were compared to WT (Fig. 4A) their positive polarity b-waves appear to be completely missing. However, upon closer inspection of these mutant responses at two low flash luminances (-3.6, -2.4 log cd sec/m<sup>2</sup>), where the a-wave and slow PIII are small in amplitude, we found a b-wave like response, albeit small (~15-20μV), that was consistently present in Gpr179<sup>nob5</sup> but not in Trpm1<sup>−/−</sup> mice (Fig. 4B). When quantified, the b-wave like response was significantly larger in Gpr179<sup>nob5</sup> than Trpm1<sup>−/−</sup> mice (t-test; P < 0.001; Fig. 4C).
Figure 4. Gpr179\textsuperscript{nob5} rod ERGs have a small b-wave. (A) Representative rod ERGs recorded from WT (black), Gpr179\textsuperscript{nob5} (blue) and Trpm1\textsuperscript{−/−} (red) mouse retinas to strobe flash stimuli presented to the dark-adapted retina. Note that the positive polarity b-wave of the WT ERG is missing in Gpr179\textsuperscript{nob5} and Trpm1\textsuperscript{−/−} mice. Values to the left of the waveforms indicate flash luminance in log cd sec/m\textsuperscript{2}. (B) ERG responses obtained from Gpr179\textsuperscript{nob5} and Trpm1\textsuperscript{−/−} mice to a -3.6 log cd sec/m\textsuperscript{2} flash. Colored traces indicate the responses from 5 different Gpr179\textsuperscript{nob5} mice (left) and 7 different Trpm1\textsuperscript{−/−} mice (right). The offset black trace in each panel is the average of all mice. (C) Average (± sem) b-wave amplitude (difference between pre-stimulus baseline and the largest positive deviation from the baseline recorded between 100 and 300 msec after flash presentation) of responses shown in (B). Note that in GPR179\textsuperscript{nob} mice there is a response that is absent in the Trpm1\textsuperscript{−/−} mice.
GPR179 sets the sensitivity of mGluR6 cascade modulation of TRPM1

Our observations that both mGluR6 and TRPM1 proteins are expressed in the OPL of Gpr179\textsuperscript{nob5} retina (Fig. 1), as well as the presence of a small b-wave in these mice (Fig. 3), suggest that mGluR6 and its cascade still gate the TRPM1 channel, although the sensitivity of the system is significantly reduced. To address this question, we tested several elements of this hypothesis using whole cell patch clamp recordings of rod DBCs in a retinal slice preparation. We used the methods described by Nawy and colleagues (Nawy, 2004; Shen et al., 2009) in which retinal slices were bathed in 4 \( \mu \text{M} \) L-AP4 to maximally activate the mGluR6 cascade and close TRPM1 channels. Puffs of CPPG, a mGluR6 antagonist, then were used to deactivate the cascade, mimicking the events evoked by light. We held rod DBCs at +50mV to minimize run-down and, as a consequence, outward currents are evoked by CPPG puffs.

