Title

The Effects of Neurosteroids, such as Pregnenolone Sulfate and its receptor, TrpM3 in the Retina.

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The Effects of Neurosteroids, such as Pregnenolone Sulfate, and its receptor, TrpM3 in the Retina.

By

Corey Webster

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Committee in charge:

Professor Marla Feller, Chair
Professor Diana Bautista
Professor Daniella Kaufer
Professor Stephan Lammel

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Abstract

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Professor Marla Feller, Chair

Pregnenolone sulfate (PregS) is the precursor to all steroid hormones and is produced in neurons in an activity dependent manner. Studies have shown that PregS production is upregulated during certain critical periods of development, such as in the first year of life in humans, during adolescence, and during pregnancy. Conversely, PregS is decreased during aging, as well as in several neurodevelopmental and neurodegenerative conditions. There are several known targets of PregS, such as a positive allosteric modulator NMDA receptors, sigma1 receptor, and as a negative allosteric modulator of GABA-A receptors. Recently a transient receptor potential channel, TrpM3 has been shown to be activated by PregS. TrpM3 is a heat sensitive (between 33-40ºC), non-selective cation channel that is outwardly rectifying. PregS has been shown to increase the frequency of post-synaptic currents in the hippocampus and developing cerebellum, induce calcium transients in a subset of retinal ganglion cells, and enhance memory formation in rodents. Furthermore, PregS mediated TrpM3 activation induces calcium dependent transcription of early immediate genes, suggesting that activation of this channel may produce lasting effects on cells and systems in which it is activated. Because PregS is abundant during critical periods of development, we hypothesized that it may play a significant role during development. Furthermore, the role of PregS or its receptor TrpM3, has not previously been well characterized in the retina. To address this question, in this dissertation, we examine the role of the neurosteroid PregS and its receptor, TrpM3, on retinal waves, which are characteristic of specific stages of synaptic development and connectivity. Briefly, we show that PregS induces a TrpM3 dependent prolonged calcium transient, which is absent in the TrpM3$^{-/-}$ animals and increases the correlation of cell participation in retinal waves. We also show that TrpM3 increases the frequency of post-synaptic currents, indicating a mechanism of action presynaptic to retinal ganglion cells, but that TrpM3 is expressed primarily in RGCs and Müller glia. Taken together, our results indicate that both PregS and TrpM3 are important in modulating spontaneous synaptic activity during development.

For Professor Marla Feller

Science begins in darkness.
With a question and then a crucible,
A will to resolve the impossible,
To push forward into an abyss of limitless murk,
Trying to find a handle, or create one.
To shine a light in the dark.
To focus and steer a beam to illuminate all that had never before been seen.
As for me, I am eternally grateful,
To have found a mentor bold enough,
To have built that laser.
So that I could see in the dark.

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Chapter I: Introduction and Motivation of Thesis

The retina is a model system for studying development.

The retina is an ideal model system to better understand the role of spontaneous activity during development. The temporal sequence and cellular mapping of synaptic connectivity during development, from the light sensitive photoreceptor layer to the retinal ganglion 'output' layer of the retina, is well characterized. Prior to eye opening, the retina undergoes tremendous synaptic sculpting and refining (Katz and Shatz, 1996). This period coincides with wavelike patterns of correlated activity in the retina, which also occur in several other sensory systems, such as the cochlea, spinal cord, cerebellum, hippocampus, and neocortex, and in several species, which point to spontaneous correlated activity as an essential component of neural circuit formation.

Retinal waves and synaptic connectivity occurs during each wave period.

In the weeks prior to eye opening, synaptic connectivity in the retina coincides with distinct neurotransmitter mediated retinal waves. Early in development, between P0 and P10 in mice, retinal waves are mediated by acetylcholine via the starburst amacrine cells that project onto retinal ganglion cells and form synapses during this period (Feller 1996, Wong 1999). After P10, bipolar cells are among the last cells in the retina to differentiate and begin to form glutamatergic synapses with retinal ganglion cells, inducing glutamate mediated stage III retinal waves. Several factors determine various properties of retinal waves, such as the wave velocity, wave width, cellular correlation, cellular synchrony with each wave. GABA, adenosine via the PKA-cAMP pathway, and acetylcholine all contribute to wave dynamics. cAMP levels have been shown to modulate the width, speed, frequency of retinal waves, and degree to which cells are correlated with each wave (Stellwagen et al. 1999). Interestingly, when one eye is injected with cAMP agonist to increase its retinal wave activity, its territory in the lateral geniculate nucleus is larger compared to the uninjected retinal projections. However, bilateral intraocular injection of cAMP agonists did not alter eye specific segregation (Stellwagen et al. 2002). This finding suggests that relative activity of each retina is instructive of retinogeniculate projections, rather than absolute activity levels. Further evidence of retinal waves being instructive of the refinement of synaptic connections between the retina and lateral geniculate stem from studies of mice lacking B2 nicotinic acetylcholine receptors (β2-nAChR-KO). These mice lack stage II cholinergic retinal waves but do possess waves similar to stage I gap junction mediated high velocity waves, and they also show altered eye specific segregation (Reviewed by M. Feller, 2009). Thus, not only the presence of retinal waves, but also their character, drives synaptic refinement of retinogeniculate projections.

Neurosteroids and their receptors

Neurosteroids are a relatively new class of neuroactive compounds, first identified about two decades ago. They are both produced and target cells within the CNS. Neurosteroids may act as modulatory ligands for a number of different receptors, some identified, some not yet known. Neurosteroids are synthesized in neurons, glial cells and oligodendrocytes of the CNS from cholesterol via the side chain cleavage enzyme cytochrome p450, which converts cholesterol into pregnenolone (Reviewed by Robel and Baulieu et al. 1994). Pregnenolone is a neurosteroid that does cross the blood-brain barrier and is a precursor to numerous other steroid and neurosteroid compounds that are derived by enzymatic reactions with pregnenolone, such as progestogens, androgens, glucocorticoids, and mineralocorticoids. While pregnenolone has been shown to exert effects on agonizing CB1 receptors, acting as an allosteric endocannabinoid, addition of a sulfate moiety on pregnenolone by the sulfotransferase, SULT2B1, which is found in various regions of the brain and retina, converts the precursor into pregnenolone sulfate, which has myriad effects on neuronal and glial receptors, and is the focus of this dissertation. Pregnenolone sulfate has been shown to act on numerous targets in the brain, such as possessing positive allosteric modulation activity on NMDA receptors, negative allosteric modulation of AMPA, kainate and glycine receptors, as an agonist of sigma receptors. Recently, Wagner et al (2008) discovered that Pregnenolone sulfate directly activates TrpM3 in pancreatic cells, which then are induced to produce insulin. Since much of the work on Pregnenolone sulfate in various regions of the brain was done prior to this finding that it targets TrpM3, it would be of great interest to revisit this work in the context of TrpM3 modulation.

Neurosteroids in development

Neurosteroids produced or administered during development have been shown to produce an array of behavioral and physiological changes lasting throughout adulthood. For example, PregS administered to Sprague-Dawley rat pups from postnatal day 1 to 14 resulted in reduced anxiety in adult female rats but not male rats and reduced locomotor activity in adult male rats (Jorge et al. 2005). Further evidence to support the importance of PregS, as well as the hormones synthesized from it, is that the P450scc homozygous knockout mice, which lack the enzyme necessary for converting cholesterol to Pregnenolone all die within two days of birth due to lack of glucocorticoids and mineralcorticoids (Hu et al. 2002). Humans heterozygous for a loss-of-function mutation in P450scc, which results in 60% reduction in pregnenolone producing enzymatic activity, presented with adrenal insufficiency and gonad dysfunction (Kolli et al. 2019). Other hormones synthesized from Pregnenolone, such as progesterone have been shown to facilitate dendritic growth, dendritic spine formation, and synaptic density in Purkinje neurons in cerebellar slices from newborn rats or in neonatal rats, and there is a burst of neurosteroidogenesis in the cerebellum that coincides with Purkinje neuron maturation (Sakamoto et al. 2001, Sakamoto et al. 2002). Another neurosteroid, allopregnanolone, synthesized from progesterone and reaches its highest concentration in the nervous system during pregnancy, has been shown to regulate $GABA_A$ subunit expression, and at its highest concentrations, suppresses physiological activity of specific neural networks. The author hypothesizes that the increased concentrations of neurosteroids, such as allopregnanolone, may be associated with maternal cognitive, psychiatric and physiological changes during pregnancy (Herbison, 2001). When administered intraperitonially to neonatal rats, allopregnanolone

increased the percent of parvalbumin expressing interneurons while reducing the overall number of neurons in the medial prefrontal cortex in adults (Gizerian et al. 2004).

If neurosteroids, such as PregS are important in regulating developmental phenomena, such as neural circuit refinement, then we would expect these compounds to be present at high concentrations early in development, and perhaps again during periods of rapid refinement, such as adolescence. Indeed, in a study of 659 human subjects, plasma concentrations of PregS at birth are highest (86 +/- 38ug/dl), and then steadily decreased during the first month of life (53.8+/- 31.3 ug/dl at age 30 days), by the end of the 1st year was a fraction of its newborn concentration (3.7 +/- 2.8 ug/dl) and then stably reduced (1.7 +/- 1.6 ug/dl) until adulthood when it increases slightly (5.3+/- 2.6) throughout mature adulthood (De Peretti, 1983). Furthermore, pregnenolone has been shown to increase in concentration starting at week 28 of gestation and peaking at week 36 both in umbilical artery plasma as well as amniotic fluid (Hill et al, 2010).

Neurosteroids, such as pregnenolone sulfate, are synthesized by neurons and glia within many regions of the CNS, particularly, the retina, hippocampus and cerebellum. Pregnenolone synthesis was first identified in the isolated retina by gas chromatography. P450scc, required to synthesize pregnenolone from cholesterol, was identified in the retinal ganglion cell layer, and blocking this enzyme by aminoglutathimide reduced the concentration of pregnenolone. Conversely, compounds that increase cellular cAMP increased the production, and thus, concentration of pregnenolone in isolated retinas (Guarneri et al., 1994). During postnatal development in the rat, pregnenolone has been shown to be synthesized by Purkinje cells of the cerebellum. Cytochrome P450scc enzyme was broadly distributed in the cerebellum early in development (P0) prior to differentiation of the first Purkinje cells, but then localized primarily to Purkinje soma by the first week of development. The authors suggested that pregnenolone synthesis coincides strongly with development of Purkinje cells (Ukena et al. 1998). Thus, neurosteroids are synthesized within regions of rapid neuronal differentiation and development and are present at relatively high concentrations in the nervous system during various stages of development.

Neurosteroids play a role in memory formation, hippocampal LTP

In addition to its roles in development, Pregnenolone Sulfate has been shown to increase long-term potentiation in hippocampal slice recording experiments. Sliwinski et al (2004) showed that physiologically relevant concentrations (300nM) of Pregnenolone Sulfate can enhance LTP in CA1 pyramidal neurons. They show that this effect is induce by ‘accentuating’ the activity of NMDA receptors. Whereas, Chen et al (2007) found that PregS induced phosphorylation of the NMDA receptor subunit, NR2B, extracellular signal regulated kinase 2 (ERK), and cyclic AMP response element binding protein (CREB), suggesting that the PregS effects on LTP may be lasting, in part due to phosphorylation events. Earlier reports had indicated that *in vivo* injection of PregS to the amygdala or hippocampus resulted in post-training memory enhancement to a foot shock stimulus (Flood et al. 1995). It has long been hypothesized that PregS, which increases in concentration in the hippocampus during stress, and is the precursor to other stress related hormones, such as corticosteroids, and allopregnenolone, is part of the cellular and

physiological mechanism by which memory for stressful or traumatic events is enhanced (reviewed by Girdler et al. 2007).

Role of PregS in Disease, particularly developmental disorders/diseases (Autism, pediatric siezures, post-partem depression).

There is some evidence to support a role of neurosteroids in diseases and disorders related to both aging and development. During aging, there is a strong decrease in neurosteroid levels, particularly PregS and DHEAS, which act very similarly in humans, and this decline in neurosteroid levels coincides with the period during which age related neurodegenerative diseases initiate (Valee et al. 2001). Furthermore, in addition to the age-related decline in neurosteroid levels, many neurodegenerative conditions themselves are associated with even further reduction in PregS and DHEAS, such as Alzheimer Disease. For example, DHEAS levels were 50% lower in AD patients compared to age matched (mean age 73) healthy controls (Yenase et al. 1996). And, more recently, a clinical trial has been conducted to establish whether PregS plasma levels may be a potential biomarker for early stages of Alzheimer's Disease (Clinical Trial Number: NCT00912886). The presence of the AD risk factor apolipoprotein gene variant, ApoE4 has been shown to severely impact lipid and cholesterol metabolism and trafficking in cells both in human and animal models (Mann et al. 2004). This aberrant processing of cholesterol by the AD risk factor gene ApoE4, or the increase in oxidized lipids secondary to disease process, likely reduces the available cholesterol in the correct locations within the cell (mitochondria) for conversion to PregS by p450scc as well as other neurosteroids. As this dissertation will show, PregS has strong impact on synaptic activity, as well as the likelihood for Hebbian plasticity events where higher frequency presynaptic activity is coupled to prolonged postsynaptic calcium influx.

Neurodevelopmental disorders result from aberrant growth and development of the CNS that effects brain function, emotional processing, learning, self-control and memory. Autism spectrum disorder (ASD) encompasses a range of neurodevelopmental disorders that result in impaired social communication and interaction, repetitive behavioral patterns, restricted interests or hobbies and is typically identified by age one to two years. Prepubertal children with ASD have been shown to have increased saliva concentrations of several steroid hormones, including PregS, particularly in older male children (Majewska et al. 2014). Furthermore, ASD displays high comorbidity with muscular dystrophy. A genome wide screen for ASD found a correlation between ASD and duplications of exons 31-44 of a muscular dystrophy gene, *DMD*. When the DMD mutation was combined with a very rare exon 1-9 deletion of TrpM3, this was found to be correlated to ASD (Pragnamenta et al. 2011). [can expand here about lipid processing in DMD mutants].

In addition to the role of neurosteroids in neurodevelopmental conditions such as ASD, there is also evidence that neurosteroid concentrations, particularly that of PregS, are altered in other developmental diseases, such as certain childhood epilepsy conditions and schizophrenia. A mutation in the protocadherin gene, *PCDH19*, which encodes a calcium dependent cell adhesion protein, results in severe pediatric seizures as well as delayed cognitive development. Interestingly, steroidogenesis, both at baseline and following adrenocorticotropin challenge, is highly reduced in female children with *PCDH19* epilepsy; in particular, PregS shows 100 fold

lower baseline serum levels compared to age matched controls (Trivisano et al. 2017), despite that PregS has been shown to have epileptogenic activity. Clinical trials are currently underway to determine whether an allopregnanolone analogue, ganaxolone suppresses pediatric seizures in female children with *PCDH19* epilepsy (Clinical Trial #: NCT02358538). [PCDH19 and later onset of SCZ]. It has been reported that levels of certain neurosteroids, such as pregnenolone and allopregnanolone, in regions of the brain such as the posterior cingulate and medial prefrontal cortex exceed levels found in plasma serum by 10 fold. Levels of pregnenolone in the posterior cingulate gyrus of schizophrenia patients was more than two fold higher than the median of age matched control patients (Marx et al. 2006). It is unknown whether the aberrant neurosteroid levels are correlative or causative of the disease, or perhaps a side effect of the drug clozapine, which a number of subjects were on in this study. However, a phase 1 clinical trial was conducted to assess the efficacy of pregnenolone treatment on the cognitive and negative effects of schizophrenia. Results demonstrated that pregnenolone treatment increased serum pregnenolone, pregnenolone sulfate and allopregnanolone levels, and greatly improved the negative symptoms of schizophrenia as well as the global cognitive improvement scores, but did not improve positive symptoms in the disease (Marx et al. 2009). Thus, neurosteroid treatment may represent a viable novel therapeutic mechanism in a number of conditions, including schizophrenia, pediatric epilepsy, and Alzheimer's disease.

The Role of Trp Channels in Development

Transient receptor potential (Trp) channels represent a broad array of cation channels responsive to varied stimuli, such as temperature, pressure, pungent plant chemical irritants, such as capsaicin found in chilli-peppers and menthol. While much is known about the role of Trp channels in mechanotransduction, temperature sensation, and pungent compound sensing, much less has been documented about their role in development. Increased temperature, or fever, during the first trimester is known to lead to birth defects. Trp channels sense temperature and pass monovalent cations and calcium in response to increased temperature. In one of the first reports of Trp channel involvement in development, Hutson et al showed that antagonists to TrpV1 and TrpV4 protected against birth defects following increased temperature during development, and that non-invasive magnetogenetic activation of these channels caused craniofacial and cardiac birth defects in chicks and zebrafish (Hutson et al. 2017). Further evidence that Trp channels may play a significant role in development is the finding that *Xenopus* neurons that lack TrpC1, or the receptor for netrin-1 which activates TrpC1 indirectly, showed ablated growth cone turning and lacked netrin-1 gradient dependent Ca^{2+} intracellular elevation (Wang and Mu-Ming Poo, 2005). A similar study showed that TrpC1 mediates growth cone turning via brain-derived-neurotrophic factor (BDNF) (Li et al. 2005). Another indication that Trp channels may play a role during development is that according to a study that isolated the transcriptome of each major cell type in the brain, TrpM3 ranked 17 out of greater than 20,000 genes profiled as most differentially expressed in astrocytes between the ages of P8 and P17, which is the window during which much synaptic refinement occurs (Cahoy et al. 2008). Due to the finding that TrpM3 may be associated with the neurodevelopmental disorder, ASD, and that aberrant regulation of its ligand is also associated with several neurodevelopmental conditions, as well as that little is known about TrpM3 during development, a better

understanding of TrpM3 activation and signaling during development would seem highly beneficial and fruitful.

In this dissertation, we have explored the role of the potent signaling molecule and precursor to all steroid hormones, Pregnenolone Sulfate during the period of retinal development that coincides with cholinergic or glutamatergic retinal waves, as well as assembly and refinement of synapses between bipolar or amacrine cells and retinal ganglion cells. We show that a receptor for PregS, TrpM3 is present during this window of development in the inner plexiform layer (IPL) where formation of synapses occurs, and in a subset of retinal ganglion cells, as well as in Müller glia. We also show using voltage clamp recordings in RGCs that post-synaptic current frequency, but not amplitude, both excitatory and inhibitory, are increased in the presence of PregS, suggesting that PregS acts to facilitate synaptic transmission presynaptic to RGCs. Additionally, we show increased RGC participation in waves as well as prolonged calcium transients following addition of PregS in WT retinas; however, TrpM3 KO retinas completely lacked the prolonged calcium transient response, although other qualities, such as wave frequency and amplitude were preserved in the TrpM3 KO retinas.

Chapter II: New hypotheses regarding the role of Trp channels and neurosteroids in function of RGCs, development, and disease

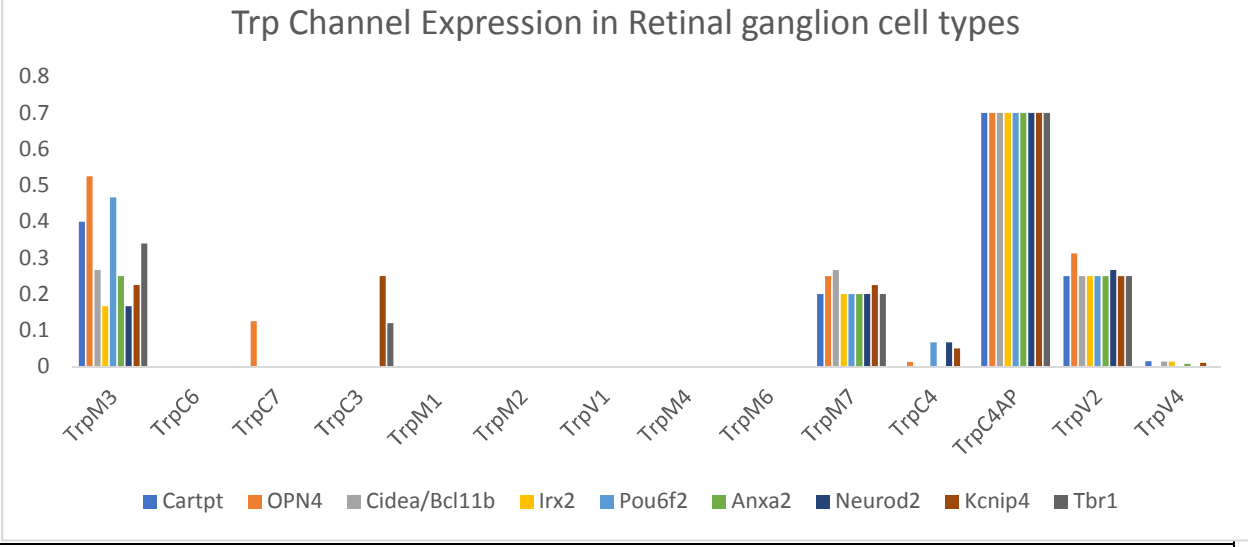


Figure 2.1.A. Single cell RNA-seq followed by t-SNE to identify clusters of retinal ganglion cell types from Rheame et al. *Nature Communications.* 2018. 40 subclusters were identified, which were grouped into 9 different known retinal ganglion cell types based on co-expression of the genes identified above. All the Trp channels profiled in the experiment are listed with average gene expression in FKBP.