We examined whether the TRPM1 channel could be gated by the mGluR6 cascade by exogenous application (puffs) of CPPG onto the dendritic terminals of synaptically isolated Gpr179\textsuperscript{nob5} rod DBCs. Fig. 5A shows representative responses from WT and Gpr179\textsuperscript{nob5} rod DBCs to 0.6mM CPPG puffs 200 msec in duration. In WT rod DBCs there is a robust outward current. The response, while present in Gpr179\textsuperscript{nob5} rod DBCs was significantly smaller than WT (Fig. 5B; two-way ANOVA: \( P < 0.001 \)). Increasing puff duration to 1 sec produced no discernible change in WT responses (two-way ANOVA: \( P > 0.05 \); data not shown), but significantly increased the response in Gpr179\textsuperscript{nob5} rod DBCs (Fig. 5B; two-way ANOVA: \( P < 0.001 \)). Despite this increased Gpr179\textsuperscript{nob5} response, the amplitude was still significantly smaller than in WT (two-way ANOVA: \( P < 0.001 \); Fig. 5A, B). We attempted to increase stimulation further by increasing the concentration of CPPG to 3mM. At both 200 msec and 1 sec puff duration, WT responses remained similar to those evoked by 0.6mM CPPG (Fig. 5C,D), indicating that the mGluR6 cascade was maximally activated by 0.6mM and 200 msec.
puffs. In Gpr179\textsuperscript{nob5} rod DBCs, increasing the CPPG concentration to 3mM yielded responses similar in magnitude, regardless of puff duration. They also were similar to the response evoked by 0.6mM/1 sec CPPG puffs. This suggests that the Gpr179\textsuperscript{nob5} rod DBC response can be saturated at the same concentration as WT but requires longer duration puffs (Fig. 5B,D). Regardless of the conditions, the Gpr179\textsuperscript{nob5} rod DBC responses never reached the amplitude recorded in WT (Fig. 5C,D; two-way ANOVA: 200 msec: $P < 0.001$; 1 sec: $P < 0.001$). Together these data suggest that while GPR179 is not required to gate TRPM1, it sets the sensitivity of the mGluR6 cascade and modulation of TPRM1 in rod DBCs.
Figure 5. CPPG evokes a small amplitude response in \textit{Gpr179}\textsuperscript{nob5} rod DBCs, which increases with stimulus intensity. (A) Representative voltage clamp responses of WT and \textit{Gpr179}\textsuperscript{nob5} rod DBCs evoked by puff application of the mGluR6 antagonist CPPG (0.6 mM; 200 msec or 1 sec). (B) Histogram compares the average peak response amplitudes of WT and \textit{Gpr179}\textsuperscript{nob5} rod DBCs. WT responses to 200 msec and 1 sec puffs did not differ and were combined. Regardless of duration \textit{Gpr179}\textsuperscript{nob5} response amplitudes were significantly smaller than WT. \textit{Gpr179}\textsuperscript{nob5} response amplitudes significantly increase when puff duration increased from 200 msec to 1 sec. (C) Representative voltage clamp responses of WT and \textit{Gpr179}\textsuperscript{nob5} rod DBCs evoked by puff application of 3 mM CPPG for either 200 msec or 1 sec. (D) Histogram compares the average peak response amplitudes of WT and \textit{Gpr179}\textsuperscript{nob5} rod DBCs. WT responses did not increase with increased puff duration (200 msec to 1 sec). Regardless of duration \textit{Gpr179}\textsuperscript{nob5} response amplitudes were significantly smaller than WT. Increased puff duration did not produce larger response amplitudes in \textit{Gpr179}\textsuperscript{nob5} rod DBCs suggesting that they are saturated under these conditions. (E) Representative voltage clamp responses of WT and \textit{Gpr179}\textsuperscript{nob5} rod DBCs evoked by a 1 sec puff of the TRPM1 channel agonist, capsaicin (10 µM). (F) Histogram compares the average peak response amplitudes of WT, \textit{Gpr179}\textsuperscript{nob5} and \textit{Trpm1}\textsuperscript{-/-} rod DBCs. \textit{Gpr179}\textsuperscript{nob5} response amplitudes are significantly larger than \textit{Trpm1}\textsuperscript{-/-} although significantly smaller than WT rod DBCs. The number of rod DBCs in each experimental group is shown within each bar of the histograms.
Because TRPM1 is expressed in Gpr179\textsuperscript{nob5} retina we characterized whether the channel could be gated directly by examining rod DBC responses in WT and Gpr179\textsuperscript{nob5} to the TRPM1 agonist capsaicin. As observed previously (Shen et al., 2009), capsaicin evoked robust responses in WT rod DBCs (Fig. 5E). In contrast, capsaicin evoked responses in Gpr179\textsuperscript{nob5} rod DBCs were significantly smaller than WT. As a control, we examined capsaicin evoked responses in Trpm1\textsuperscript{-/-} rod DBCs and found they were significantly smaller than present in Gpr179\textsuperscript{nob5} rod DBC (Fig. 5F; one-way ANOVA: vs WT: $P < 0.001$; vs Trpm1\textsuperscript{-/-}: $P < 0.05$). These data are consistent with our immunohistochemical results and suggest that the TRPM1 channel is present in Gpr179\textsuperscript{nob5} rod DBCs and can be modulated either by the mGluR6 cascade or directly by capsaicin. Because strong stimuli (both pharmacological and light in the ERG results) are required and the response never reaches the amplitude seen in WT cells we conclude that GPR179 modulates the sensitivity of the cascade. The data also address an issue noted previously, that the rod DBC response to capsaicin is primarily via modulation of TRPM1 channels with a minor contribution from an “off-target” non-TRPM1 channel (Morgans et al., 2009).