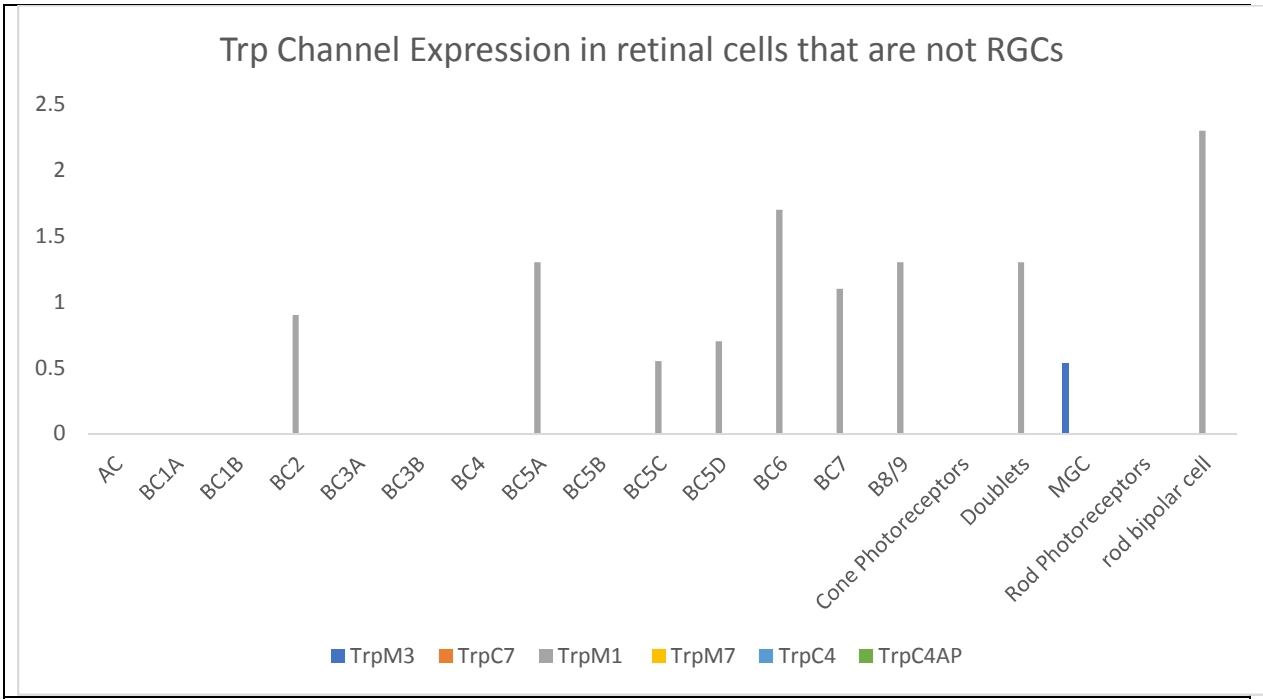


Figure 2.1.B. Data from Shekhar et al. Cell. 2016. Single cell RNA-seq followed by t-SNE to identify cell types. Retinal ganglion cells were not included in this data set. All the Trp channels that were included in the data set are visualized as median expression by cell type. Although there were outliers of cells expressing a particular Trp channel, those outliers were often not represented by the median data point. AC = amacrine cell, BC = bipolar cell, MGC = Müller glia cell.

Trp channel expression in the retina

There have been several different pieces of literature aimed at delineating the location or cell type where each Trp channel is expressed. While at first glance, this knowledge may seem esoteric, there are interesting patterns that have emerged. The most extensive characterization of Trp channel subtype was carried out by Gilliam et al. (2011) where they show by RT-PCR, in-situ hybridization, and anti-body staining the enrichment of 12 different Trp channels in the retina. The following Trp channels were identified in bulk retinal tissue by RT-PCR: TrpC1 at low levels, TrpC2 high levels, TrpC3 moderate, TrpC4 moderate, TrpC5 moderate, TrpC6 high levels, TrpC7 high levels; TrpM1 high levels, TrpM2 low levels, TrpM3 high levels with two splice variants, TrpM4 high levels, TrpM5 very low levels, TrpM7 moderate levels, TrpM6 and TrpM8 not detected; TrpV1moderate levels, TrpV3 moderate levels, TrpV3 low levels, TrpV4 high levels, TrpV5 very low levels, TrpV6 very low levels, TrpA1 very low levels. By in situ hybridization they further elucidate the retinal layer in which each Trp channel is expressed. The following regions of localization were identified: TrpC1 photoreceptor and some in the ONL, TrpC3 expressed at low levels in diffuse manner, TrpM1 expressed very strongly in bipolar cells in the INL, TrpM3 also is expressed in INL, although the distribution is different than the bipolar cell expression of TrpM1, and also lightly in the retinal ganglion cell layer.

In addition to the Gilliam characterization of Trp channel distribution throughout the retina, recent single cell RNA-seq data sets can provide more detailed information about the precise cell types in which specific Trp channels are enriched. Figures 2A and 2B are data from single RNA-seq experiments in the retina, the first looks across all different retinal ganglion cell types that cluster into 9 different clusters according to t-SNE analysis, representing the differential expression of marker genes in different retinal ganglion cell types (Rheaume et al. 2018. Dataset also available at https://health.uconn.edu/neuroregeneration-lab/rgc-subtypes-gene-browser/). The other useful single cell RNA-seq data set in the retina is from Shekhar et al, 2016, which consists of transcriptomic expression data across most other cell types in the retina that are not retinal ganglion cells (amacrine cells, bipolar cells, rod photoreceptors, cone photoreceptors, and Müller glia). Between these two single-cell RNA-seq data sets, we can gain a better sense of which cell types in the retina express which Trp channel subtypes, and to a large extent, without comparing across the disparate data sets, the relative expression of each Trp channel by cell type. Very interestingly, we see that some Trp channel types are ubiquitously expressed across retinal ganglion cell types, such as TrpC4AP, while other TrpC subtypes are not robustly expressed in retinal ganglion cells according to this data set, with some exceptions. TrpC6 is expressed at very low levels in OPN4+ retinal ganglion cells, namely iPRGCs. TrpC3 also shows some expression in Kcnip4+ and Cidea+ retinal ganglion cells. These TrpC variants were not identified in other non-RGC cells in the retina. Other Trp channels that were expressed across nearly all retinal ganglion cell types are TrpM3, TrpM7, and TrpV2. In OPN4+ ipRGCs, these channels had 2-4 fold higher expression levels compared to TrpC7, which has been characterized in ipRGCs (Berg et al., 2019). In cell types other than retinal ganglion cells, TrpM1 was robustly expressed in many bipolar cell types, consistent with physiological data which will be further discussed. TrpM3 was also shown to be robustly expressed in Müller glia, but not other non-RGC subtypes in this data set.

A note should be added about the high expression of TrpC4AP, which appears in high expression levels in all retinal ganglion cell types, but not other cell types within the retina: virtually nothing is known about this Trp channel's function in the retina, however, it has been shown that SNPs of TrpC4AP are present in 36% of late onset Alzheimer's disease, with a low GWAS subject size (Reviewed in Betram and Tanzi, 2009). Its GO category indicates that it may function as a phosphatase.

Known function of various Trp channels in the retina, compared to expression studies.

TRP channels were first discovered in *Drosophila* where they function to induce a light response in photoreceptors (Montell et al. 1989; Hardie et al. 1992; Phillips et al. 1992). Since then, they have been much more broadly characterized for their roles in mechanotransduction, thermosensation, osmolarity regulation, response to natural products such as capsaicin and menthol. Functionally, several Trp channels have been identified and characterized in the mammalian retina. The enigma surrounding the identity of an ion channel that was responsible for the depolarization of ON-bipolar cells in response to light prompted work leading toward the characterization of TrpM1 in bipolar cells. Upon stimulation by light, photoreceptors, produce inverse polarity responses to synaptic glutamate release in ON vs OFF bipolar cells in that ON

bipolar cells depolarize, while OFF bipolar cells hyperpolarize in response in response to light. It was known that ON and OFF bipolar cells express distinct glutamate receptors: ON bipolar cells express metabotropic glutamate receptors (mGluR6); OFF bipolar cells express ionotropic glutamate receptors (iGluRs). Thus, in darkness, photoreceptors tonically release glutamate, which activates mGluR6, and results in closure of a non-selective cation channel. Conversely, upon light stimulus, synaptic glutamate is reduced, which deactivates mGluR6, which is coupled to said nonselective cation channel, inducing a depolarization, which was discovered to be TrpM1 (Morgans et al. 2009). The authors demonstrated that TrpM1 is found in dendrites of ON-bipolar cells; $TrpM1^{-/-}$ animals lacked b-wave electroretinogram (ERG) responses, indicating that these mice lack bipolar cell responses; whole cell patch clamp experiments revealed that $TrpM1^{-/-}$ rod bipolar cell chemically induced light depolarizations were abolished, demonstrating that TrpM1 is coupled to mGluR6 activity in ON-bipolar cell. This was a great breakthough in the phototransduction signaling pathway in the retina and positioned a Trp channel at the center of this important biological function.

Trp channels and the PLR

The pupillary light reflex is a reflex that controls the level of pupillary constriction or dilation in response to intensity of ambient light levels, luminance. In effect, the pupillary light reflex determines the amount of light received by photoreceptors in the retina. The circuitry controlling the pupillary light reflex is well characterized and dependent on intrinsically photosensitive retinal ganglion cells (ipRGCs) located in the retinal ganglion cell layer of the retina. These cells are melanopsin (OPN4) positive, which is a protein that not only is active in phototransduction, but it also acts as a photoisomerase, converting *all-trans* retinal to *11-cis*-retinal without the help of additional cell types, such as Müller glia, as required by rod and cone photoreceptor pigments. Melanopsin containing ipRGCs, upon stimulation by photopic (bright) light levels are thought to be involved in non-image forming functions of the retina, such as the pupillary light reflex, circadian rhythms, and control of retinohypothalamic projections. These retinohypothalamic projections from ipRGCs may impact the hypothalamic pituitary axis (HPA) function as well as modulate the production of certain steroid hormones and this function will be discussed further as it relates to feedback to the neurosteroid sensor, TrpM3, expressed in ipRGCs. ipRGCs project to the suprachiasmatic nucleus (SCN) of the hypothalamus, ventrolateral preoptic nucleus of the hypothalamus, intergeniculate leaflet of the thalamus, olivary pretectal nucleus (OPN) of the midbrain, and directly to the amygdala. Projections to the OPN send axons to the Edinger-Westphal nucleus which in turn send their parasympathetic axons through the oculomotor nerve (CNIII), which innervate the ciliary ganglia, which finally control the iris sphincter muscle which allows for pupillary dilation or constriction.

OPN positive ipRGCs are among the first cells in the retina to send projections to the brain during development and do so prior to the rod and cone synaptic connections made during development onto bipolar cells (Hoon et al. 2014). Since ipRGCs modulate the PLR directly, in addition to indirect control by rods and cones once they are synaptically connected, the PLR during early stages in development is thought to be a measure of ipRGC activity specifically. The first evidence that ipRGCs control the PLR came from Lucas et al (2003) who demonstrated that mice lacking the gene that expresses melanopsin, a marker for ipRGCs, had a highly reduced pupillary light reflex to photopic light levels despite having normal pupillary constriction to

carbachol, a positive control for the parasympathetic activation of the ciliary muscle. Upon establishing that ipRGCs modulate the PLR, much work was done to further identify ion channels that act in the PLR pathway endogenous to ipRGCs. A few years after the identification of ipRGC control of the PLR, it was shown that a broad antagonist of Trp channels, 2-aminoethoxydiphenylborane (2-APB) and show that this inhibits the PLR. The authors attributed the effect of 2-APB to a selective inhibition of the TrpC canonical Trp channel types because they identified TrpC1, TrpC3, TrpC4, TrpC6, TrpC7 by immunocytochemistry; however, they did look for other Trp channels that may be expressed in ipRGCs (Sekaran et al. 2007). Evidence for canonical Trp channel activation, and the specific phototransduction pathway in ipRGCs will be further discussed, but the subject is broached here to leave open the possibility that there may be involvement of non-canonical Trp channels in ipRGC activation.

While Trp channels that are not of the canonical (TrpC) type have not been thoroughly studied in non-image forming ipRGC functions or ipRGC activation, there are some indications that the Trp channels of the melastatin type may provide a complementary activation pathway in these cells. First, as will be further discussed, much of the pharmacology conducted to detect Trp channel involvement in ipRGC activity also targets other Trp channel types, such as TrpM (Klose et al. 2010). Second, recent single-cell or FACS sorted transcriptomic studies which identify all genes expressed in OPN4 positive ipRGCs in an unbiased manner reveal high levels of expression of TrpM3 relative to other TrpC channel types in ipRGCs (Rheaume et al. 2018; Siegart et al. 2012) as shown in Fig2A. Finally, we have begun to characterize the role of TrpM3 in ipRGC mediated animal behaviors, and photoaversion. Others have characterized the role of TrpM3 in PLR (Hughes et al., 2012). It was noted by microarray analysis in mutant mice lacking functional rods and cones (*rd/rd cl*) that two Trp channels appeared to transcriptionally compensate for the lack of rod/cone activity, namely TrpM1 and TrpM3. Interestingly, mice lacking TrpM3 showed attenuated PLR equal to the $OPN4^{-/-}$ response. Additionally, the $TrpM1^{-/-}$ more profoundly lacked a PLR than either then $OPN4^{-/-}$ or *rd/rd cl* mice. No colocalization between Beta-gal, a marker for cells that express TrpM3 in the mutant animal and melanopsin was seen (Hughes et al. 2012). However, the beta-gal expression in this work appears different than the pattern that we observed, in that they are only detecting the very brightly labeled Müller glia end feet, whereas, with better image resolution and contrast, the more faintly labeled RGCs can be identified with these methods.

Signal transduction of TrpC in ipRGCs.

Pak, Grossfield and White as well as Hotta and Yoshiki reported in 1969 a set of genetic mutants in *drosophila* that showed defective electroretinograms, demonstrating for the first time that there were multiple steps in the phototransduction pathway in *drosophila*, and that some steps were downstream in the rod/cone phototransduction pathway. Subsequently, the *drosophila* TRP gene was identified as activated by an IP_3 dependent pathway in phototransduction (Montell, 1989). This, of course, was an exciting moment in science, as it was the first identified Trp channel, and has spurred decades of fruitful work into various modes of Trp channel mediated signal transduction.

In the decades that followed the discovery that a Trp channel is required for certain aspects of phototransduction in *drosophila*, intrinsically photosensitive retinal ganglion cells had been identified (ipRGCs) and studied for their role in nonvisual photic responses, such as pineal melatonin synthesis, the pupillary light reflex, and circadian rhythms. It was also known that ipRGCs expressed melanopsin (OPN4), which is an opsin similar to invertebrate opsins that are G-protein coupled and utilize 11-cis-retinal as their chromophore. In a seminal work, Panda and colleagues (2005) were able to show that oocytes injected with melanopsin mRNA produced photocurrents that were blocked by antibodies against Gαq/Gα11 G proteins. They further reasoned that since the *drosophila* phototransduction pathway involved the *drosophila* TRP channel, which was very sequentially homologous to the canonical mammalian Trp channel family, TrpC, that determining whether a TrpC could be activated by the melanopsin G-protein coupled cascade in oocytes would point to the involvement of a possible TrpC channel in mammalian systems as well. Indeed, upon inection of TrpC3, they show stong TrpC light dependent activation that was blocked by the Trp channel antagonist, Lanthanum (La^{3+}). They also suggested that the voltage/current relation in ipRGCs was very similar to that of the non-selective cation channel, TrpC3. This discovery triggered a great amount of work towards identifying the putative TrpC channel responsible for light dependent depolarization in ipRGCS.

This suggestion that a TrpC channel is responsible for the light activated depolarizing current in ipRGCs has been followed by numerous different studies. The first attempt (Warren et al. 2006) to identify a TrpC channel in ipRGCs utilized pharmacology and immunohistochemistry to suggest that TrpC6 is responsible for the light-dependent depolarizing current. They first show that the ipRGC response is blocked by lanthanides and ruthenium red. La^{3+} has been shown to block a number of other Trp channel types, including TrpM (reviewed in Zholos et al. 2010). However, ruthenium red is generally thought to be a nonselective cation inhibitor, and has been shown to block TrpV and some TrpM channels, but has not been shown to inhibit TrpC. Additionally, they show inhibition of the ipRGC response using SKF-96365, which inhibits TrpC channels, as well as TrpM8 (Zholos, 2010) and many voltage gated calcium channels, such as Cav1, Cav2, Cav3 and Cav3.1 at similar IC50s as that used for TrpC identification (Singh et al. 2010). Interestingly, Warren et al also used flufenamic acid and showed an enhancement of the ipRGC light activated depolarization. Flufenamic acid (FFA) is a compound in the fenemate class of drugs . FFA is a highly non-selective pan Trp channel antagonist that inhibits TrpC and TrpM channels. They identify TrpC6 by immunohistochemistry as a candidate TrpC channel to account for the light evoked depolarization, but then pharmacologically used an agent, FFA, that is known to inhibit TrpC6 with an IC_{50} of 17.1uM, but show this this drug potentiates the light evoked response (reviewed in Klose et al. 2010). This initial attempt to characterize the precise identity of a Trp channel that could account for the light activated depolarizing current in ipRGCs left perhaps more questions unanswered than it did perhaps resolve them.

The second piece of literature which sought to characterize the Trp channel identity in ipRGCs, as well as its contribution to intracellular calcium following light activation, did so using immunopanned isolated ipRGCs (Hartwick et al. 2007) with electrophysiology and calcium imaging as well as pharmacology. They used the pan Trp channel blockers, lanthanum and gadolinium and 2-APB, which inhibit not only TrpC but also many of the TrpM and TrpV channel varieties. In addition, the study used SKF-96365 which targets TrpC and voltage gated calcium channels, as well as two voltage gated calcium channel antagonists, verapamil and

diltiazem, all of which ablated the light evoked response in isolated ipRGCs. Finally, they used the fenemate, flufenamic acid, a pan Trp channel antagonist which has known targets in both the TrpC and TrpM family. The authors concluded that the identity of the Trp channel involved in the light evoked depolarization in ipRGCs is TrpC7, "because [flufenamic acid] blocks TrpC3 and TrpC7, but not TrpC6" according to a study by Inuoue et al. 2001. However, the more recent, rigorous, and systematic study comparing the affinity of fenemates, such as flufenamic, meclofenamic (MFA), and mefenamic acid (MEF) for various different Trp channels conclusively demonstrates that compounds such as FFA target many different Trp channels, with very similar affinity (Klose et al, 2010). Thus, the conclusion, or hypothesis, made in Hartwick et al. needs to be revisited given this updated pharmacological understanding.

At this point, one piece of literature was published questioning the presence of TrpC in ipRGCs as an ion channel involved in signal transduction. Their methodology consisted of excised patch recordings from isolated ipRGCs as well as single-cell RT-PCR. They were able to conclude that stimulation the melanopsin pathway in ipRGCs activated a Gq/11 class of G proteins, which in turn stimulate the downstream effector enzyme phospholipase C. However, regarding the role of a TrpC in the phototransduction of ipRGCs, the authors commeted:

> "However, our data may also pose a problem for the hypothesis that TrpCs are the light-gated channel in ipRGCs. DAG analogues and metabolites, which activate…TrpC channels, failed to induce a current in ipRGCs or to occlude the light-evoked current. It seems safe to say that uncertainty about the identity of the light-gated channels in ipRGCs remains the most glaring gap in our understanding of phototransduction mechanisms in these neurons (Graham et al. 2008)"

Thus, given that the pharmacological efforts to characterize the identity of the Trp channel in ipRGCs have resulted in conflicting or confusing findings, efforts to characterize the channel genetically hoped to be more fruitful. At around the same time in 2011, two papers provided genetic insight into possible the identity of TrpC channels in ipRGCs responsible for the light dependent ipRGC depolarization. Much of the literature suggested that TrpC3, TrpC6 and/or TrpC7 may be the culprit of this activation. Based on this, Perez-Leighton and Schmidt and colleagues characterized the ipRGC light evoked responses from mice lacking either TrpC6, TrpC7, or TrpC3 and showed that the melanopsin evoked light responses persist in each of these knockout lines (Perez-Leighton et al., 2011). They concluded that much of the previous pharmacology and immunohistochemistry dependent literature had been inconsistent, sighting evidence showing that two different pieces of literature had localized TrpC6 in different regions, suggesting that antibodies used weren't specific (Warren et al, 2006; Sekaran et al, 2007). They also noted that it had been demonstrated that TrpC7 was enriched in ipRGCs relative to other RGC cell types, but that ectopic expression of melanopsin still induces photocurrent in non-ipRGC 'regular' RGCs (Lin et al. 2008). The assessment that TrpC7 is enriched in ipRGCs relative to other RGC types is consistent with the recent single cell RNA-seq data, which suggests that TrpC7 may be exclusively expressed in OPN4 positive ipRGCs (Fig 1, data from Rheame et al. 2018). If true, this would point to other non-selective G protein coupled cation channels in the ipRGC activation pathway, possibly another Trp channel. The Perez-Leighton work concluded that either a) other Trp channels besides those tested are involved in the

melanopsin evoked light response, or b) the channels tested may be functioning in a heteromeric fashion, as had been demonstrated for some of the TrpC subtypes.

The second paper published in 2011 (Xue et al. 2011) showed that in the double knockout, lacking TrpC6 and TrpC7, the M1 ipRGC photocurrent was ablated. However, the individual gene knockouts of TrpC3, TrpC6 or TrpC7, had persistent photocurrents similar to WT, consistent with the Perez-Leighton study. Interestingly, although the TrpC6/TrpC7 double KO retinas lacked an M4-ipRGC photocurrent, the pupillary light reflex tested in these animals was unaffected. The authors concluded that melanopsin mediated phototransduction to activate the sphincter muscle controlling the iris must therefore diverge from the mechanism in ipRGCs. This brings up several important points: First, there may exist an alternate melanopsin mediated phototransduction pathway that does not involve the heteromeric TrpC6/TrpC7 channel; if so, in what cell types is it active? Second, the PLR is a robust determinant of ipRGC function, but an alternate behavior such as photoaversion, or circadian rhythm entrainment may portray the state of the ipRGC responsivity. Third, single cell RNA-seq data would suggest that there are several Trp channels expressed in ipRGCs (Rhease et al. 2018), some of which would be activated by the melanopsin GPCR phototransduction pathway, while others would be de-activated. That the authors didn't see the reduced PLR response as expected may point to a role for one of these other Trp channels, perhaps a necessary component of the ipRGC phototransduction pathway for the PLR and photoaversion. Finally, TrpC channels have been implicated in axon guidance in development as will be further discussed. Although this may be seemingly unrelated to the function of ipRGCs during development, there is the possibility that TrpCs may be important for the expression of other factors that contribute to ipRGC light evoked responses.

TrpM3 is modulated by GPCR

Although much of the literature on the light evoked ipRGC response thus far has focused primarily on identifying a particular TrpC or heteromeric set of TrpCs as the candidate for the light evoked depolarization in ipRGCs—a notion that arose from the very first characterization of TRP in *drosophila*, a paralogue of TrpC, more recent single-cell RNA-seq suggests that several Trp channels may play a role in the activity and ionic conductances of RGCs. According to one such data set (Rhease et al. 2018) in OPN4 positive cells, order of the abundance of Trp channel transcripts is as follows: TrpC4AP > TrpM3 > TrpV2 > TrpM7 > TrpC7 > other Trps not detectable. Note that although Xue et al. (2011) determined that TrpC6 was required for M1 light evoked activation, Berg et al. (2019) show that TrpC6 is expressed at low levels in M1-M3 ipRGCs, but that TrpC7 is highly differentially expressed in ipRGCs while TrpM3 and TrpC3 are more ubiquitously expressed in all RGCs. Although TrpC6 is required for M1 ipRGCs phototransduction, it may be expressed at very low levels. It is likely that the phototransduction of M1 ipRGCs is mediated primarily by Trp channels other than TrpC6, such as TrpM3 or TrpC3. While mRNA levels in cells may not necessarily indicate actual protein expression, that several other Trp channels in ipRGCs are much more highly expressed than those that have been extensively studied in this cell type indicates that this screen may be picking up, in a more unbiased fashion, relevant players in ipRGC phototransduction that have been largely unexplored in this context.

Another reason that TrpCs were selected for the study of their role in ipRGC phototransduction is that it was known that they were activated by the G-protein coupled receptor of Gαq type, as well as by other components of the same pathway, such as phospholipase-C (PLC) and diacylglyerol (DAG) (Venkatachalam et al., 2003). The phototransduction pathway in ipRGCs, currently an active area of investigation at different light levels and different ipRGC cell types (Sonoda et al. 2018), is generally thought to involve photoisomerization of the chromophores associated with melanopsin, followed by Gαq or Gβγ activation, leading to PLC activation, which then activates a Trp channel capable of activation by a G-protein coupled pathway. Thus, much of the literature focused on Trp channels with this known property, TrpC.

More recent work has extended the modulation of Trp channels by G-protein coupled receptors beyond the canonical Trp channels. For example, like TrpC channels, TrpM3 has been shown to require the membrane phospholipid phosphatidylinositol 4,5 pisphosphate [$PI(4,5)P_2$] (Badheka et al. 2015; Toth et al, 2015) and that stimulation of PI(4,5)P2 hydrolysis via phospholipase C (PLC) activation inhibited both heterologous and endogenous TrpM3 channels (Toth et al. 2015). Furthermore, expression of TrpM3 in planar lipid bilayers required PI(4,5)P2 for activation by PregS (Uchida et al. 2016). Recently, it was reported that TrpM3 is inhibited by Gβγ co-expression, and that co-expression of a Gβγ scavenger, or 'sink,' attenuates this inhibition (Badheka et al, 2017). Furthermore, TrpM3 co-immunoprecipitated with Gb indicating a direct modulation by this G-protein subunit. The authors also found that activation of Gq-coupled M1 acetyl-choline receptors or Gi-coupled M2 receptors resulted in inhibition of PregS mediated TrpM3 currents in whole-cell patch clamp experiments of exogenously expressed channels, and this effect was attenuated by a Gβγ scavenger. They further showed that the effect was mediated directly by Gβγ but not PLC, an alternate mediator in the Gq pathway. Additionally, Badheka et al (2017) showed that activation of dopamine receptors, specifically D2 Gi-coupled, also inhibited PregS activation in TrpM3 channels. Although this work focused on Gi-coupled receptors, such as M2 and D2, they also noted that Gq-coupled pathways that utilize the Gβγ signaling effector equally inhibit TrpM3.

Intrinsically photosensitive ipRGCs, as well as amacrine cells and other RGCs in the retina, possess gap junctions composed of connexins, likely connexin36. Connexins have been studied in other systems and have been shown to be modulated by dopamine receptors, namely D1 and D2/4. For example, in medium spiny neurons, activation of D1 selectively increased gap junction coupling by Cx36, whereas activation of D2 selectively reduces Cx36 coupling (Cummings et al. 2008). This connexin activation follows the classic pathway of D1 receptor activation. Its sequence is as follows: dopamine binds to the D1 receptor, which is also a Gq coupled GPCR, this increases cyclic AMP (cAMP) via adenylate cyclase. cAMP activates the protein kinase, PKA, which phosphorylates specific serines of its targets and modulates them. In the case of Cx36, phosphorylation of Ser293 has been shown to increase Cx36 coupling (Klothmann et al. 2008). Thus, activation of D1 receptors in ipRGCs should, if it follows the classic pathway, result in increased coupling of Cx36. Strikingly, what was observed is that in amacrine cells, activation of D1 results in dephosphorylation of Ser293 (Klothmann et al. 2008). The authors wrote the following in their conclusion:

> "The D1 receptor pathway modulates Cx36 phosphorylation in AII amacrine cells. This pathway classically activates PKA, and we have demonstrated that PKA partially mediates the effects of D1 agonist application. It is thus **paradoxical** that application of a

> D1 agonist leads to reduced phosphorylation of Cx36 at Ser293. We predict that the changes in the phosphorylation state of Cx36 in AII amacrine cells are associated with changes in the coupling state of these cells (Klothmann et al. 2008)"

Indeed, the first indication that ipRGC activity is modulated by dopamine, which in turn affects gap junction mediated cellular connectivity was presented in Kirkby et al (2013). The authors showed that in retinas lacking the β2 subunit of the nicotinic acetylcholine receptor (β2KO), retinal waves were largely absent, or erratic, but upon light activation, the β2KO showed increased wave frequency, and that wave frequency of the β2 but not WT retinas was highly dependent on dopamine. They conclude that both light and dopamine reduction, as in the condition of the β2KO, increases wave frequency. They further show that Cx36 is required for the wave-like activity, as in Cx36 and β2 double knockout they don't see waves, but they do see highly prolonged calcium transients that resemble what we see with TrpM3 agonist (chapter 2). It is also worth noting that in β2KO retinas, but not WT, selective blockade of gap junctions by 18 β -glycyrhetinic acid (18β -GA) also induced similar prolonged transients (Piccociotto et al., 1995; Xu et al., 1999). Kirkby and Feller were the first to postulate that gap junction coupling in ipRGCs is negatively modulated by D1 receptor activation and positively modulated by D2 receptor activation. This find was further asserted by Arroyo et al (2016), who showed that the cholinergic signaling during stage II retinal waves does induce dopamine release, and that acutely blocking either cholinergic signaling or D1 increased the number of light responsive cells as well as the number of neurobiotin coupled ipRGCs. When the fenemate, meclofenamic acid (MFA) was applied, a gap junction and also pan Trp channel antagonist, light induced photocurrents in ipRGCs were nearly completely ablated. If MFA were a selective gap junction antagonist, one might have expected to see a response similar to that reported in the Cx36 β2KO, or the 18β-GA experiments noted above. Indeed, TrpCs were first described in ipRGCs by the highly similar compound flufenamic acid blockade, which ablates the melanopsin mediated photocurrent (Hartwick et al., 2007). Thus, the highly astute observation that ipRGC gap junction connectivity is mediated by dopaminergic signaling in ipRGCs was seminal work in the field, though it still leaves open the question of the inverted sign of signaling from the D1 receptor to reduced connexin activity. Furthermore, given that the gap junction antagonist used also inhibits Trp channels, including TrpC6 (IC_{50} 38μM) and TrpM3 (IC_{50} 13.3μM), this certainly opens the possibility that one of these ion channels could be mediating gap junction coupling and ipRGC sensitivity directly.

Outward rectifying K2P channel contributes to ipRGC excitability in M4s.

It has been established that melanopsin mediated phototransduction in M1 ipRGCs occurs via Gq coupled mechanism involving the activation of phospholipase C, which targets TrpC6/TrpC7 heterodimer (Xu et al. 2011). However, the view that this mechanism persists in all ipRGC types, or that this mechanism provides an exhaustive explanation of ipRGC activation was recently revised when it was shown that in M4 ipRGCs the Gq coupled melanopsin cascade directly targets two-pore 'leak' potassium channel, K2P in scoptopic, dim, light conditions, but still activates TrpC6/7 channels in photopic, bright, light conditions (Sonoda et al. 2018). Interestingly, K2P channels are outwardly rectifying (Shewe et al. 2016) and the authors showed

that melanopsin phototransduction increased the intrinsic excitability of M4 ipRGCs by increasing the input resistance (Rinp), a result that would be expected if a 'leak' channel was closed upon melanopsin dependent phototransduction, the opposite of Rinp with activation of TrpC in M1 cells. They further delineated pharmacologically and electrophysiologically that the K2P channel closure upon phototransduction is mediated by PLC and increases M4 excitability, but that at high light levels, perhaps with increased PLC activation, TrpC channels activate but this activation isn't sufficient to decrease Rinp or the intrinsic excitability. Xu et al poses that this mechanism by which a Gq coupled response inhibits a K2P channel and under brighter light conditions also activates the canonical Trp channels is a molecular gate by which contrast can be detected. This was also the first indication in ipRGCs that closing an ion channel in a melanopsin dependent manner serves to increase light sensitivity.

We have now seen that in M1 ipRGCs there exists several G-protein coupled or cAMP/PKA dependent pathways by which the cells either are excited or increase gap junction coupling via either the melanopsin dependent phototransduction cascade or dopamine receptor dependent activation. However, it would seem that there are still some unanswered questions about ipRGC phototransduction. The sign inversion in the D1 transduction pathway suggests that some other factor is playing a role in gap junction connectivity. Second, TrpM3 knockout animals display reduced PLR and possibly reduced photoaversion (our experiment which needs more replicates to confirm, but I was prohibited from conducting the experiment). Third, inhibition of TrpM3 as an outward rectifying non-specific cation channel would have the effect of increasing Rinp, and perhaps increase intrinsic excitability without passing calcium. Fourth, TrpM3 has been shown to be expressed in ipRGCs by RNA-seq. Fifth, TrpM3 is inhibited by Gβγ, which is released by melanopsin upon activation, as well as by D1 receptor activation. Sixth, TrpM3 is not inhibited or modulated by PLC, as is TrpC, which is also activated by melanopsin dependent phototransduction as well as D2 receptor activation. Therefore, if TrpM3 is expressed in ipRGCs as RNA-seq data suggests that it is at higher levels than TrpCs, then upon activation by light, melanopsin releases Gβγ and PLC is activated simultaneously. The Gβγ targets TrpM3 to inhibit this outward rectifying channel, which is likely somewhat active at physiological temperatures, as well as by exogenous agents, such as neurosteroids. The inhibition of TrpM3 may accomplish two things: it would reduce the flux of calcium and it may increase intrinsic excitability. Simultaneously, light would activate PLC via melanopsin, which activates TrpC channels as we have previously seen. If this mechanism were to hold true for D1 receptor activation, similarly, D1 receptors have been shown to release βγ, which would inhibit TrpM3. It could be that inhibition of TrpM3 reduces the probability of light responses. For example, in either the β2KO or by D1 receptor antagonist, SCH-23390, light responses are increased in frequency, that is, when the Gβγ would be unbound to TrpM3. Furthermore, the modulation of connexins by dopamine receptors would have the correct sign of signaling if TrpM3 were an intermediary. In low dopamine conditions, TrpM3 should be more active because it lacks the Gβγ inhibition by D1, in which case calcium from TrpM3 could stimulate adenylyl cyclase and kinase activation, which would in turn phosphorylate connexin and increase gap junction coupling in low dopamine conditions. TrpM3 has been shown to activate the kinases required to phosphorylate target proteins, MAPK and PKA (Rubil et al. 2016). It is currently unknown whether a Trp channel can modulate a gap junction, although they certainly possess the molecular machinery to do so.

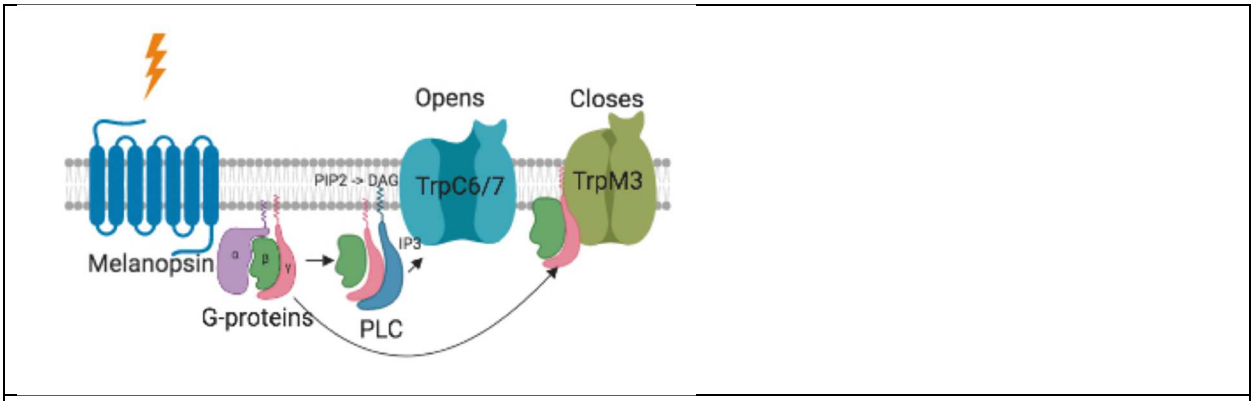


Figure 2.2.A. Revised model ipRGC excitation given TrpM3 is inhibited by $G_{\beta\gamma}$.

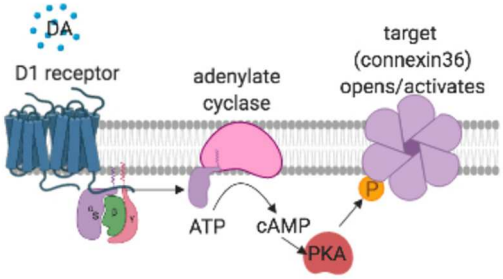


Figure 2.2.B. Canonical D1 receptor G-protein coupled connexin36 gap junction modulation.

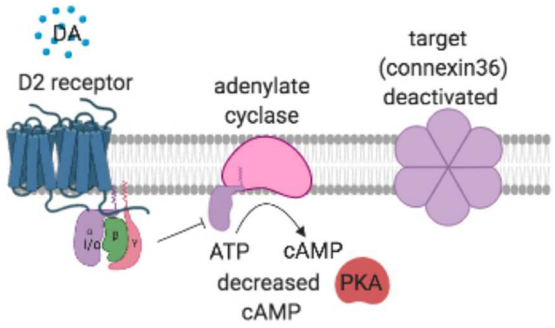


Figure 2.2.C. Rod-Cone gap junction coupling: D2 receptor G-protein coupled connexin36 gap junction modulation.

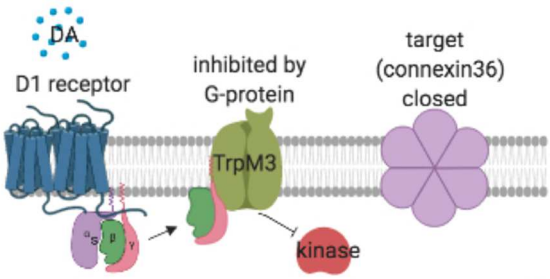


Figure 2.2.D. Model for TrpM3 mediated gap jx coupling: In ipRGCs, gap junction coupling occurs paradoxically to cononical D1 signalling. D1 receptor activation inhibits Cx36. This could occur if TrpM3 modulates Cx36 by a kinase since TrpM3 is inhibited by the G-proteins released by D1 activation, $G_{\beta\gamma}$.

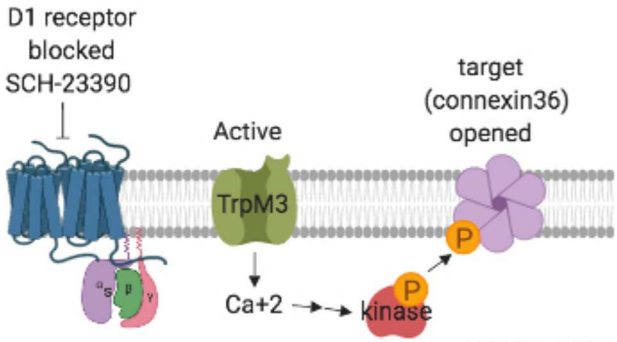


Figure 2.2.E. When D1 receptors are blocked, if TrpM3 mediates Cx36, TrpM3 is not inhibited, and is either activated constitutively or by exogenous ligands, or sensitized by Ca. This may result in kinase dependent phosphorylation of a connexin, increasing gap junction coupling.