In the above recordings, we noted that spontaneous currents in WT appeared noisier than in Gpr179\textsuperscript{nob5} rod DBCs (Fig. 6A). This suggested that the open probability of the TRPM1 channel might differ between WT and Gpr179\textsuperscript{nob5} rod DBCs, as we had noted for Nyx\textsuperscript{nob} rod DBCs (Gregg et al., 2007). To evaluate this question, we measured both the standing outward current and the current variance of rod DBCs ($V_{\text{hold}} +50\text{mV}$). The outward holding currents as well as the variance were significantly larger in WT rod DBCs compared to Gpr179\textsuperscript{nob5} (Fig. 6B,C; one-way ANOVA: holding current: $P < 0.001$; variance: $P < 0.01$). As a control we analyzed the holding current and variance in Trpm1\textsuperscript{-/-} rod DBCs and found that they were similar to Gpr179\textsuperscript{nob5} cells (Fig. 6; one-way ANOVA: holding current: $P > 0.05$; variance: $P > 0.05$). These results support the idea
that while the TRPM1 channels are correctly expressed and localized, they are in a functionally closed state compared to those in WT rod DBCs. The results continue to support the hypothesis that GPR179 is required for the high sensitivity of the modulation of the TRPM1 channel by the mGluR6 cascade.
Figure 6. *Gpr179*/*nob5* rod DBCs have decreased standing currents and TRPM1 channel open probability. (A) Representative traces of spontaneous currents from two WT and two *Gpr179*/*nob5* rod DBCs. Rod DBCs were held at +50 mV and 1.5 sec sections of each recording were analyzed to yield the data in B and C. Histograms show average (B) standing current and (C) current variance for WT, *Gpr179*/*nob5* and *Trpm1*/*-/-* rod DBCs. *Gpr179*/*nob5* and *Trpm1*/*-/-* have similar standing currents (C) and current variance (D) and both are significantly lower than WT. Combined these data indicate that the TRPM1 channel in *Gpr179*/*nob5* rod DBCs has a low open probability that is similar to TRPM1/*-/-* rod DBCs where the channel is absent. The number of rod DBCs in each experimental group is shown within each bar of the histograms.
Long duration light increments evoke an ERG b-wave in Gpr179\textsuperscript{nob5}

Since rod DBCs can respond to strong pharmacological manipulation, we hypothesized that long duration full-field light flash stimuli also might evoke an ERG b-wave. To test this we recorded ERGs from WT, Gpr179\textsuperscript{nob5} and Trpm1\textsuperscript{-/-} mice using an LED-based stimulation system to present a -1.2 log cd/m\textsuperscript{2} stimulus for durations that ranged from 10 to 1000 msec. WT responses were dominated by the b-wave (Fig. 7A), which grew in amplitude with increasing stimulus duration (Fig. 7B). The same stimuli evoked a slow b-wave in Gpr179\textsuperscript{nob5} mice, whose amplitude also increased with stimulus duration. In contrast, none of the stimulus configurations evoked a b-wave in Trpm1\textsuperscript{-/-} mice (Fig. 7) or in two other mouse models of cCSNB, Nyx\textsuperscript{nob} and Grm6\textsuperscript{nob3} (data not shown). Compared to WT, the amplitude of the response from the Gpr179\textsuperscript{nob5} retina was significantly smaller (two-way ANOVA; P < 0.001). Whereas the WT response saturated to a ~50 msec stimulus duration, the Gpr179\textsuperscript{nob5} response continued to increase through stimulus durations of ~500 msec (Fig. 7). These ERG results are consistent with the mGluR6 mediated gating of TRPM1 we observe in the patch clamp recordings of Gpr179\textsuperscript{nob5} rod DBCs.
Figure 7. Increasing flash duration increases the ERG b-wave amplitude in $\text{Gpr179}^{\text{nob5}}$ mice. (A) Representative ERG responses recorded from WT (black), $\text{Gpr179}^{\text{nob5}}$ (blue) and $\text{Trpm1}^{-/-}$ (red) mice to $-1.2$ log cd/m$^2$ stimuli of increasing duration (top to bottom; and, indicated by the stimulus trace below each set of waveforms). Note that as stimulus duration increases, a slow positive wave becomes apparent in $\text{Gpr179}^{\text{nob5}}$ mice that is absent in $\text{Trpm1}^{-/-}$ mice. Waveforms indicate the average of: WT (n = 3), $\text{Gpr179}^{\text{nob5}}$ (n = 12) and $\text{Trpm1}^{-/-}$ (n = 7) mice. (B) Average (± sem) b-wave amplitude evoked by flash stimuli of different durations for the same mice. Note that the amplitude of the $\text{Gpr179}^{\text{nob5}}$ response increases across a range of flash durations (20-500 msec) whereas WT response amplitudes are stable (≥20 msec), and $\text{Trpm1}^{-/-}$ mice lack a response at any duration.
GPR179 and RGS7/11 provide unique functional roles in the DBC cascade

Because the DBC dendritic tips of Gpr179\textsuperscript{nob5} mice lack expression of RGS7 and RGS11 (Orlandi et al 2012) and RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} DBCs also show very small light evoked responses (Cao et al 2012), it is possible that all of our observations result from the absence of the RGS7/11 complex. Because GPR179 is expressed and normally localized in RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} DBCs (Fig. 8A), we repeated our characterizations (Fig. 5 and 6) in RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} rod DBCs and determined similarities and differences to differentiate the role of GPR179 from RGS7 and RGS11. The response amplitude evoked by 0.6 mM 1 sec CPPG puffs in RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} and Gpr179\textsuperscript{nob5} rod DBCs were similar (Fig. 8B; \(P = 0.45\)). In addition, both the standing outward current and current variance were similar in RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} and Gpr179\textsuperscript{nob5} rod DBCs (Fig. 8C-E, one way ANOVA: \(P > .05\)), and these parameters in the RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} cells were significantly lower than in WT cells (one way ANOVA: \(P < 0.0001; P < 0.01\)). We conclude that RGS7 and RGS11 are responsible for modulation of the sensitivity through the mGluR6 cascade.

When we tested the ability of capsaicin to directly open the TRPM1 channel in RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} we found a surprising result. Unlike the significantly smaller capsaicin evoked response found in Gpr179\textsuperscript{nob5} rod DBCs (Fig. 5E), capsaicin evoked responses in RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} rod DBCs were similar in amplitude to WT (Fig. 8F,G; one-way ANOVA: 200msec: Gpr179\textsuperscript{nob5} vs. RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-}: \(P < 0.001\); WT vs. RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-}: \(P > .05\)). These data suggest that GPR179\textsuperscript{-/-} and RGS7\textsuperscript{-/-}/RGS11\textsuperscript{-/-} double knockouts phenocopy each other for several characteristics, including response to CPPG, standing current and current variance. However, the presence of GPR179 even in the absence of RGS7 and RGS11 results in setting the “state” of TRPM1 such that the capsaicin response is similar to WT, presumably because of its direct interaction with TRPM1.
Figure 8. Capsaicin modulation of the TRPM1 channel differentiates the roles of Gpr179\textsuperscript{nob} and \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} in the mGluR6 signaling cascade. (A) Representative confocal images of retina sections from WT and \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} double knockouts labeled with antibodies to GPR179 and PNA (a cone terminal marker) show that GPR179 is localized normally in \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} retina. (B) Representative voltage clamp responses of \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} and Gpr179\textsuperscript{nob5} rod DBCs evoked by a 1 sec, 0.6 mM CPPG puff. The histogram compares the response amplitudes and shows that \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} and Gpr179\textsuperscript{nob5} are similar. (C) Representative traces from voltage clamp recordings of WT, \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} and Gpr179\textsuperscript{nob5} rod DBCs held at +50 mV. Analyses are similar to those described in Figure 6. (D) Both standing current and (E) current variance are similar in \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} and Gpr179\textsuperscript{nob5} rod DBCs and significantly smaller than in WT. (F) Representative voltage clamp responses WT, \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} and Gpr179\textsuperscript{nob5} rod DBCs to capsaicin puffs (200 msec; (10 µM). (G) Histograms compare peak response amplitudes for rod DBCs and show that WT and \textit{RGST\textsuperscript{7}/RGS11\textsuperscript{18}} are similar, whereas Gpr179\textsuperscript{nob5} rod DBC responses are significantly smaller. The number of rod DBCs in each experimental group is shown within each bar of the histograms.
Discussion

Visual function in starlight depends on the modulation of mGluR6, which gates the TRPM1 channel in rod DBCs. While the last four years, have seen more growth in this area than the previous 40, many details of the cascade mechanism remain poorly understood. Our results show that a new seven transmembrane protein, GPR179 along with RGS7/11 are required for maximal sensitivity of the cascade and that GPR179 is required for modulation of TRPM1 gating. Our immunohistological and protein biochemistry results support our functional assessments. GPR179 colocalizes with mGluR6 (Peachey et al., 2012b), TRPM1 and nyctalopin (Fig. 1) placing it in a prime location to be part of the gating mechanism controlling TRPM1 activity, and GPR179 and TRPM1 physically interact (Fig. 3).