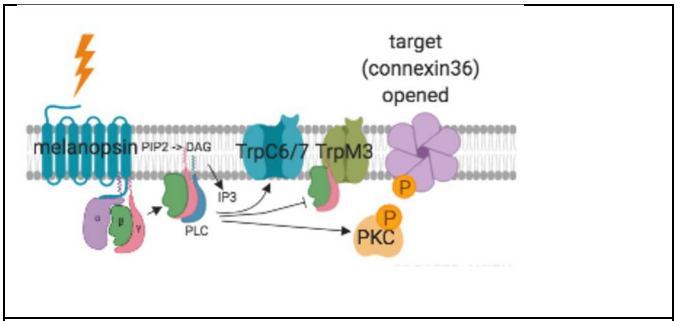


Figure 2.2.F. Model for melanopsin activation with TrpM3: Upon light activation, melanopsin activates G-proteins, which activate TrpC6/7 via PLC and simultaneously inhibit TrpM3 via $G_{\beta\gamma}$. PLC activates PKC, which may phosphorylate Cx36 to increase gap junction coupling by light stimulation. Strong or persistent activation melanopsin may induce TrpM3 opening via its Ca sensitivity from TrpC activation.

Melanopsin activation by 490nm wavelength light, and the phototransduction pathway that ensues has been extensively studied. It is generally accepted that the opsin, 11-cis-retinal, photoisomerizes once bound to the opsin protein, melanopsin, upon the absorption of a photon. This activates a melanopsin mediated G-protein coupled pathway, specifically a G-αq pathway. Melanopsin in ipRGCs signals via Trp channels shown to be anchored by a PDZ scaffolding (Goel et al., 2002). Upon activation by light, Gq is activated to act on phospholipase C (PLC), which induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and then formation of diacylglycerol (DAG), which is further metabolized by diacylglycerol lipase (DAGL). PIP2, PLC, and DAG mediated activation of PKC can activate Trp channels, particularly TrpV1 (Zygmunt et al. 2013), but also activate TrpC (Reviewed by Hankins, 2008). However, if the RNAseq data sets are correct (Rheame et al. 2018; Berg et al. 2019) and TrpM3 is also expressed in OPN4 positive ipRGCs, then TrpM3 is inhibited by Gβγ G-proteins, which would result in the inhibition of this ion channel upon activation of melanopsin by light. This has not been shown experimentally, though the effects of closing a TrpM3 channel in response to light could range from insignificant to similar to those seen when K2P channels are deactivated in M4 ipRGCs by a similar mechanism (Sonoda et al. 2018), particularly since both TrpM3 and K2P channels share the property of outward rectification. The one property of TrpM3 that is not shared by K2P is the strong propensity to pass calcium upon activation, as we will see in the next chapter, which could also have profound effects on cellular physiology.

Gap junctions, specialized electrical connections between two cells, often of the same type, allow for ions, molecules, and electrical signals to flow swiftly between directly connected cells, thereby increasing the array of available options for intercellular communication. Gap junction coupling in the retina between the many different cell types of neurons and glia is fairly well characterized (Reviewed in Bloomfeld and Volgyi, 2009). In the retina, gap junctions in several cell types are mediated by dopamine receptors. Rod-cone electrical synapses, have been shown to be mediated by D2 dopamine receptors, such that during the day when dopamine levels are high, D2 receptors are maximally activated, and this results in reduced gap junction coupling (Ribelayga et al. 2008). The authors further proposed that gap junction modulation by dopamine

was controlled by circadian rhythm, rather than light levels in rods, which increases sensitivity of rods for dim light at night but not during the day.

The molecular cascade by which D2 receptors in rods and cones increase gap junction coupling is known and follows canonical D2 receptor signaling. D2 dopaminergic receptors are Gαi/o GPCRs; thus, they are typically thought of as inhibitory GPCRs. The Gαi/o inhibits andenylate cyclase upon activation. Thus, when stimulated by dopamine, D2 receptors will decrease cellular concentrations of cAMP, further reducing the activity of certain kinases, particularly PKA (Ledonne et al. 2017). Cellular machinery that requires phosphorylation by PKA to be active are turned off upon D2 receptor activation, as is the case for gap junctions in rods upon stimulation by dopamine.

Other cells in the retina also display dopaminergic control of gap junction coupling, namely ipRGCs (Kirkby et al., 2013; Arroyo et al., 2016) as well as AII amacrine cells (Klothmann et al., 2008). However, unlike rod-cone gap junctions, blockade of D2 receptors in ipRGCs or AII amacrine cells results in reduced gap junction coupling, whereas blockade of D1 receptors increases gap junction coupling in a manner completely paradoxical to canonical signaling pathways of D1 and D2 receptors. For example, Kirkby and Feller (2013) showed that the frequency of retinal waves in WT retinas was unchanged by either D1 or D2 receptor antagonists. However, in β2KO retinas, which lack cholinergic mediated waves, and are thought to have dopaminergic and gap junction mediated waves, although the extracellular dopamine concentrations are likely lower in β2KO than WT due to the highly reduced correlated spontaneous activity. Because D2 receptors have higher affinity for dopamine than D1 receptors, the authors argue that activity in the β2KO is likely related to D2 activation. The β2KO wave frequency is reduced in both dark and light conditions compared to WT, likely because of the lack of cholinergic signaling. However, unlike the WT retinas, in the β2KO, wave frequency is increased by light. Furthermore, they showed that inhibition of D2 receptors by raclopride further decreases wave frequency below either light or dark levels in the β2KO. Conversely, inhibition of D1 receptors by SCH23390 greatly increases wave frequency in the β2KO above both dark and light levels. The authors conclude that gap junction connectivity is increased by D2 receptors and decreased by D1 receptor activation, in direct contrast to the canonical pathways of D1 and D2 receptor GPCR mediated gap junction coupling as in rods and cones.

D1 receptor activation was shown to activate PKA, a kinase known to phosphorylate Cx36, however in AII amacrine cells, D1 receptor activation results in reduced Cx36 phosphorylation, which reduces activity of Cx36 (Klothmann et al., 2008). However, it was subsequently shown that a phosphatase, PP2A, may be activated by PKA following D1 receptor activation, resulting in dephosphlorylation of Cx36 (Klothmann et al. 2009). However, if PP2A dephosphorylates Cx36 Ser293 to reduce gap junction connectivity in response to D1 receptor activation, then that would imply that Cx36 is constitutively phosphorylated, or activated, in dark and/or very low dopamine conditions. However, it has been demonstrated that photopic light increases AII amacrine gap junction coupling (Bloomfeld et al., 1997), which may be mediated by D2 receptor signaling from dopaminergic amacrine cells (Reviewed by Bloomfeld et al., 2009), or it may be mediated by gap junctions between ipRGCs and AII amacrine cells (Reifler et al., 2015). Whether the increase in gap junction coupling by light is directly mediated by D2 receptors or by melanopsin in ipRGCs remains to be shown, however, either would indicate that Cx36 is not

constitutively phosphorylated during dark adaption, or in low dopamine concentrations. The premise that D2 receptor activation, which is activated by relatively low dopamine concentrations, results in increased gap junction coupling suggests that connexin phosphorylation is coupled to receptor activation, rather than some constitutive phosphorylation that must be actively dephosphorylated upon activation by D1 -> PKA -> PP2A. Thus, although a phosphatase may be active during D1 activation, an alternative mechanism that could result in the sign flip of the connexin phosphorylation pathway is necessary.

An ion channel that passes calcium, has been shown to activate several kinases upon activation, and is inhibited by the G-proteins Gβγ in response to D1 receptor activation, as well as is outwardly rectifying would be a good candidate to explain the sign switch in response to D1 activation leading to reduced Cx36 coupling in ipRGCs. TrpM3 has been shown to be expressed in ipRGCs by genetic methods, and animals lacking TrpM3 lack the melanopsin dependent PLR (Hughes et al., 2012). Preliminarily we show that TrpM3 p6 animals may have deficient photoaversion in photopic conditions (Figure 2.3). Upon direct activation by agonists, such as PregS or CIM0216, TrpM3 acts via MAPK to induce early immediate gene expression, such as c-fos (Rubil et al., 2015). TrpM3 is also thought to activate PKC in pancreatic cells to stimulate insulin production (Reviewed by Thiel et al., 2013). If active in ipRGCs, TrpM3 would likely stimulate PKC activation, which has been shown to phosphorylate and activate connexins (Reviewed by Solan et al, 2009). Furthermore, parallels can be drawn between the melanopsin activation mediated inhibition of the outwardly rectifying K2P in M4 ipRGCs at scotopic light levels, which, in combination with TrpC activation at photopic light results in cellular contrast sensitivity in dim and bright light (Sonada et al., 2018). The addition of TrpM3 to the ipRGC phototransduction cascade, as well as dopamine dependent gap junction coupling would serve to explain several points: 1. D1 activation inhibits TrpM3, and if gap junction coupling is mediated by calcium influx from TrpM3, this may explain the lack of gap junction coupling upon D1 activation. 2. D2 activation also stimulates gap junction coupling via a molecular pathway similar to light triggered melanopsin activation, via Gβγ and possibly PKC activation, which may directly activate both TrpC as well gap junction coupling, in either case TrpM3 would be inhibited from passing outwardly rectifying currents. 3. There is a dynamic range between dark/low dopamine, which would result in D2 activation vs dark/D2 receptor block. This model does not account for a constitutive phosphorylation which would be required of a phosphatase dependent model. 4. Many of the gap junction blockers thus tested, such as meclofenamic acid, also block Trp channels, such as TrpM3, which have prevented experimentally observing this model; however, in low dopamine conditions, such as in the β2KO, and with Cx36KO, prolonged transients similar to those observed via TrpM3 activation are seen, suggesting a TrpM3 – like calcium transient in CX36KO that is not seen with the Trp and connexin antagonist (MFA).

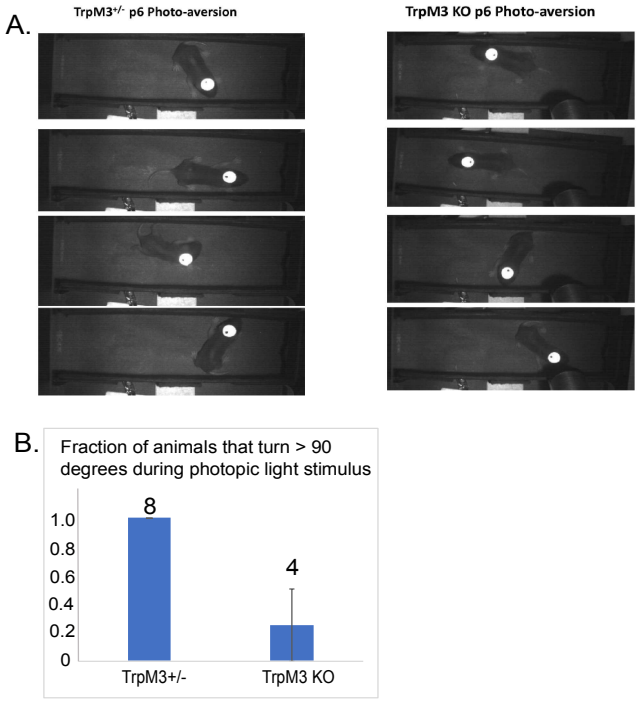


Figure 2.3. Preliminary data suggesting that the TrpM3 $^{-/-}$ may have a deficit in photoaversion at photopic light levels. A. The top left column are 4 representative p6 TrpM3$^{+/-}$ mice' response to a photopic light stimulus. The light is on the left side of the box, and the images shown are the maximal head angle during a 1.5 min period during which the light is turned on after a 1hr dark rearing period. The right column shows the response of the TrpM3 KO p6 pups. B shows the fraction of mice in each group that turned greater than 90 degrees during the 1.5 min stimulus period. Number of mice tested are 8 het and 4 KO.

Putative Revised model of ipRGC activation involving TrpM3 inhibition by Gβγ

Thus, I propose a revised model of ipRGC light activation and gap junction coupling as follows: Upon activation by photoisomerization of retinal, the melanopsin G-proteins, Gβγ are released to stimulate PLC, which in turn activates TrpC and simultaneously Gβγ deactivates TrpM3, closing the outward rectifying nonspecific cation channel. This may have the effect of increasing input resistance on the cell, unless gap junctions are simultaneously activated by the same G-protein mediated pathway, which may ultimately activate PKC to phosphorylate connexins. This all could happen in a dynamic fashion, such that lower melanopsin activation levels perhaps close TrpM3 first, then at slightly higher levels, activate TrpC and at still greater activity, activate connexins. D2 receptor activation would have an extremely similar pathway of connexin activation, and should also activate TrpC and close TrpM3, although this effect has only been

shown in the literature indirectly as low dopamine levels where D2 receptor activation would predominate lead to increased light sensitivity, while blocking D2 at low dopamine levels reduces light sensitivity (Kirkby and Feller, 2013). Conversely, D1 receptor activation induces Gs/olf coupled G-protein activation. In this case $G_{\beta\gamma}$ is still present and upon activation blocks TrpM3 (Badheka et al., 2017). However, the Gs/olf g-protein will tend to activate adenylate cyclase to increase cellular cyclic AMP levels, rather than activate PLC dependent pathways required to activate TrpC (Reviewed by Nishi et al., 2011). So, in the case of D1 receptor activation, TrpM3 is blocked, TrpC is also not active unless melanopsin is also activated by light, and if gap junctions are coupled to Trp channel activation, connexin will be dephosphorylated in this state, and thus uncoupled. A mechanism such as this would also explain the increased light sensitivity and gap junction coupling seen when D1 receptors are blocked and also light is present. I think this model provides a more complete and likely picture of the light and dopamine modulation of both gap junction coupling and light sensitivity than our current model involving D1 mediated phosphatase activation, as both light and dopamine contribute to cellular signaling dynamics in either overlapping or complementary signaling pathways.

Melanopsin is expressed in dopamine rich regions of the brain

In the revised model both light driven melanopsin activation and D2 receptor activation, both pathways involve largely the same molecular mediators: melanopsin or D2 -> Gβγ -> PLC -> PKC phosphorylation of a connexin -> increased gap junction coupling and light sensitivity. Whether this pathway is objectively true in ipRGCs or other cell types remains to be seen, however, melanopsin is known to be expressed throughout the brain where its primary function remains unknown as light doesn't penetrate the cranium. According to the Allen Brain Institute Human Brain Atlas RNAseq data set, which quantifies expression of a particular transcript based on brain location (Hawrylycz et al., 2012), OPN4, the gene for melanopsin, is expressed primarily in dopamine rich regions in the human brain, including striatal loci such as the caudate, putamen, nucleus accumbens, claustrum, as well as hippocampal structures such as the dentate gyrus as well as the basolateral nucleus of the amygdala. This strongly suggests that melanopsin is present in dopaminergic cells of some sort. Indeed, DRD1, or the D1 receptor, has a Pearson correlation coefficient of 0.574 and is ranked as the 34th most correlated transcript with that of melanopsin according to the Brain Atlas data set, out of over 17,000 probes. Interestingly, M1 acetylcholine receptors also had high Pearson coefficient of 0.587 and ranked as the 15th most correlated transcript with melanopsin. It is known that melanopsin follows a Gq coupled pathway of activation leading to PLC activation, as does the M1 acetylcholine receptor. This data suggests that melanopsin in the brain may augment or complement D1 and/or M1 receptors in some way.

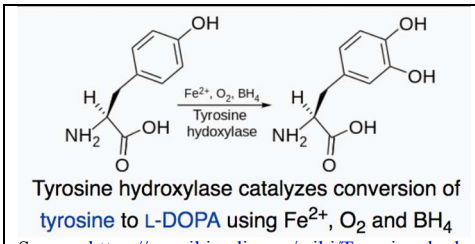
D1 neurons produce copious ROS

In order to hypothesize about the function of melanopsin in dopaminergic neurons, particularly of the striatum and hippocampus, we must first examine some of the basic biochemistry as well as correlated pathological conditions of D1 containing neurons. Dopaminergic neurons in the striatum have been shown to both produce and respond to copious reactive oxygen and reactive nitrogen species (Reviewed by Halliwell, 1992). These highly reactive molecules include

hydrogen peroxide (H_2O_2), Nitric oxide and its oxyradical (NO), superoxide and its oxyradical (O_3), singlet oxygen, among many other oxidizing agents. One of the primary sources of reactive oxygen species in dopaminergic neurons is in the synthesis of dopamine. Tyrosine hydroxylase (TH) is an enzyme found in dopaminergic neurons that catalyzes the reaction of tyrosine to L-DOPA, a precursor of dopamine. TH utilizes a Fenton Chemistry reaction of the general form:

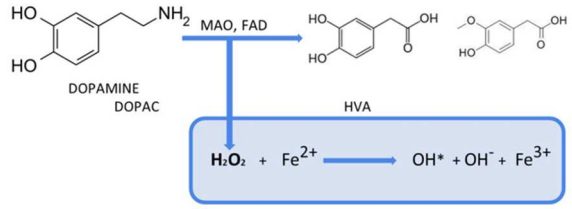
Fenton Reaction: O_2 or $H_2O_2 + Fe^{2+} \rightarrow O^- + O^*$ or $OH^* + OH^- + Fe^{3+}$ (where* signifies a radical)

These highly reactive oxygen intermediates are used in the production of dopamine precursors, but can also leak, progressively generating increased intracellular concentrations of ROS with the production of dopamine. The other enzyme that dopaminergic neurons utilize in the dopamine lifecycle is monoamine oxidase, which similarly generates ROS by a Fenton reaction with intracellular iron. Other sources of ROS generation in dopaminergic neurons include mitochondria. In cell types, such as the dopaminergic neurons of the striatum and hippocampus that are highly active, energy demand is high. Mitochondria produce ATP through oxidative phosphorylation via the electron transport chain (ETC). However, the ETC can become leaky, generating superoxide in the process of ATP production. This occurs increasingly with age. Thus, because dopaminergic neurons, particularly in the striatum and hippocampus, are very active and energy demand is high, as well as that they produce ROS in the life cycle of dopamine production and degredation, dopaminergic neurons are high producers of ROS in the brain.



Tyrosine hydroxylase catalyzes conversion of tyrosine to L-DOPA using Fe^{2+}, O_2 and BH_4

Source: https://en.wikipedia.org/wiki/Tyrosine_hydroxylase



Source: Chahede et al.

Not only are dopaminergic neurons high producers of ROS, but they are also thought to be extremely sensitive to ROS. Production of ROS as a key underpinning of neurodegenerative conditions that involve dopaminergic neuron death, such as Parkinson's was first described after human patients presented with Parkinsonian symptoms after ingesting a compound, MPTP, a contaminant in heroin synthesis, which blocks the mitochondrial permeability pore, resulting in excessive ROS generation (Burns et al., 1985; Liberatore et al., 1999). Since this discovery, MPTP has become a cornerstone in animal models of Parkinson's disease (Smeyne et al., 2005). However, MPTP toxicity likely poisons mitochondria equally across all cells of the CNS, so why it damages dopaminergic cells more so than other cell types remains an open question. This may indicate in increased sensitivity for excessive ROS in dopaminergic cells; perhaps they have a biochemical pathway that allows for dopaminergic neurons to respond differentially to varying levels of ROS that other cell types in the brain lack.

We have seen thus far that melanopsin serves as a G-protein coupled receptor, capable of modulating several ion channels, as well as gap junctions in concert with both D1 and D2

receptor mediated dopamine signaling, as well as controlling the excitability of cells upon stimulation by light. We have also seen that OPN4, the gene that expresses melanopsin, is expressed in the ipRGCs of the retina, where it is light responsive, but also intriguingly it is very highly expressed in dopaminergic regions, such as the striatum, hippocampus, and amygdala and is highly correlated in expression to D1 dopamine receptors (Allen Human Brain Atlas). In order to determine whether melanopsin may have an alternate function outside of light sensing in dopamine rich regions of the brain, we must first dive into the basic function of the melanopsin chromophore in the presence of light, both in ipRGCs as well as in the retinal photoreceptors, rods and cones.

Although the life cycle of the chromophore in photoreceptors versus melanopsin positive cells is different, both utilize the polyene chromophore, retinal, which is derived from Vitamin A (von Lintig, 2000). Bound to the opsin protein, retinal generally exists in one of two forms, as 11-*cis*-retinal or all-*trans*-retinal. Upon absorption of a photon of light of the correct wavelength, which depends on local residues of the opsin, the π electrons of the C_{11}-C_{12} bond are excited. Upon excitation by light, the π electrons undergo a momentary photoelectric effect (Einstein, 1905), or photo-oxidation, however as the minimum amount of energy required to strip retinal of those electrons has generally not been reached by absorption of photons in the visible light range, the unpaired electrons settle momentarily as a C_{11}, C_{12} carbon radical transition state, which allows the C_{11}-C_{12} σ bond to rotate freely, effectively resulting in a photoisomerization between the 11-*cis*-retinal to all-*trans*-retinal (Kubli-Garfias et al., 2015). This photoisomerization between the *cis* and *trans* states of retinal induce a conformational change in the opsin protein, resulting in signal transduction upon absorption of photons of specific wavelengths. This reaction drives vision via photoreceptors as well as the PLR, circadian rhythms and other non-image forming behaviors driven by ipRGCs in the retina.