At low light levels, the concentration of glutamate in the synaptic cleft is high and the mGluR6 receptor is maximally activated, which maximally activates a G-protein cascade that results in the exchange of Gα0-GDP to the active Gα0-GTP bound form, which releases the Gβγ dimer. The exact mechanism of TRPM1 gating, and whether this is accomplished by Gα0-GTP or Gβγ is an active research area (Koike et al., 2010a; Koike et al., 2010b; Shen et al., 2012). Application of Gβγ or activated Gα0 directly via the patch pipette to mouse rod DBCs in a retinal slice suggest that Gβγ closes the channel while addition of activated Gα0 has minimal impact (Shen et al., 2012). These authors argue that Gβγ either directly or indirectly closes TRPM1 channels. This model predicts the light response deactivates Gβγ, presumably by reformation of the trimeric G protein complex. This would suggest that a large pool of Gα0-GDP must be available to rapidly reform the trimeric G-protein complex once Gβγ ceases the interactions needed to inhibit TRPM1. This could explain why RGS7 and RGS11 are required and present in excess, indicated by the fact that a mouse without RGS11 and a hypomorphic allele of RGS7 had near normal ERG b-waves (Mojumder et al., 2009; Chen et al., 2010). A
significant change in the ERG b-wave was only demonstrated when both genes were completely eliminated (Cao et al., 2012; Shim et al., 2012). The competing model of TRPM1 gating is that activated Gα0 closes the channel, and that the RGS proteins are required to rapidly inactivate Gα0-GTP.

The Gβγ model predicts that the standing inward current seen in DBCs reflects a balance between the constant activation of Gβγ due to tonic activation of mGluR6 and its inactivation by rebinding to Gα0-GDP. In the Gpr179 Nob5 mutants, which lack RGS7 and RGS11 at the DBC tips (Orlandi et al., 2012), and the RGS7+/−/RGS11+/− mutants, the model predicts an increase in active Gβγ because there is reduced ability to hydrolyze GTP to GDP bound to Gα0. The consequence of this would be a decrease in TRPM1 open probability and in the DBC standing current. Under our experimental conditions of maximal activation of mGluR6, mimicking “dark” adapted conditions, we see both of these features, a dramatically decreased standing current and decreased open probability of TRPM1 in rod DBCs from Gpr179 Nob5 and RGS7+/−/RGS11+/− rod DBCs (Fig. 6 and Fig. 8). This effect is not the result of the absence of TRPM1 from the rod DBC signal transduction complex because; 1) immunohistochemistry shows TRPM1 is localized normally in Gpr179 Nob5 and in RGS7+/−/RGS11+/− retinas (Fig. 1 and Cao et al. (2012)); 2) there are responses evoked in rod DBCs by: (a) long duration flashes in the ERG of Gpr179 Nob5 mice and (b) high concentration/long application of CPPG in Gpr179 Nob5 rod DBCs (Fig. 5 and Fig. 8 B); and 3) activation of TRPM1 by capsaicin in Gpr179 Nob5 rod DBCs. The rod DBCs of Gpr179 Nob5 and RGS7+/−/RGS11+/− mice share many characteristics and one pivotal difference; the RGS7+/−/RGS11+/− DBCs have a normal response to capsaicin. There are two possibilities that could explain this result. First, there could be more TRPM1 channels present on the DBC dendrites. Second, the presence of GPR179, which interacts with TRPM1, could by a currently unknown mechanism set the “state” of the TRPM1 channel such that capsaicin is able to
maximally activate the channel and that this is different than the requirements of gating by the mGluR6 cascade. We favor the latter hypothesis for several reasons. An apparently simple test of the first mechanism would be to quantify, using western blots, the level of TRPM1 in the \textit{GPR179}^{-\text{f}} and the \textit{RGS7}^{-\text{f}}/\textit{RGS11}^{-\text{f}} retinas. However, this does not assess the amount of channel at the tips of the DBCs because TRPM1 is present in intracellular compartments throughout the DBCs (Fig. 1) and (Morgans et al., 2009; van Genderen et al., 2009; Cao et al., 2011; Pearring et al., 2011; Peachey et al., 2012a; Xu et al., 2012). The fact that the maximal CPPG response in \textit{Gpr179}^{nob5} and the \textit{RGS7}^{-\text{f}}/\textit{RGS11}^{-\text{f}} rod DBCs is similar suggests similar levels of TRPM1 are linked to the mGluR6 cascade in these mutants. Therefore in the \textit{RGS7}^{-\text{f}}/\textit{RGS11}^{-\text{f}}, which express GPR179, we propose that the state of the TRPM1 channel is fundamentally different than in the \textit{GPR179}^{nob5} DBCs and this allows capsaicin to activate it to normal levels. What this means at the molecular level is currently unclear but likely critical to understanding how TRPM1 is gated in DBCs.