The life cycle of the chromophore is different in photoreceptors versus in melanopsin positive ipRGCs. Upon photoisomerization by light, all-*trans*-retinal is not able to be converted back to 11-*cis*-retinal in photoreceptors for further phototransduction reactions so it must be transported to a Müller glia cells or the retinal pigmented epithelial cells for conversion back to 11-*cis*-retinal. Because ipRGCs utilize melanopsin rather than rhodopsin or c-opsin, all-*trans*-retinal photoconverts back to 11-*cis*-retinal in the dark. In photoreceptors all-trans-retinal is transported to retinal pigmented epithelial cells by esterification that allows the Schiff base to be transported across membranes. Within the RPE cells, an iron oxegenase, RPE65, catalyzes the oxidative cleavage of, essentially, the all-*trans*-retinoid π bond in carotenoid form, followed by electron rearrangement and dehydration, which restores the 11-*cis*-retinal. This reaction is also thought to be a Fenton reaction, similar to the mechanism involved in the production of dopamine, whereby in the presence of Fe^{2+}, O_2 forms a molecular oxygen radical, which oxidizes the π electrons, resulting in an epoxide transition state. Under favorable molecular conditions, this epoxide is temporary, and the electron rearrangement and dehydration results in isomerization from all-*trans* retinal to 11-*cis*-retinal (Reviewed by Jahng, 2012).

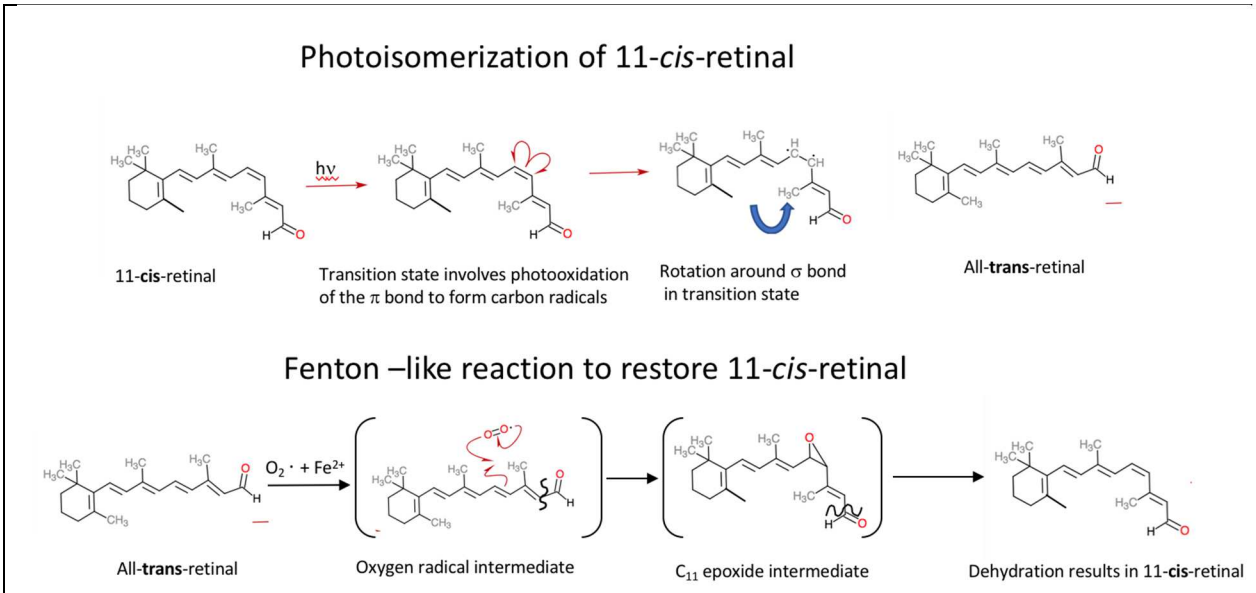


Figure 2.4: Photoisomerization of 11-cis-retinal to All-trans-retinal. Fenton-like reaction that restores 11-cis-retinal occurs in the pigmented epithelium. This reaction underscores the reactivity toward ROS that could occur as a result of oxidative stress within melanopsin positive cells.

Now we have established that the chromophore, retinal, has a particularly reactive π bond between C_{11}-C_{12} which allows for both photo-isomerization as well as Fenton oxidative coupling resulting in isomerization by different cellular processes. Retinoids can be found intracellularly in the form of retinal, retinol, and retinoic acid; All of these have been shown to confer anti-oxidant activity owing to their hydrophobic chain of polyene units, which can quench singlet oxygen, neutralize thiyl radicals, and bind to and stabilize peroxyl radical forms of reactive oxygen species. They have been shown to have a dynamic range of reactive oxygen scavenging activity where at low levels of oxidative tension will auto-oxidize and are thus most aptly suited for neutralizing oxidants at physiological levels of reactive oxygen species concentrations (Hiramatsu et al., 1990, Reviewed by Palace et al., 1999). In cells containing iron, such as dopaminergic neurons and ipRGCs, as well as RPE, it is likely that ROS causes isomerization of various forms of retinal, in particular all-*trans*-retinal to 11-*cis*-retinal. There is some evidence in the literature that similar hydrophobic polyenes, such as fatty acids, undergo cis to trans isomerization upon oxidation by reactive nitrogen species, such as nitrogen dioxide radicals (Balazy et al., 2008). Furthermore, retinol has been observed to exhibit spectral shifts and reduction in extinction coefficient in an O_2 dependent manner, which appeared to be catalyzed by the presence of Fe^{2+}, as in a Fenton reaction, and this spectral change was inhibited by the addition of catalase, an H_2O_2 scavenger, or an oxygen radical scavenger, both of which would disrupt the Fenton oxidation. This is direct evidence that retinoids undergo spontaneous oxidation in the presence of iron and oxidants in a Fenton-like manner involving oxygen radicals, or reactive oxygen species (Fisher et al., 1972). The behavioral ramification of a Fenton or ROS isomerization of the chromophore in melanopsin, proceeding not in the presence or absence of light, but rather, in response to physiological concentrations of reactive oxygen or reactive nitrogen species would be significant.

If true that the alternate function of melanopsin is as a cellular redox sensor, and we imagine that in a state of high oxidative stress, such as a disease state, then by Le Chatlier's principle we might expect either the state of the chromophore to exist predominately in its oxidized isomer form, or that the oxidants may completely reduce the extinction coefficient of the chromophore, as seen in Fisher et al. (1972). Either case would likely result in reduced sensitivity of melanopsin to light. Thus, we would expect to find symptoms of this oxidative stress to include reduced pupillary light reflex or altered circadian rhythms. Indeed, studies have indicated that in Parkinson's disease, which is characterized by excessive production of ROS in the striatum, which spreads to other areas, a significant increase in latency and decrease in amplitude of the maximum pupillary response was seen even in PD patients that were largely asymptomatic (Giza et al., 2011). The authors suggested that the PLR may be used in early detection of PD. However, deficient PLR is not specific to PD. Alzheimer's disease patients have also demonstrated reduced PLR (Tales et al., 2001; Prettyman et al., 1997). Although Schizophrenia (SCZ) is not generally associated with altered parasympathetic response, there is evidence of the contribution of reactive oxygen to the pathology of SCZ (Fendri et al, 2006), as well as indication that the PLR is altered in SCZ, which the authors conclude is not due to an underlying neurodegeneration condition, but represents the 'accelerating aging' of the CNS in SCZ (Granholm et al., 2004). Even in conditions not directly associated with neurodegeneration, such as diabetes mellitus without neuropathy, 78% of diabetic patients showed prolonged pupillary constriction latency PLR, while 39% showed prolonged dilation latency (Marcus et al., 1987). Diabetes is well documented to involve extensive production of reactive oxygen species leading to progression of its pathology (Reviewed by Kaneto et al., 2010). While not technically a disease, the process of aging largely involves a progressive and cumulative increase in endogenous oxidants (Reviewed by Ray et al., 2012). Furthermore, the increase in mitochondrial ROS with age follows a linear correlation (Brawek et al., 2010). Pozzessere et al., (2014) showed a linear correlation between baseline pupil diameter and constriction velocity with age in human subjects. Thus, across many highly unrelated diseases with disparate pathology, two common factors are an early and robust increase in reactive oxygen species production, and deficits in pupillary light reflex, which would be predicted if ROS directly modifies the chromophore driving the light response in ipRGCs.

Nitrate activates PLR in melanopsin dependent manner

There is some evidence that reactive nitrogen species activates or sensitizes melanopsin to light stimulus. First, it has been shown that nitrogen dioxygen radicals (NO_2*) or their precursors, peroxynitrite, nitrous acid, nitrogen trioxide, facilitate the reaction of cis-arachidonic acid to trans-arachidonic acid, which is a polyene of similar saturation as cis-retinal. Thus, it is possible that the species and concentration of oxygen radical can determine the direction of isomerization. Matynia et al. (2016) made several important observations in dissecting the pathway that leads to photoallodynia, or photoaversion, usually in the context of migraine headache. First, they showed that melanopsin is expressed in the trigeminal nerve and induces a light dependent response it the absence of connectivity from either photoreceptors or melanopsin expressing ipRGCS either by optic nerve crush or in TG neuron isolated patch clamp experiments. They also showed that this TG neuron photoresponse was melanopsin dependent, as $OPN4^{-/-}$ TG neurons lacked the light response. Second, they showed that severing the optic nerve conferred a

lack of PLR or photoaversion response, as well as lack of cortical (V1) responses and essentially rendered the mice 'blind.' However, upon administration with nitroglycerin, a compound which releases nitric oxide radicals, and which is thought to contribute to the pathology of migraine headaches, they observed high degree of photoaversion in the presence of nitroglycerin after nerve crush, and this photoaversion was dependent on the presence of OPN4. As OPN4 is not expressed in smooth muscle or endothelial cells, they conclude that nitroglycerin sensitizes OPN4 mediated photoaversion in neurons that are expressed in trigeminal ganglia. These neurons have dendrites that extend to the cornea and lacrimal glands, thus, they may still be light active. If translatable, this work would indicate that other melanopsin expressing neurons, such as those in the striatum, hippocampus, or retina, are sensitized by reactive nitrogen species, perhaps by the all-*trans*-retinal radical isomerization mechanism previously discussed. As it further pertains to the physiology of melanopsin expressing ipRGCs, as well as other OPN4+ cell types, perhaps in the striatum or elsewhere, it has been demonstrated that reactive nitrogen species modulates gap junction coupling via a G-protein coupled/ protein kinase dependent pathway, similar to that elicited by activation of melanopsin (Patel et al., 2006). Taken together, this would implicate reactive oxygen and reactive nitrogen species as potent modulators of melanopsin, particularly in neurons that express melanopsin not exposed to light, such as those of the striatum, and that this modulation could have significant effects on neuronal excitability and downstream pathway activation, such as by modulating gap junction coupling.

ROS, and disease effects on ipRGC mediated Circadian rhythms

ipRGCs project to brain regions known to modulate circadian rhythms, such as the suprachiasmatic nucleus. If reactive oxygen or reactive nitrogen species modulate the activity of melanopsin, or perhaps dysregulate it at high concentrations, then we would predict that pathological phenotypes that are characterized by an increase in ROS production, dysfunctional mitochondria, and activated NADPH oxidase would also impinge on the function of melanopsin, both retinal cells, such as ipRGCS, as well as dark melanopsin cells, such as dopaminergic neurons in the striatum. Furthermore, since ROS overproduction occurs at very early stages pathologically, and may represent a fundamental pathological event common in many diseases, both neurodegenerative and neuropsychiatric as well as systemic, such as cancer and diabetes (Reviewed in Rego et al., 2003; Reviewed in Waris et al., 2006), we would expect that in these conditions there may also be a disruption in circadian rhythms secondary to possible ROS or RNS mediated melanopsin modulation. First, it has been demonstrated that melanopsin (OPN4) is required for diurnal circadian rhythms (Panda et al., 2002). Indeed, several pathological conditions exhibit altered circadian rhythms at very early disease stages that cannot be explained by altered neurotransmission alone. In Alzheimer's disease, altered circadian rhythms proceed at very early stages, likely prior to the onset of neurodegeneration and as the disease progresses atrophy to the suprachiasmatic nucleus is noted. Furthermore, these altered circadian rhythms have been shown to exacerbate deposition of amyloid-β in mouse models (Reviewed by Musiek et al., 2015; Wu et al., 2007). Currently, blue light designed to re-entrain circadian rhythms is under clinical investigation for Alzheimer's disease and mild cognitive impairment (Figeuiro et al. NCT03933696). Parkinson's disease (PD), a neurodegenerative condition that results in the degeneration of tyrosine hydroxylase containing dopaminergic neurons, also presents with

circadian rhythm disruption (CD) in very early stages of the disease, prior to degeneration of motor pathways in PD, and this CD has been shown to exacerbate disease progression in animal models (Lauretti et al., 2017). Huntington's disease patients and mouse models also exhibit altered circadian rhythms, resulting in the modulation of circadian clock genes in the SCN (Morton et al., 2005). While systemic tumors have generally not been documented to accompany altered neurotransmission, both murine models and human cancer patients often display increased ROS production, as well as altered circadian rhythms (Reviewed by Mormont et al., 1997), which are known to both increase the risk of tumorigenesis as well as exacerbate the progression of cancer. It has also been proposed that controlling circadian rhythms may pose a therapeutic intervention in cancer treatment and prevention (Reviewed by Gery et al., 2010). There is also an age induced decline in normal circadian rhythm phase similar to that associated with circadian rhythm deficits in patients with probable Alzheimer's disease (Harper et al., 2005).

Thus far, we have examined both the PLR and circadian rhythm deficits of numerous disparate pathological conditions, many of which do not involve alterations in neurotransmission or parasympathetic responsivity, but do all have in common increased inflammation and ROS/RNS generation, which could be acting directly on the C_{11}-C_{12} isomerization of 11-*cis*-retinal or all-*trans*-retinal such that modulation of melanopsin containing neurons is both an early indication and a common symptom across many diseases involving increased ROS production. This modulation could be concentration dependent, such that physiological concentrations of ROS induce a particular conformation, whereas, high pathological concentrations of ROS render melanopsin insensitive. The species of ROS may also determine the direction of modulation.

Pregnenolone in circadian rhythms, and TrpM3 in the retina

In addition to the myriad ways in which the precursor for all steroid hormones, PregS, peaks during significant periods of development as previously discussed, it has also been shown to be regulated by circadian rhythms. Sjoberg et al (1979) demonstrated that in healthy men levels of PregS peaked in the early morning hours and was strongly correlated to the production of cortisol temporally. PregS has been shown to be synthesized in the retina in retinal ganglion cells, as well as other regions, such as the hippocampus, in an activity-dependent manner (Guarneri et al., 1994; Shibuya et al., 2003). It is possible that if the enzymes required to synthesize PregS are located in ipRGCs, or in the suprachiasmatic nucleus (de Tezanos et al., 1999), and is produced in direct response to light dependent melanopsin activation of ipRGCs. In this dissertation, we demonstrate that the addition of PregS (50uM) to retina preparations during stage III glutamatergic retinal waves, which have been shown to facilitate circuit assembly and refinement during development (Arroyo and Feller, 2016; Huberman et al., 2008; Wong 1999) increases post-synaptic current frequency at both inhibitory and excitatory synapses, and this appears to occur in a presynaptic manner due to the increased frequency but not amplitude of post-synaptic currents. We also show an increase in cellular participation in waves with exogenous PregS, which could be due to increased presynaptic neurotransmitter release during wave events, or it could be due to increased gap junction coupling via a calcium dependent mechanism. We show a prolonged calcium increase in RGCs subsequent to PregS addition, which is TrpM3 dependent as it is lacking in the $TrpM3^{-/-}$ retinas. While we didn't follow the

downstream implications of the TrpM3 dependent prolonged calcium transients in RGCs via PregS, others have shown that TrpM3 activation either by PregS, or a synthetic ligand CIM0216, induces gene transcription of early immediate genes c-Fos, c-Jun, and the transcription factor, AP-1, which regulates cell growth, inflammatory cytokine production, and wnt signaling pathways (Rubil et al., 2016; Qiao et al., 2016). In other cell types, such as the pancreatic cells, PregS also induces rapid influx of calcium, but this TrpM3 dependent calcium signaling induces the production of insulin, demonstrating the crosstalk between circadian rhythm modulated PregS and insulin production (Wagner et al., 2008).

Intentional misalignment of circadian rhythms in both humans and laboratory animals, either by shift work, excess stress, or experimental paradigm results in aberrant steroid hormone regulation, such as highly increased cortisol levels at night, a reversal of normal elevations, and generally increased insulin levels (Scheer et al., 2009). Furthermore, it has been shown that flight attendants who frequently crossed time zones showed cognitive defects and reduced temporal lobe volume (Cho et al., 2001). Several studies of shift workers have revealed increased incidence of cancer, psychological disorders, metabolic syndrome, diabetes, and cardiovascular diseases among these individuals (Woo et al., 2008; Pietroiusti et al., 2010). It is noteworthy that 30-50% of cancers are thought to be hormone receptor dependent. Thus, one mechanism by which many aspects of physiology could be affected by circadian rhythms is that of the PregS/TrpM3 neurosteroid and receptor pair. If PregS is produced in an activity dependent manner, specifically by the activity of ipRGCs either in the retina or SCN or other regions, then this diurnal activity would induce TrpM3 dependent gene transcription of factors that are involved in inflammatory immune responses, cell cycle and cell growth, and many other cellular processes. Additionally, the activity dependent PregS production acts as a precursor to other biologically important hormones, such as cortisol, estrogens and androgens. In the absence of robust light dark cycle to drive activity as well as silencing of ipRGCs, PregS, and thus hormones, gene transcription, and inflammatory factors may continue to be produced, as we see in studies of disrupted circadian rhythms. Pregnenolone signaling, and perhaps also TrpM3 activity mediated gene transcription, may be an important checkpoint in ensuring that hormone signaling, cell-cycle, inflammatory mediators occur in a circadian entrained manner; an imbalance of which results in any of numerous pathological conditions. If we consider our hypothesis that an underlying pathology that generates ROS can desensitize ipRGCs to light conditions, reducing circadian entrainment, this would also have a detrimental effect on PregS concentration in the CNS. Indeed, as previously discussed, reduced PregS is associated with cognitive decline in several conditions, including Alzheimer's disease, Schizophrenia, Autism, pediatric epilepsy, and aging. PregS production, or lack thereof, as a readout of ipRGC activity could pose a feed forward mechanism further exacerbating an underlying condition, resulting in reduced PregS dependent synaptic activity and gene transcription.

Source of ROS in the brain

As we have discussed, ROS represent a common denominator across multiple disease pathologies in their earliest forms, often prior to the onset of symptoms, such as cognitive impairment or motor deficits. Elevated local ROS induces DNA damage which can play an important role in many cancers, and influences cellular processes such as proliferation, apoptosis, and senescence, which all contribute to the onset of disease, including diabetes and

neurodegenerative conditions, as well as the rate of aging process (Waris et al., 2006). Indeed, further indication that ROS are a central cellular process in the development of a disease state is the evidence that ROS increases linearly with age (Kim et al. 1996). In a longitudinal clinical trial assessing the drug metformin, an insulin receptor blocking drug shown to have dramatic effects on reducing cellular oxidation, increasing anti-oxidants, and reducing inflammation, as well as increased longevity in animal studies, one of the clinical outcomes addressed in the trial is whether the time between the onset of the first disease diagnosis and the second disease state is increased (Barzilai et al., 2016). If reducing oxidant load is a mechanism to prevent the onset of any disease following the first diagnosis, this would also point to ROS as a potent mediator of the switch from healthy cellular state to that of a diseased state. Causes of increased ROS in different cell types can be multifactorial, however, several studies indicate that psychological stress potently increases ROS in the brain. One study showed that repeated restraint stress decreased the expression of ROS scavengers, such as superoxide dismutase, catalase, and glutathione, and this decrease in ROS scavengers was mimicked by injection of corticosterone (Zafir et al., 2009). Furthermore, Jenkins et al (Reviewed 2014) argued that production of corticosteroids activates the b-adrenergic receptor, which then induces the production of ROS, or lack of scavengers, and this leads to DNA damage resulting in increased cancer risk. In addition to the increase in cortisol with acute stress, in humans the neurosteroids DHEA-S (Morgan et al., 2004) and PregS (Sabeti et al., 2007) are also increased in response to acute psychological stress. It has been proposed that the increase in PregS is an important factor in the increase in hippocampal synaptic plasticity and memory for traumatic events (Sabeti et al., 2007). Interestingly, PregS ameliorates the conditioned fear response in rodents (Noda et al., 2000) so the ratio of converted PregS to corticosteroid may represent a biochemical threshold between an optimal stress for growth purposes (ie synaptic plasticity) versus stress that causes cellular damage and conversion to disease state. The increase in psychological stress related PregS production, which is either converted to corticosteroid to further capitulate the stress response, or converted to other hormones, or utilized as PregS as a neurosteroid as described in this dissertation, increases ROS production via the β-adrenergic receptor or other mechanisms such as mitochondrial dysfunction or NADPH oxidase activity, represents a fundamental mechanism for the formation of numerous disease states. In addition to ROS generation, the β-adrenergic receptor may represent a disease node in which its expression is highly correlated to the expression of many other disease relevant genes, such as TREM-2, APBB1, APBB2, BACE-1 (AD), PSENEN (PD), BRCA1 (breast cancer) among several others (Allen Human Brain Atlas correlates dataset). The ensuing ROS generation may also act on melanopsin by the previously proposed mechanism of desensitizing melanopsin to light, thereby destabilizing circadian rhythms, which further misaligns the production of PregS, and its conversion to Cortisol, estrogens or androgens, and further increases the tendency toward cellular damage.

In summary: Oxidation, or oxygen radicals, flow analogously to a river, activating many branch points following the original source. Under optimal flux of radicals, the oxidation state performs in perfect harmony and balance with all the downstream branches. However, upon excessive activation by a stressor, particularly a traumatic event(s), the β-adrenergic receptor, which likely serves as a checkpoint from which many other inflammatory conditions, leading to chronic neurodegenerative conditions, cancer, and numerous other diseases from which people suffer.

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Chapter 3: eNeuro Manuscript

Steroid-sensitive TrpM3 channels modulate spontaneous activity in the developing retina

Abstract:

Melastatin transient receptor potential (TrpM) channels are both heat sensitive and, in the central nervous system, function as receptors for the neurosteroid pregnenalone sulfate (PregS). The expression and function of TrpM3 has been explored in adult retina, though its role during development is unknown. We found that during the second postnatal week, TRPM3 immunofluorescence labeled distinct subsets of inner retinal neurons, including a subset of retinal ganglion cells (RGCs), similar to what has been reported in the adult (Brown et al., 2015). Labeling for a TRPM3 promoter-driven reporter confirmed expression of the TRPM3 gene in RGCs and revealed additional expression by nearly all Müller glial cells. Voltage clamp recordings from developing RGCs reveal that PregS induced a robust increase in the frequency but not amplitude of spontaneous synaptic events, similar to what was observed at glutamatergic synapses onto Purkinje cells (Zamudio-Bulcock and Valenzuela, 2011). Using two-photon calcium imaging, we show that PregS induced a prolonged calcium transient in a subset of RGCs. These PregS-induced prolonged calcium transients were absent in TrpM3 knockout mice and were also induced by application of the synthetic TrpM3 agonist CIM0216. Moreover, we observed that PregS modulated retinal wave activity, inducing an increase in the percentage of cells that participate in waves without altering wave frequency. Together, these results indicate that functional TrpM3 channels are present in circuits that impact spontaneous activity during retinal development.

Significance statement:

TrpM3 is a heat sensitive ion channel found throughout the CNS, and has been shown to function as a receptor for the neurosteroid, Pregnenolone Sulfate. The role of TrpM3 during development has been characterized in the hippocampus and cerebellum, but not previously in the developing retina. We show that in the developing retina, TrpM3 responds to PregS by producing prolonged calcium transients in a subset of retinal ganglion cells, increases the number of retinal ganglion cells that participate in spontaneous retinal waves, and increases the frequency of spontaneous synaptic current onto retinal ganglion cells. Thus, we show that TrpM3 and the endogenous neurosteroid, PregS, function in modulating spontaneous activity in the retina during development.

Introduction:

Melastatin transient receptor potential (TRPM) channels are polymodal cation conductances that are found throughout the body, including the peripheral nervous system where they play a key role in heat and pain sensation (Vriens et al., 2011) ((Vriens and Voets, 2018). TrpM3 is a cation

channel with a high permeability to calcium (Grimm et al., 2003; Oberwinkler and Philipp, 2014), with the extent of permeability dependent on which splice isoforms are expressed (Oberwinkler et al., 2005). In addition to being activated by heat, TRPM3 is strongly activated by the neurosteroid pregnenolone sulfate (PregS) (Wagner et al., 2008) as well as the synthetic small molecule CIM0216 (Held et al., 2015a). Activation of TrpM3 via PregS or CIM leads to a dramatic increase in intracellular calcium, which in turn strongly activates signaling cascades that alter gene transcription (Thiel et al., 2017).

The role of TrpM3 in the central nervous system is less well-explored (Thiel et al., 2017). TrpM3 is present in the adult mouse retina, where it is expressed by retinal ganglion cells as well as cells in the inner nuclear layer (Brown et al., 2015), including Müller glial cells (Gilliam and Wensel, 2011)((portals.broadinstitute.org/single_cell/study/SCP3/retinal-bipolar-neuron-drop-seq). TrpM3 knockout (KO) mice have normal retinal structure and electroretinogram (ERG) responses but an attenuated pupillary light reflex (Hughes et al., 2012), suggesting a role for TrpM3, either directly or indirectly, in modulating retinal ganglion cell activity but not that of photoreceptors. Interestingly, TrpM3 may play a critical role during development. For example, in the developing cerebellum, TrpM3 is present at glutamatergic synapses, and activation of TrpM3 by PregS potentiates synaptic transmission (Zamudio-Bulcock and Valenzuela, 2011), suggesting a functional role in spontaneous activity of developing circuits.

Though the endogenous ligands for TrpM3 are not yet fully determined (Thiel et al., 2017), the neurosteroid pregnenolone sulfate (PregS) is a strong candidate (Wagner et al., 2008). PregS is synthesized within the nervous system (Kimoto et al., 2001; Harteneck, 2013), including the retina (Cascio et al., 2015). Moreover, significantly lower concentrations of PregS are needed to activate TrpM3 in elevated temperatures (Vriens et al., 2011), indicating a synergism between ligand and temperature sensing that has been described in other channels such as TrpV1 (Neelands et al., 2008). In addition to activating TrpM3, PregS has excitatory effects on neural circuits by a variety of other mechanisms (Harteneck, 2013), including as a negative allosteric modulator of GABA-A receptors, as a positive allosteric modulator of NMDA receptors, and through interactions with AMPA, kainate (Yaghoubi et al., 1998), glycine (Wu et al., 1997) and nACh receptors (Smith et al., 2014). PregS-induced depolarization of isolated RGCs is absent in the TRPM3 KO (Brown et al., 2015), indicating that PregS is a specific agonist of TRPM3 in the retina. However, the impacts of PregS on intact retinal circuits are unknown.

Here we use the mouse retina to explore a role for PregS and TrpM3 signaling during development. Prior to eye opening and the maturation of vision, the retina exhibits retinal waves, a term used to describe spontaneously generated, correlated bursts of action potentials that spread across the retinal ganglion and inner nuclear cell layers (Wong, 1999; Blankenship and Feller, 2010). Retinal waves drive propagating neural activity in the superior colliculus and the visual cortex, where they play a key role in the formation of retinotopic maps and ocular dominance columns (Huberman et al., 2008; Ackman and Crair, 2014; Arroyo and Feller, 2016). We use immunohistochemistry, two photon calcium imaging, and whole cell voltage clamp recordings to determine the impact of TrpM3 agonists and genetic deletion on spontaneous synaptic signaling as well as on retinal waves.

Materials and Methods

Mice:

All experiments were performed on mice aged postnatal day P8-P15 of either sex from C57BL/6 WT (Harlan Laboratories) or TrpM3 KO (Vriens et al., 2011) (NIH-1697: LexKO 380). Animal procedures were approved by the Institutional Animal Care and Use Committees and conformed to the National Institutes of Health *Guide for the care and use of laboratory animals*, the Public Health Service Policy, and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

Immunohistochemistry

Animals were deeply anesthetized with isoflurane and decapitated. Eyes from mice aged postnatal day 8 (P8) to P15 were dissected in freshly made ACSF at room temperature by cutting just behind the ora serrata, then removing the lens. Eyecups were fixed for 20 minutes in ice cold 4% PFA in 0.1 M phosphate buffer, pH 7.4 (PB). The eyecups were washed in PB, then sunk in sequentially increasing concentrations of sucrose in PB (10%, 20%, 30% w/v) and immersed in 30% sucrose solution overnight at 4°C. The tissue was cryoembedded in OCT tissue-embedding compound over dry ice and methanol, and then sectioned perpendicular to the eye cup in 16 µm sections using a Cryostat. Sections were mounted on Super-Frost glass slides, air-dried, and then stored at -80°C or used immediately for staining.

Several antibodies were used. First, rabbit antiserum against amino acids 1543-1672 of mouse TrpM3 (accession AEE80504) was produced as previously described (Brown et al., 2015). For the anti-TrpM3 and anti-calretinin double labeled sections, a 1:200 dilution of affinity purified anti-TrpM3 and (1:20 dilution) of mouse monoclonal anti-calretinin (sc-365956; Santa Cruz Biotechnology, Dallas, TX) were used, followed by a 1:2000 dilution of anti-Mouse Alexa 488 and anti-Rabbit Alexa 594 secondary antibodies. Second, for the EAAT1 and LacZ double labeled sections (Figure 1B), rabbit anti-EAAT1 (Abcam ab416) 1:500 dilution and chicken anti-B galactosidase (Abcam 9361) 1:300 in 0.5% donkey serum, 0.5% Triton X-100 and 0.2% sodium azide in PBS were used as primary stain overnight at 4°C. Finally, Rabbit anti-RBPMS 1:500 (Phospho Solutions, #1830) and chicken anti-B galactosidase 1:300 were used for the RBPMS and LacZ double labeling, followed by anti-rabbit Alexa 594 and anti-chicken Alexa 488 secondary antibodies.

The TrpM3 and calretinin double labeled sections were imaged on a Leica TCS SP8 X confocal microscope using a 63X/1.40NA oil immersion objective. Staining intensity versus distance between the outer plexiform layer (OPL) and inner limiting membrane (ILM) was conducted using the image-processing software, FIJI. Six 10 µm by 70-80 µm rectangular ROIs, oriented such that the length spanned the region of intensity between the OPL and ILM, were placed in the field randomly. The ROI Manager and Plot Profile functions were used to generate average fluorescence intensity values as a function of time. Custom scripts in Python were used to normalize and graph the data. Sections for EAAT1/Lac Z double label and RBPMS, LacZ double label were imaged on a Zeiss 780 AxioExaminer confocal microscope with either a 40X/1.4NA oil immersion or 63X/1.4NA oil immersion objective. Images were adjusted for brightness and contrast and pseudocolored using Leica software and FIJI (NIH ImageJ).

Acutely isolated retinas

Animals were deeply anesthetized with isoflurane and decapitated. After enucleation of the eyes, retinas were dissected in oxygenated (95% O_2/ 5% CO_2) ACSF (in mM, 119 NaCl, 2.5 KCl, 1.3

$MgCl_2$, 1 K_2HPO_4, 26.2 $NaHCO_3$, 11 D-glucose, and 2.5 $CaCl_2$) at room-temperature under infrared illumination. Isolated retinas were mounted RGC side up on nitrocellulose filter paper (Millipore, Billerica, MA), and transferred to a recording chamber of an upright microscope for imaging and simultaneous electrophysiological recording. The whole-mount retinas were continuously perfused (3 ml/min) with oxygenated ACSF at 32-34°C for the duration of the experiment.

Two-photon calcium imaging.
Retinas were bolus loaded with Cal-520 AM (AAT Bioquest, Sunnyvale, CA) using the multicell bolus loading technique (Stosiek et al., 2003) as previously described (Blankenship et al. 2009). Cal-520 was prepared at a concentration of 906.6μM in 20% Pluronic in DMSO solution, which was then diluted 1:10 in ACSF, pH 7.4, and sonicated and filtered at 0.45 μm to remove particulates. Boroscilicate glass micropipettes (Sutter Instruments) were pulled to an approximate lumen of 1-2 μm (Narishige, PC-10) and used for pressure injection of the dye (World Precision Instruments, PV-820 Pneumatic PicoPump) at a pressure of 10-20 psi with the pipette positioned just under the inner limiting membrane. Two-photon calcium imaging of neurons in the retinal ganglion cell layer was performed on a custom built two-photon microscope with an ultrafast pulsed laser (Chameleon Ultra, Coherent, Santa Clara, CA) tuned to 920 nm using a 60x objective (Olympus 60X, 1 NA, LUMPlanFLN). The microscope was controlled by ScanImage software (version 3.8, www.scanimage.org). Scan parameters were: 128 × 128 (6 Hz), or 256 × 256 (3 Hz), at 1 ms/line. 10-minute imaging epochs were used and retinas were maintained at 33°C for the duration of the experiment.

Image Processing
Image stacks were imported into FIJI (Schindelin et al. 2012) and ROIs were manually identified by selecting RGCs from the average intensity across the entire movie. A custom python script was used to calculate $\Delta F/F_0$, using the first frame that did not have a calcium transient as F_0. Following a median filter between 3 adjacent frames, calcium transients were classified as prolonged if they exhibited integrated responses that were above a threshold defined by the integrated responses during control waves.

Electrophysiological Recordings

Whole cell voltage clamp recordings were made from hemisectioned retinas continuously superfused in oxygenated ACSF (32-34°C) at a rate of 2-4 ml/min. Retinas were visualized under infrared illumination (870 nm). The inner limiting membrane was removed using a glass recording pipette. Borosilicate glass recording pipettes (Sutter Instruments) were pulled (PP-830; Narishige) with tip resistance of 3-6 MOhm and filled with cesium gluconate internal solution containing (in mM): CsMeSO4 110, NaCl 2.8, HEPES 20, EGTA 4, TEA-Cl 5, ATP-Mg 4, GTP-Na3 0.3, Phosphocreatine-Na2 10, QX-314 5, pH adjusted to 7.2 and with an osmolarity of 290 mOsm/kg H_2O. The liquid junction potential correction for this solution was -13 mV. Voltage-clamp recordings were obtained from somas of retinal ganglion cells (holding potential of -60 mV) as 10 minute gap free recording using pCLAMP10 recording software and a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA), sampled at 20 kHz and low-pass filtered at 2 kHz.

Pharmacology

For pharmacology experiments, imaging and/or electrophysiology recording was conducted under baseline conditions for at least 10 minutes prior to application of pharmacological agents. Pharmacological agents were added to oxygenated ACSF, maintained at 33°C, and bath perfused onto the retina for 2.5 minutes following the start of imaging or recording to obtain both stable baseline and change due to the agent in the same imaging/recording epoch. Pharmacological agents were used in the following concentrations: Pregnenolone sulfate 50 μM (Sigma-Aldrich, St. Louis, MO), Mefenamic acid 50 μM (Sigma-Aldrich), CIM0216 10-50 μM (Tocris,Minneapolis, MN).

RESULTS

TrpM3 in the developing retina is localized to inner retinal processes and RGCs

In the adult retina, TrpM3 is localized to processes in the inner plexiform layer (IPL), a subset of retinal ganglion cell somas, and potentially other cells including displaced amacrine cells (Brown et al., 2015). TRPM3 mRNA and TRPM3 promoter-driven expression of a reporter has been demonstrated in Müller glia (Gilliam and Wensel, 2011; Hughes *et al.*, 2012). To characterize the localization of TrpM3 during development, we used two approaches. First, we used a custom antibody targeted against the C-terminus of mouse TrpM3 (Brown et al., 2015). To demarcate synaptic layers in the developing IPL, we co-stained for calretinin, a calcium binding protein localized to a subset of amacrine and ganglion cells whose processes define IPL sublamina right after eye-opening (Figure 1). From P9 to P15 we observed punctate TRPM3 immunofluorescence throughout the IPL as well as labeling of RGCs, many of which were also positive for calretinin (Figure 1A). Faint labeling was seen over some inner retinal neurons. TRPM3 immunofluorescence increased between P9 and P15, with the labeling at P13 closely resembling that of the adult (Brown et al., 2015).

Second, we used a TrpM3 KO mouse, which contains β-galactosidase (β-Gal) in the place of TrpM3 coding sequence (Hughes et al., 2012) (Figure 1B). Similar to studies in the adult retina, we observed high levels of β-Gal in the IPL, and flanking RGCs, consistent with Müller glia end feet and lateral processes, as well as some β-Gal in RGCs. We observed co-localization of β-Gal with EAAT1 immunofluorescence, which labels a glial glutamate transporter, consistent with strong expression in Müller glial cells. In addition, somatic staining for β-Gal in the GCL co-localized with the RGC marker RBPMS. Together, these data indicate that, during development, TrpM3 is expressed in developing RGCs, Müller cells, and potentially other INL neurons.

PregS increases the frequency of spontaneous synaptic events in RGCs

Activation of TrpM3 increases the frequency of spontaneous synaptic events recorded in Purkinje cells in the developing cerebellum (Zamudio-Bulcock and Valenzuela, 2011). To determine whether activation of TrpM3 similarly impacts synaptic transmission in the retina, we performed whole cell voltage clamp recordings from RGCs wherein we compared the frequency and amplitude of spontaneous synaptic events. Addition of PregS increased the frequency (control: 5.8 +/- 2.8 Hz,; PregS: 16.79 +/- 8.8 Hz), but not the amplitude of sEPSCs (control: 10.8 +/- 3.2pA; PregS: 10.8 +/- 3.1 pA, n=8 cells from 6 mice) (Figure 2A,C,E). A similar increase in frequency (control: 16.6 +/- 3.8 Hz ; PregS: 34 +/- 5.4 Hz) but not amplitude (control: 18.4 +/- 10.5pA; PregS: 18.1 +/- 8.1 pA, n=8 cells from 6 mice) following addition of PregS was observed from sIPSC currents (Figure 2B,D,F). Taken together, the electrophysiology data point

to a presynaptic mechanism of action of TrpM3. This increase in frequency was absent when the the TrpM3 antagonist Mefenamic acid (50 μM) was applied with PregS for both sEPSCs (6.5 +/- 5.53, n = 8 from 6 mice) and sIPSCs (19.8 +/- 4.8Hz, n = 7 from 6 mice), with no effect on amplitude (sEPSC, 9.1 +/- 3.0 pA, n= 8; sIPSC, 13.4 +/- 5.3 pA, n = 8 from 6 mice). Hence, TrpM3 alters neurotransmitter release properties presynaptic to RGCs.

Activation of TrpM3 induced prolonged calcium transients but does not significantly alter retinal wave frequency.

As the retina develops, retinal waves are mediated by different retinal circuits (Blankenship and Feller, 2010). Given the strong expression of TrpM3 during the second postnatal week (Figure 1) and the impact on glutamatergic synapses (Figure 2), we focused on retinal activity during glutamatergic waves, which occur between P11 and P15.

To determine the impact of TrpM3 activation on the retina, we bath applied TrpM3 agonists and measured the responses using 2-photon calcium imaging of the ganglion cell layer of retinas isolated from P11-12 mice loaded with the organic calcium indicator Cal-520 (Figure 3). Addition of the neurosteroid PregS (50 μM) induced large and prolonged calcium transients in a subset of RGCs (Figures 3A & B, 46.2 +/- 30.1% cells undergo prolonged transients, n = 6 experiments from 4 retinas). RGCs with large transients were evenly distributed throughout the field of view (Figure 3A). Note, these prolonged transients were not synchronized across the field of view as one might expect if they were due to direct activation of RGCs. Rather in a subset of cells, the prolonged transients occurred simultaneous with waves, indicating a synergistic interaction (Figure 3A).