Experiments described here and elsewhere show that the known components of the mGluR6/TRPM1 cascade are closely associated and many physically interact (Cao et al., 2011; Pearring et al., 2011; Orlandi et al., 2012). These interactions are likely to be critical to system performance and understanding the details of these interactions and how they likely change during light stimulation will be critical to a full understanding of the DBC signaling system. The importance of the physical arrangement of the cascade components is highlighted by the fact that RGS7 and RGS11 are still expressed in \textit{Gpr179}^{nob5} mice, but are not localized correctly to the tips of the DBCs (Orlandi et al., 2012). There are likely to be many other proteins critical to complex function. One such molecule is LRIT3, a protein of unknown function, but when absent results in cCSNB in humans (Zeitz et al., 2013) with a phenotype consistent with disrupted DBC signal transduction.
In conclusion, we show that GPR179 interacts with TRPM1 and one of its functions is to localize RGS7 and RGS11 near the signaling complex (Orlandi et al., 2012). A second critical function is to set the “state” of TRPM1 such that it responds optimally to deactivation of the mGluR6 cascade. Understanding this “state” at the molecular level may provide important clues as to how TRPM1 is gated by the mGluR6 cascade.

References


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Personal Data

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Dept. Biochemistry and Molecular Biology
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Date of Birth: March 4, 1986
Citizenship: U.S.A.

Education

2004-2008 Eastern Kentucky University, Richmond, KY.
Majors: Biology and Chemistry

2008-2013 University of Louisville, Louisville, KY. Department of Biochemistry and Molecular Biology Ph.D.

2014 Expected start date, January 6, 2014. Duke University, Durham, NC.
Department of Neuroscience Postdoctoral Scholar

Research Experience

2005-2007 Principal Investigator: Eric E. Dueno
Synthesized supramolecular molecules and obtained crystal structures for each. Research goal was to identify novel molecules with potential applications in medicinal host-guest chemistry.

2009-2013 Principal Investigator: Ronald Gregg

Identified and cloned the gene responsible for bipolar cell dysfunction (Gpr179) in a novel congenital stationary night blindness mouse model. Further characterized the protein to better understand the glutamate signaling cascade in depolarizing bipolar cells.

**Awards**

2008 Eastern Kentucky University Honors Program Scholar

2012 University of Louisville Neuroscience Day Graduate Student Poster First prize.

2012 ARVO Foundation/Retina Research Foundation/Joseph M. and Eula C. Lawrence Travel Grant

2013 Most Outstanding Graduate Student, University of Louisville School of Medicine Fall Commencement

2013 Graduate Dean’s Citation, University of Louisville

**Societies**

ARVO (Association for Vision and Research in Ophthalmology)

**Invited Seminars**

2013 23rd Annual Louisville Neuroscience Day. “GPR179, an orphan G protein-coupled receptor, is critical to rod mediated vision”

**Publications**

**Papers (peer reviewed)**

formamide hexasolvate, C$_{52}$H$_{40}$O$_{16}$·6C$_{3}$H$_{7}$NO. \textit{Acta Cryst.} E63, o3533-o3534. doi:10.1107/S160053680702107


Manuscripts


Book Chapters


Abstracts


4. Thomas A. Ray, Jillian N. Pearring, Pasano Bojang, Jr., Susana Contreras-Alcantara,

5. Thomas Ray, Dr. Eric E. Dueno. Synthesis of Functionalized Quinoxaline-Cavitands. Kentucky Academy of Science 2006 Annual Meeting, Morehead State University, Morehead, KY.

**Classroom Teaching**

2009-2010 Teaching assistant for Methods in Biochemistry and Molecular Biology

**Service Activities**

2010-2011 Department of Biochemistry and Molecular Biology new faculty search committee.