We repeated the experiments in TrpM3 KO retinas and found that PregS did not induce prolonged calcium transients (Figure 2C, 3.8 +/- 5.1 % cells, n = 7 experiments from 4 retinas). Moreover, bath application of the TrpM3 synthetic ligand CIM in wild type retina induced similar prolonged transients, however in a significantly higher percentage of cells (Figure 2D, 88.8 ± 7.0 %, n=6 experiments from 4 retinas). CIM is highly selective for TrpM3, but is thought to activate a different conductance state (Held et al., 2015a), which could potentially explain the stronger effect on RGCs (Figure 2E). Together these data indicate that the PregS-induced prolonged transients were via activation of TrpM3 channels.

Next, we monitored the effect of PregS on several features of retinal waves (Figure 4). Addition of PregS did not significantly change the frequency of retinal waves or the amplitude of the calcium transients per wave. PregS increased the total percent of RGCs that participate in waves in WT retinas. We observed a similar trend in TrpM3 KO mice but it was not significant; however, cell participation was high in control conditions in TrM3 KO mice, possibly indicating a ceiling effect. No waves were observed in CIM (n=6 experiments, from 4 retinas), and hence quantifying wave properties was not possible under these conditions.

To test whether endogenous activation of TrpM3 modulated retinal waves, we measured the effect of the TrpM3 antagonist, Mefenamic acid (Mef, 50 μM, Zamudio-Bulcock and Valenzuela, 2011) on wave dependent calcium transients (Fig 4A, right). The presence of Mef dramatically decreased the number of cells that participated in waves, effectively blocking all wave-like activity. However, the blockade of wave-induced transients by Mef persisted in TrpM3 KO indicating there is a TrpM3 independent effect of Mef, in addition to antagonizing

TrpM3. In the patch-clamp experiments (Figure 2), Mef application with PregS did not impact the frequency or amplitude of EPSCs and IPSCs onto RGCs compared to control (see Figure 4), indicating that this off-target effect may be due to modulation of voltage-gated calcium channels (Teramoto et al., 2005) which mediate the calcium response.

DISCUSSION

We have demonstrated that the multimodally regulated ion channel TrpM3 modulates spontaneous activity in the developing retina. First, we show that TrpM3 is present in RGCs during development and show evidence for additional expression by Müller glia. Second, using two-photon calcium imaging, we show that addition of the TrpM3 agonist, PregS causes a prolonged increase in intracellular calcium concentration triggered by retinal waves, a phenomenon that is absent in the TrpM3 KO and mimicked by the TrpM3 synthetic agonist, CIM. Finally, using whole cell voltage clamp recordings from RGCs, we found that addition of PregS increased the frequency of spontaneous EPSCs and IPSCs, indicating that activation of TrpM3 alters signaling presynaptic to RGCs. These data imply that TrpM3 and its ligand, the neurosteroid, PregS, modulate spontaneous synaptic activity during a critical time of development.

TrpM3 affects retinal signaling via postsynaptic mechanisms

In the adult retina, TrpM3 is expressed in a distinct subset of RGCs as determined by antibody staining (Brown et al., 2015), reporter expression (Hughes et al., 2012), and functional assays on dissociated RGCs (Brown et al., 2015). Interestingly, TrpM3 KO mice exhibit an altered pupillary light reflex (Hughes et al., 2012), a light-evoked pupil constriction mediated by intrinsically photosensitive retinal ganglion cells. Though the pupil constriction in response to light stimulation is rapid and maintained throughout the exposure to light, the pupils did not reach a full constriction and had a significantly attenuated post-stimulus response – both phenotypes that are reminiscent of mice lacking melanopsin. A TrpM3 promoter-driven reporter was not found to co-localize with melanopsin containing intrinsically photosensitive RGCS (ipRGCs) (Hughes et al., 2012) indicating its impact on PLR may be indirect, perhaps via TrpM3 expression in glial cells or within the ciliary body (see below).

We provide several lines of evidence indicating that TrpM3 is present in RGCs during development. First, antibody staining indicates a subpopulation of cells in the ganglion cell layer express TrpM3, similar to the pattern in adult. Second, visualization of cells in TrpM3+/- mice, in which a β-Gal cassette replaces the coding sequence of TrpM3 (Hughes et al., 2012), replicates the staining pattern in the GCL that we observed with the antibody. Third, application of the TrpM3 agonist, PregS, causes prolonged calcium transients associated with retinal waves, an effect absent in the TrpM3 KO.

We observed an interesting synergism between activation of TrpM3 via PregS and retinal waves. Bath application of PregS did not cause a synchronous depolarization of all RGCs, but rather appeared to cause a prolonged depolarization when a wave co-occurred, with different cells being affected by different waves. We speculate that this synergism may be due to the multiple

sites of activation/modulation of TrpM3 channels (Held et al., 2015b; Thiel et al., 2017). For example, waves provide a strong depolarization and/or an influx of calcium that could facilitate the opening of the channel in the presence of PregS. Insights into the molecular mechanisms underlying the source of this synergism would require a deeper understanding of the channel. However, these data indicate that the presence of neuronal hormones can have a dramatic effect on the amplitude and duration of calcium transients in developing neurons.

TrpM3 affects retinal signaling via presynaptic mechanisms and/or glial signaling

TrpM3 is not confined to RGCs—instead, there is also extensive immunolabeling in the inner nuclear layer (Brown et al., 2015), as well as expression in Müller glia (Gilliam and Wensel, 2011)(. We found a similar staining pattern in the developing retina. Moreover, we demonstrated that the TrpM3 reporter, β-gal, co-localizes with EAAT, a marker for Müller glial cells, indicating strong expression in Müller cells during development.

The function of INL/glial TrpM3 in the adult retina is not known. TrpM3 KO mice have normal ERGs, indicating normal function of the outer retina (Brown et al., 2015). During development, we observed that TrpM3 agonist PregS increased the frequency of spontaneous IPSCs and EPSCs recorded from RGCs, suggesting there was an increase in excitability presynaptic to RGCs. A similar TrpM3-associated increase in EPSC frequency was observed in the developing cerebellum (Zamudio-Bulcock and Valenzuela, 2011) and the adult hippocampus (Mameli et al., 2005) consistent with a presynaptic site of action. Whether the increased I/EPSC frequency in retina occurs directly via activation of bipolar cells or indirectly via Müller glia remains to be determined.

A third possibility is that the strong depolarization of some RGCs is facilitating a retrograde influence on presynaptic release by a diffuse messenger, such as NO or reactive oxygen species. Such retrograde signaling has been implicated in retinal development (Du et al., 2009; Johnson and Kerschensteiner, 2014).

Implication for TrpM3 signaling in neural circuits

PregS has been implicated in a variety of physiological roles in the adult hippocamous. For example, PregS has been shown to modulate hippocampal plasticity, increasing synaptic activity in an age dependent (Mameli et al., 2005), and stress-dependent manner (Flood et al., 1992; Barbaccia et al., 1996) . In other studies, intracranial PregS injection has been shown to increase the survival of newborn neurons in the dentate gyrus (Mayo et al., 2005; Yang et al., 2011). Although it is not yet known whether these PregS dependent phenomena occurs via TrpM3 or other actions, a truncation mutation of TrpM3, coupled to mutation of the *dmd* gene, frequently associated with muscular dystrophy, is associated with autism spectrum disorder (Pagnamenta et al., 2011). Moreover, PregS has the highest expression of all hormones in amniotic fluid (Bicíková et al., 2002), opening the possibility that TrpM3 signaling via PregS may be important for brain development. Taken together, we demonstrate that the neurosteroid, PregS, acting via TrpM3 modulates spontaneous firing in the developing retina during a critical stage in development.

Figures:

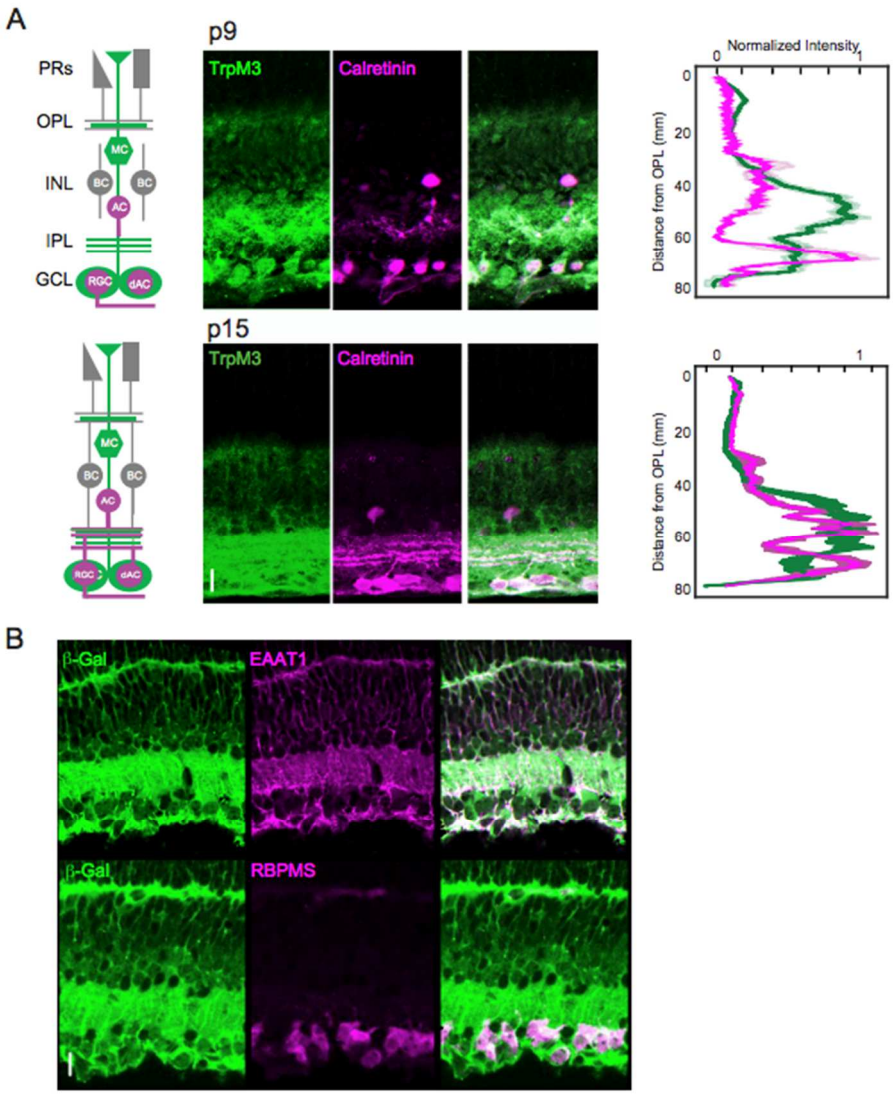

Figure 3.1. TrpM3 expression in postnatal retina A. Left: Schematic of retinal circuit at P9 and P15. Middle: Immunofluorescence for TrpM3 and calretinin at two developmental ages. Calretinin is present in amacrine cells, displaced amacrine cells, and some retinal ganglion cells. Right: Image intensity as a function of distance along the cross-section from the OPL for both TrpM3 (green) and calretinin (magenta). Bold lines are the average intensity from 6 ROIs and the light regions represent standard deviation. Scale bar is 20µm. B. TrpM3 co-localizes with Müller glia and RGCs. P12 TrpM3+/- retinas containing a β-Gal cassette in place of exon 17 of the coding sequence of TrpM3 were used to identify cells that express TrpM3 by immunofluorescence. Top: β-Gal co-localizes with EAAT1, a marker for Müller glia. Bottom: β-Gal is also present in the RGC layer, co-localizing with the RGC label, RBPMS.

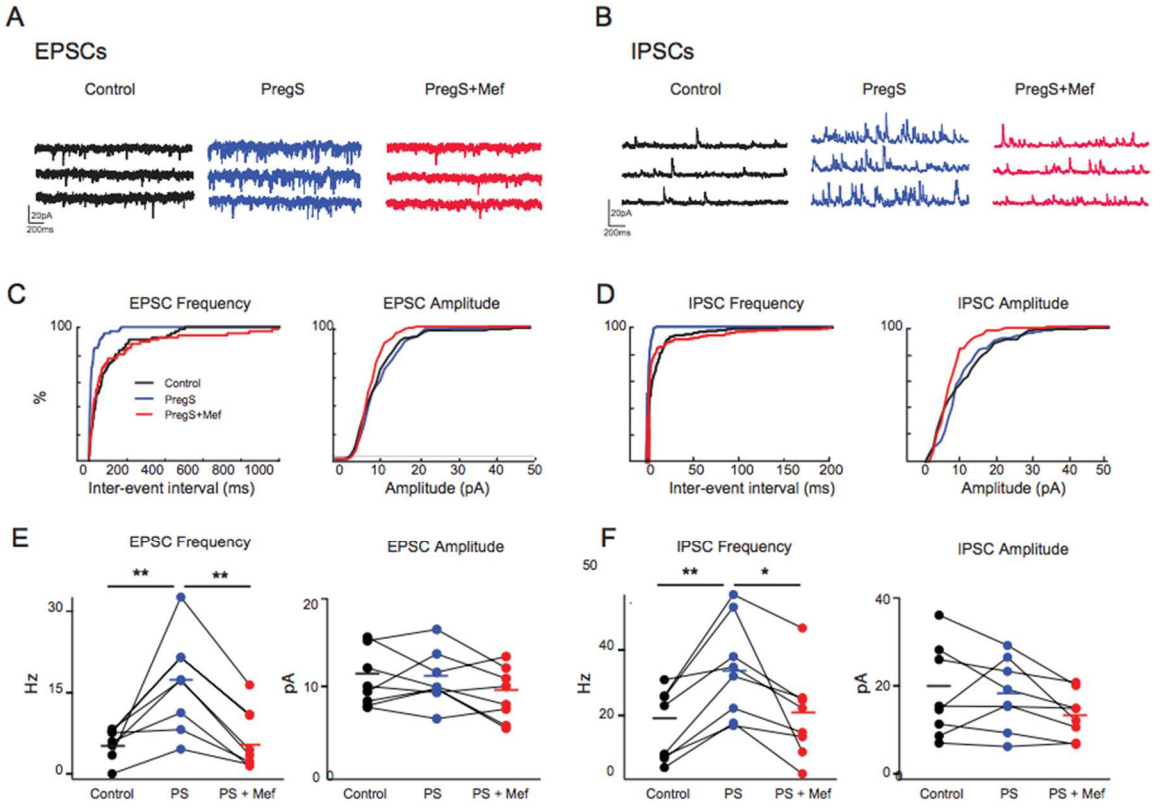

Figure 3.2: Addition of PregS increased the frequency but not amplitude of sEPSC and sIPSCs onto RGCs. A. Example spontaneous excitatory postsynaptic currents (sEPSC) recorded from a RGC in control conditions (left), in the presence of 50µM PregS (Center) and in the presence of PregS and 50µM Mef (Right). B. Same as (A) but holding the cell at 0 mV to isolate spontaneous inhibitory postsynaptic currents (sIPSCs) C/D. Cumulative probability distribution of inter-event interval (left) and amplitude of sEPSCs (C) and sIPSCs (D). Black – control conditions, blue = PregS, red = PregS and Mef E/F. (E) EPSC and (F) IPSC summary plots summarize the effects of PregS and Mef on frequency (left) and amplitude (right) of PSCs (n = 8 RGCs in each condition, grey circles and error bars are mean +/- SD. ANOVA with Tukey HSD, ** $p < 0.01$, * $p < 0.05$.

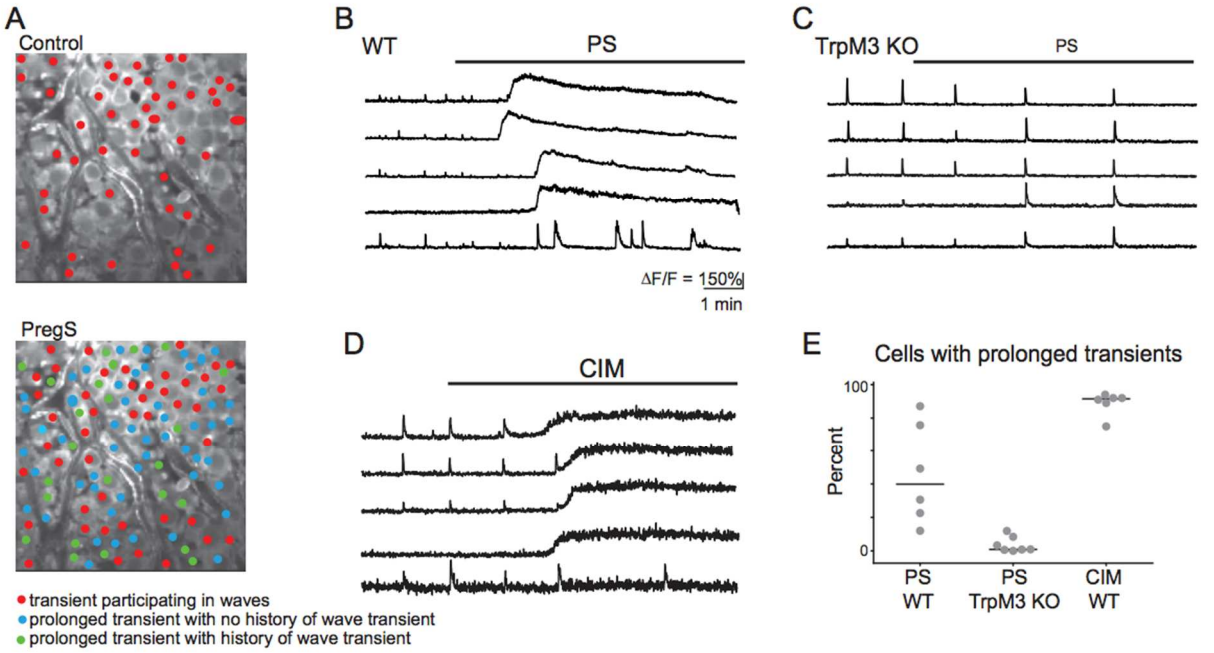

Figure 3.3. Activation of TrpM3 induces prolonged calcium transients in retinal ganglion cells A. XY plane of ganglion cell layer of two-photon fluorescence image of retina loaded with Cal520. Red dots: neurons that participate in retinal waves in control conditions; blue dots: neurons that exhibit prolonged calcium transient in response to PregS (50μM) added to the bath. B-D. Sample ΔF/F traces from representative cells in the image field of view for WT retinas exposed to PregS (B), TrpM3 mice exposed to PregS in the bath (C), and WT mice exposed to synthetic ligand CIM (D, 10 μM) in the bath. E. Average percent of cells within field of view that exhibited prolonged calcium transients in three conditions. Each circle represents one FOV, bars are average. WT PregS: 46+/- 30%, n = 6; TrpM3 KO Pregs: 3.4 +/- 5.7%, n = 7; WT CIM: 88.8 +/- 6.9%. One-way ANOVA F = 23.8 (p = 4.5e-5), Tukey HSD p values are: PregS WT vs TrpM3 KO p = 0.012, PregS WT vs CIM WT p = 0.006, PregS TrpM3 KO vs WT CIM p = 0.001.

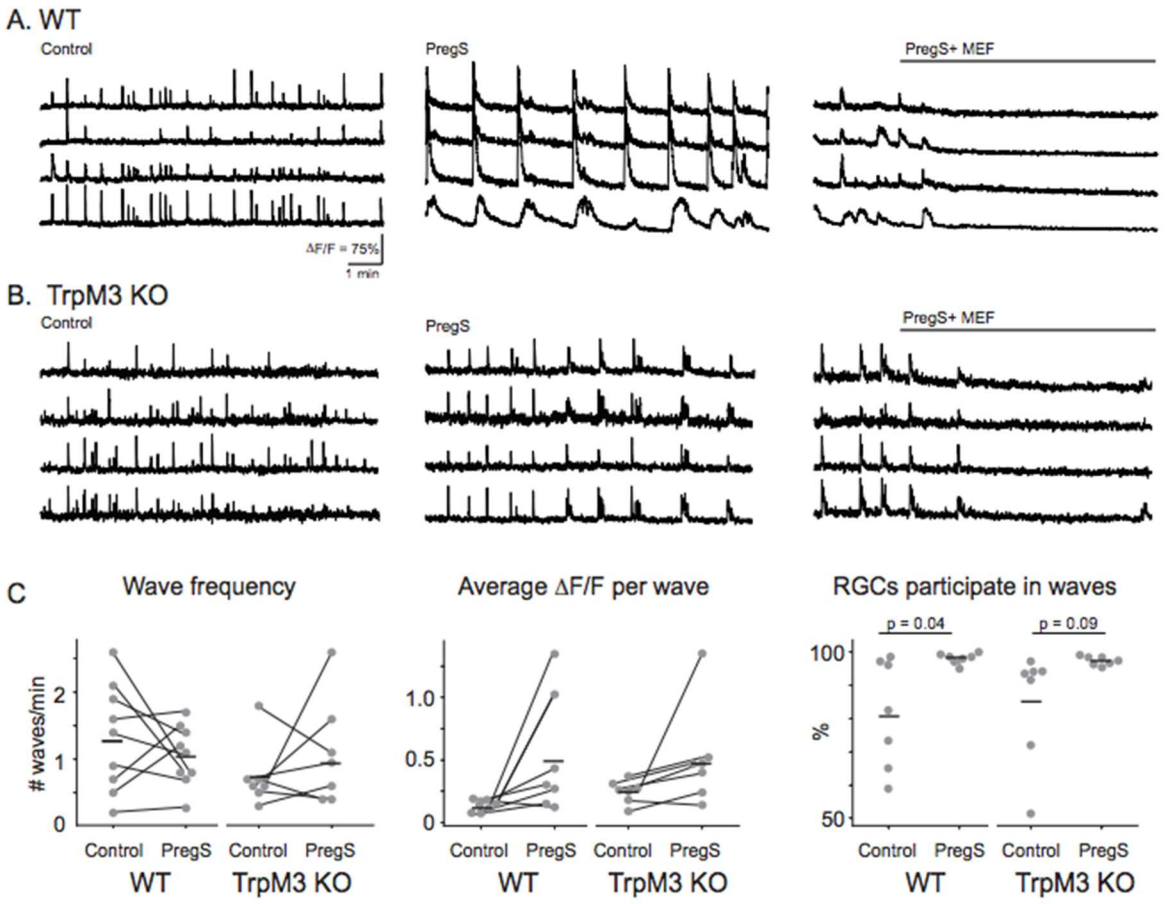

Figure 3.4. Impact of TrpM3 activation on retinal waves A. Sample ΔF/F traces from representative cells in the ganglion cell layer in control ACSF (left), in the presence of PregS (50µM, middle) and during the wash in of PregS in combination with Mef (50µM, right). Total imaging time 10 minutes B. Same as A in TrpM3 KO mice. C. Summary data indicating impact of the PregS on wave properties: Left – wave frequency control: 1.3 +/- 0.8 waves/min, n= 9 experiments, from 5 retinas; PregS: 1.1+/- 0.5 waves/min, n = 8 experiments from 5 retinas, paired t-test, p = 0.36 ; TrpM3 KO control: 0.7 +/- 0.5, n = 7 experiments from 4 retinas; TrpM3 KO PregS: 1.1 +/- 0.8, 7 experiments from 4 retinas, paired t-test, p = 0..35); Middle ΔF/F average per wave: control: 12 +/- 4%, n = 8 experiments from 5 retinas; WT PregS: 47 +/- 46%, n = 8 experiments from 5 retinas, p = 0.07, paired t-test; TrpM3 KO control: 24.7 +/- 9%, n = 7 experiments from 4 retinas; TrpM3 KO PregS: 51.2 +/- 39%, n = 7 experiments from 4 retinas, p = 0.11, paired t-test. Right: Total percent of RGCs that participate in at least one wave during the experiment: WT control: 81.7 +/- 16%, WT PregS: 98 +/- 1.7%, n = 7 experiments from 5 retinas, p = 0.04, paired t-test; TrpM3KO control: 84.6 +/- 17%; TrpM3KO PregS: 97.4+/- 1.3%, n = 7 experiments from 4 retinas, p = 0.09, paired t-test).

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Chapter 4: Development of optogenetic tools to activate NADPH Oxidase (NOX2) in neurons

What is life, but a flow of electrons? In the previous chapter, I described evidence that the transfer of electron radicals, in the form of reactive oxygen species generation and its subsequent targeting of various proteins and cellular structural scaffolding, poses a possible fundamental underlying cause of neurodegeneration as well as aging, cancers, and many other deleterious conditions. This flow of electrons likely also mediates normal physiological function, as I'll describe in this chapter.

Because reactive oxygen species production is highly upregulated and correlated with numerous pathological conditions, such as neurodegeneration and psychiatric disorders, but causality has not been established, I aimed to design an optogenetic tool that would produce reactive oxygen species in neurons upon activation by light. One of the first indications that proteins could be engineered to be controlled optogenetically came from the Lim lab (2013) where they used the plant system PhytochromeB, which upon stimulation by red light 650nm causes the PhytochromeB component to bind to a phytochrome interacting factor (PIF) protein in the presence of phycocyanobilin, a compound found in certain plants. Infrared light at 750nm or darkness is sufficient to deactivate or separate the PhytochromeB and PIF components. The Lim lab cleverly hypothesized that the proximity of proteins is what drives the interaction, rather than the presence or concentration of the reactants. Thus, driving proteins into close proximity should be sufficient to initiate the reaction between those to interactors. They tested this hypothesis by creating an optogenetic SOS^{cat} by fusing SOS^{cat} to the PIF protein and attaching PhyB to the membrane using a CAAX motif. Both were tagged with fluorescent reporters YFP and RFP respectively. Upon translocation to the membrane, SOS is known to activate the MEK/ERK pathway, involved in cell cycle and proliferation. The Lim lab showed for the first time that they could engineer an optogenetic SOS (they called OptoSOS) to activate the MEK/ERK pathway in a manner that was temporally and spatially controlled with red (ON) or infrared (OFF) light. They show that light activation induced nuclear ERK translocation, proliferation of NIH3T3 cells, and differentiation of PC12 cells.

The PhyB-PIF system, while incredibly useful, is a three-component system requiring the addition of the exogenous compound phycocyanobilin, which could add increased variability and is not practical for *in vivo* experiments. Strickland et al. (2012) engineered tunable light induced dimerization tags (TULIPS) based on a synthetic interaction between the LOV2 (light oxygen voltage) domain of *Avena sativa* phototropin 1 (AsLOV2) and an engineered PDZ domain. PDZ domains are found in many membrane bound proteins, such as PSD95, an important post-synaptic scaffold. LOV domains are very small, 125 residue photosensory domains based on a Per-ARNT-Sim (PAS) core that binds a flavin cofactor. The AsLOV2 has a flanking N and C terminal α helix, the A'α and Jα helix respectively. Upon stimulation with blue light (<500nm), the Jα helix undocks from the AsLOV2 core and unfolds, exposing its PDZ binding domain. Strickland and colleagues engineered optical control of the G-protein coupled MAPK pathway by fusing the engineered PDZ domain to Ste5ΔN, which brings a complex that activates FUS1, a growth arrest promoter, into proximity with the GPCR bound to G_{α} and $G_{\beta\gamma}$. The authors show

that activation of the GPCR pathway in budding yeast by <500nm light stimulates growth arrest and polarization in the light treated LOV-ePDZ transfected cells, but not in dark or control construct transfected cells. They also showed that they can selectively induce membrane recruitment of their optogenetic proteins to a small spot using a steerable laser to produce a spot of laser light about 0.5-1um in diameter, allowing a high degree of both spatial and temporal control of the trafficking of selected engineered proteins.

Angela Brennan, a post-doc in Ray Swanson's lab (2009) showed that NADPH oxidase, a membrane complex that consists of p47phox, a cytosolic protein that translocates to the membrane where it binds to the membrane component of NOX2 and in the presence of NADPH activates the NADPH oxidase complex to produce superoxide, which is often rapidly converted to hydrogen peroxide. Brennan showed that this process occurs in an NMDAr dependent manner in hippocampal neurons and requires the intermediate, PKCζ or PKCδ, which have been shown to phosphorylate p47phox, which then drives it to the membrane NOX2 complex. Brennan also ruled out mitochondria as a primary source of ROS upon NMDAr activation, instead showing that NOX2 is the primary source of activity dependent ROS generation in neurons. It was largely based on this work that I designed an optogenetic variant of NOX2, which I call 'OptoNOX.' I transfected neurons with a membrane targeted ePDZ fused to mCherry. Alternatively, neurons express SAP97 endogenously, which contains a highly homologous PDZ domain to which the asLOV2 could bind. I fused asLOV2 to the p47 C terminus with an eGFP reporter between the two componants. The design of the construct was such that upon activation with blue light (<500nm) the Jα helix would unbind the core of the asLOV2, exposing its ePDZ binding domain. This induces binding to either an endogenous PDZ, such as that on SAP97, or to a transfected ePDZ-mcherry-CAAX construct that would induce translocation of the p47phox to the membrane, bringing it into proximity with the NOX2 membrane component. As in the Strickland paper, the membrane translocation of p47phox should happen within seconds of light activation. Figure A below shows the design of the constructs in complex with NOX2. Figure B shows representative images from an imaging experiment conducted on an automated high throughput microscope where 4 fields were captured across 24 wells, 3 wells per condition, and the conditions included dark treatment, negative control constructs and 3 versions of the OptoNOX construct, including the two-component p47-asLOV2 and ePDZ, as well as the p47-asLOV2 only constructs. Dihydroethidium fluorescence, a probe for hydrogen peroxide in cells, detects upregulated ROS in the neurons containing the single and two component OptoNOX systems, and the percent of ROS positive cells is higher than a similar PHY-PIF version of the optogenetic NOX2, which was still elevated above the control p47-GFP transfected neurons. Furthermore, in figure D, a different assay for ROS generation was utilized, namely the genetically encoded ROS indicator, Hyper3. In this case, again there was an increase in the number of neurons in the same field that showed a turn on effect of Hyper3 in response to blue light activation of OptoNox for 1minute. At this stage in the project, I was beginning to assess morphological changes over time by remotely stimulating OptoNOX. I built an Arduino controlled LED array (Adafruit #2026 32x32 LED Matrix) with which I could control the brightness, duration, and frequency of light stimulation of neurons in an incubator environment. Though the data in figure E were collected following stimulation for 1min 2x per day between DIV 9-DIV15, if I had been able to continue the project, I imagine I would have been able to more precisely gauge the exact quantity of light, or NOX2 activation, is required to induce such

morphological changes, or if there is a maximum/minimum NOX2 activation for healthy morphological changes versus pathological. In any case, following 1min 2xday of ambient white light stimulation, there was a noticeable difference in the morphology of neurons expressing the OptoNOX variants versus the p47-GFP + cpPDZ control expressing neuron morphologies in that those expressing the OptoNOX appeared to have a higher density of dendritic spines. Thus, it could be that some NOX2 activation is very beneficial, at least for cells in culture conditions. We aimed to use the OptoNOX tool to selectively activate NOX2 *in vivo* particularly in regions of the brain, such as dopaminergic neuron rich areas such as the substantia nigra, or excitatory pyramidal neuron regions such as the hippocampus, which are particularly susceptible both to ROS accumulation and also to ensuing neurodegeneration in disease conditions. The goal of this project was to determine whether ROS alone, rather, the activation of NOX2 could directly affect the health of neurons, whether NOX2 activation drives the formation of certain neurodegenerative conditions.

One of the primary motivations for the generation of an optogenetic NOX2 stemmed from a data set that Carl Onak had collected in the Chang lab profiling which proteins had cysteine modifications, sulfenylations, secondary to NMDAr activation of neurons. This subsequently induces NOX2 activation, which produces hydrogen peroxide that ultimately modifies cysteines in proteins in much the same manner to that of protein phosphorylation. Because the mass spectroscopy data is yet unpublished, but can be found in the dissertation of Carl Onak (2014), it will be beyond the scope of this thesis. However, nitric oxide production also occurs in an activity dependent manner due to nNOS activation, and like ROS, NO also targets cysteines through S-nitrosyl-Cys modification. In a study carried out very much in parallel to the Onak study, except that the authors profiled NOS dependent protein modifications, Raju et al (2015) also found that many presynaptic vesicle fusion proteins are modified by reactive nitrogen species. Presynaptic proteins that are modified by activity dependent S-nitrosylation secondary to nNOS activation include: AP-1, AP-2, CaMKII, clatherin heavy chain, huntington, Rab DGP, Synaptophysin, Syntaxin, Vesicle fusing ATPase. Because the Onak work also identified numerous presynaptic vesicle fusion proteins, some of which overlap, many don't, we hypothesized that both means of modifying cysteines in these essential presynaptic proteins, either by S-nitrosylation, or by cysteine sulfenylation, may serve to modulate the release of neurotransmitters presynaptically, either in the up or down direction. Prior studies demonstrate that synaptic plasticity is modulated by ROS in a dose dependent manner. Kamsler et al. (2004) showed a concentration dependent modulatory affect of H2O2 on the induction and expression of long-term potentiation or long-term depression. Higher concentrations of H2O2 (0.5-5mM) reduced EPSP amplitudes. However, very low concentrations (1μM) increased the EPSP slope two-fold following high frequency stimulation (HFS). The authors concluded that plasticity modulation by ROS is mechanistically different at high versus low concentrations. NOX2 also appears to be required for spatial or contextual memory formation, since mice lacking the catalytic NOX2 component (gp91-/-) performed significantly worse in the Morris Water Maze test (Kishida et al., 2007; Serrano et al., 2004). On the other extreme, mice that lacked gp91, but also overexpressed amyloid precursor protein as a model of AD (Swedish mutation) failed to develop oxidative stress markers or behavioral deficits (Y Maze test), however the amyloid beta plaque load remained equivalent between the WT mice and those lacking NOX2, suggesting perhaps an even more critical role for NOX2 dependent ROS in the context of AD progression than previously believed (Park et al., 2008). All together, these data point to the critical role of

NOX2 in healthy synaptic plasticity, memory formation, as well as its potentially detrimental role in disease progression.

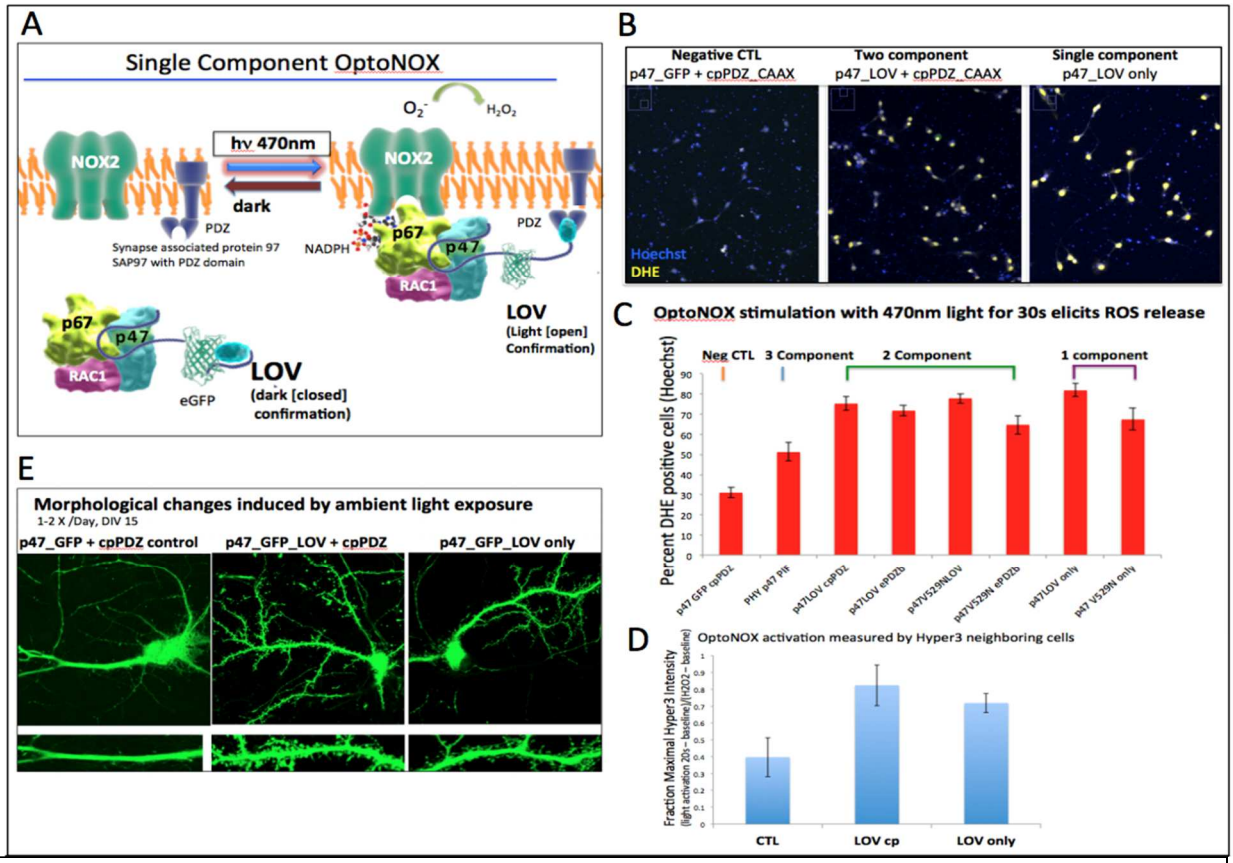

Figure 4.1: Schematic and validation of OptoNOX. A) Design of OptoNOX signel component genetically encoded optogenetic NOX2. p47 cytosolic domain is fused to GFP and LOV, which targets endogenous PDZs in neurons in the presence of blue light. B) Hippocampal neurons (DIV12) were transfected with the 3, 2, or single component OptoNOX and activated for 30s with 470nm light using an upright epifluorescence microscope. After 15min incubation in the dark, neurons were imaged using an IMX automated microscope (27 frames per condition, N=3) for DHE, Hoechst, and GFP. The transfection efficiency was 5-10%. C) Percent of DHE positive cells relative to Hoechst. D) ROS production was also monitored using the genetically encoded ratiometric sensor, Hyper3. Neighboring neurons increased Hyper fluorescence by 2 fold in the 2 or single component systems. E) Low light twice per day for 1min between DIV 9-15 stimulates dendritic spine formation in hippocampal neurons.

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Chapter 5: Brief Discussion

In this dissertation I have discussed two correlated fields in neuroscience: the notion that reactive oxygen species may serve to modulate the activity of light sensitive melanopsin as well as dendritic spine morphology, as well as the role of neurosteroids during synapse formation and development via the temperature sensitive mechanotransductive channel, TrpM3. In our manuscript, 'Steroid sensitive TrpM3 channels modulate spontaneous activity in the developing retina,' discussed in chapter 3, I show that TrpM3 mediates large calcium transients in response to the neurosteroid, Pregnenolone Sulfate. PregS also induces increased synaptic transmission, both inhibitory and excitatory, in the developing retina during a time when such correlated activity may facilitate postsynaptic connectivity in the lateral geniculate or superior colliculi. Furthermore, mice lacking TrpM3 appear to have a reduced photoaversion response at P6, suggesting that TrpM3 may partially mediate this response initiated in intrinsically photosensitive retinal ganglion cells.

In chapter two I hypothesize that the role of TrpM3 in ipRGCs is to modulate their response to both melanopsin activation, as it has been shown to be inhibited by $G_{\beta\gamma}$, which is released from its bound position on melanopsin upon activation (Quallo et al., 2017). This TrpM3 inhibition may act in a manner similar to K2P channel in M4 ipRGCs, closing the outward rectifying channel so that contrast sensitivity is enhanced (Sonoda et al., 2018). In chapter two I also hypothesize that the role of melanopsin not only in the retina, but also in dopaminergic rich regions of the brain as a redox sensor. If this turns out to be the case then the PLR may be used as a proxy for retinal, or possibly brain, redox status.

Finally, in Chapter 4, I show results from an optogenetic construct that I designed and constructed which generates reactive oxygen species via NADPH oxidase by bringing the cytosolic component of NOX2, p47, into contact with the membrane bound ROS generator, gp91, using either the PHY-PIF system or the AS-LOV2. I show that short exposures to light over several days was sufficient to alter dendrite morphology in cultured neurons.

The role of both reactive oxygen species and neurosteroids have been studied in detail during the process of aging. ROS is known to increase linearly with age (Levine et al., 2001), while pregnenolone sulfate is known to decline with age as well as with neurodegeneration (Vallee et al, 1997). Conversely, PregS levels are high throughout developmental periods, but oxidative stress is not thought to produce damaging effects in younger organisms. The ongoing question remains as to whether neurosteroids could drive oxidative stress levels down in aging, or whether the reduced levels of neurostroids often seen in cases of autism or pediatric seizures could contribute to the increased ROS in these pediatric cases.

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