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Publication Date
2015

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Neuropeptide releasing amacrine cells modulate multiple microcircuits in the inner retina

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Helen Elaine Vuong

2015
ABSTRACT OF THE DISSERTATION

Neuropeptide releasing amacrine cells modulate microcircuits in the inner retina

by

Helen Elaine Vuong
Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology
University of California, Los Angeles, 2015
Professor Patricia E. Phelps, Co-Chair
Professor Nicholas C. Brecha, Co-Chair

Amacrine cells form the most diverse group of interneurons in the retina. There are at least 30 identified types, which are differentiated based on their stratification patterns, neurotransmitter content, and soma and dendritic field sizes. Despite knowing the morphology of each amacrine cell type, our understanding of the connectivity, intrinsic, and functional properties of a majority of the amacrine cell types remain unclear. This study aims to investigate the intrinsic properties of neuropeptide-expressing amacrine cells, as well as their role in modulating inner retinal microcircuits. These studies will focus on two neuropeptide-expressing amacrine cells examined are the somatostatin-expressing (SRIF) and vasoactive intestinal polypeptide-expressing (VIP) amacrine cells.

Previous studies have shown SRIF and VIP amacrine cells play a role in regulating dopamine levels or modulating GABA signaling in the inner retina. In this study we tested how SRIF
amacrine cells can modulate the cells that comprise the light adaptation network: dopamine-expressing (DA) amacrine cells and melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs). In addition, we used a novel transgenic mouse line to map the intrinsic electrophysiological properties and synaptic partners of VIP amacrine cells.

In order to address these questions about SRIF- and VIP-amacrine cells in the network of cells in the inner retina, I employed a combination of anatomical, pharmacological, and electrophysiological manipulations in multiple transgenic mouse lines. The 3D modeling generated from antibody-labeled whole mount retinas showed the relationship of the processes of SRIF- and DA-amacrine cells, as well as the processes between SRIF amacrine cells and melanopsin ipRGCs. Using pharmacology and whole patch clamp protocols I showed SRIF, acting through specific SRIF receptor subtypes (sst$_{2A}$ and sst$_{4}$), effectively increases K$^+$ currents, decreases Ca$^{2+}$ currents, as well as regulate the spontaneous firing rate of both cell types. In addition, SRIF can directly inhibit the intrinsic light response of melanopsin ipRGCs.

Using the VIP-tdTomato transgenic mouse line we detail the ion channel composition of VIP-amacrine cells, which include delayed inward rectifying K$^+$ channels, verapamil-sensitive L-type Ca$^{2+}$ channels, a hyperpolarizing activated K$^+$ channel ($I_h$), and TTX-sensitive Na$^+$ channel currents. The recorded VIP amacrine cells showed varying combination of these ion channels, suggesting there may be multiple subtypes of VIP amacrine cells. Finally, using a puff protocol, we showed VIP amacrine cells receive inhibitory inputs mediated by GABA and glycine. In addition, they receive excitatory input from type 2 OFF- and type 6 ON-cone bipolar cells, likely through activation of an ionotropic glutamate receptor subtype, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor.
The dissertation of Helen Elaine Vuong is approved.

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University of California, Los Angeles
2015
Dedication

I would like to thank my advisors, Nicholas C. Brecha and Steven A. Barnes, for their support as my research and career mentors. I have learned an incredible amount of information and have grown so much from this experience. I would also like to say thank you to my committee members Lynn Gordon, Patricia Phelps, and Stephanie White for their insightful guidance.

There are many people to thank, all of whom have helped, encouraged, and supported me through my graduate career and the process of writing this dissertation. Some of these amazing people include past and present members of the Brecha Lab Family, including Claudia Hardi, Arlene Hirano, Jennifer Huynh, Kristopher Sheets, Hinekura Te Hapukino, Luis Pérez de Sevilla Müller (aka. “Luisito”), Janira de los Santos, Allison Sargoy, Xue Liu, Xiaoping Sun, and Allen Rodriguez. These incredible individuals have seen me through the good and bad times, and have truly made this experience fun and unforgettable.

Finally, I dedicate this dissertation to my parents, Dianna Ly and Vinh Vuong, my brothers, Tony Ly and Alexander Vuong, my best friend, Jennifer Huynh, and Drake Williams, without whom this journey would not have been possible.
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Chapter 1

Anatomical organization and functional implications of the retinal neurome
Introduction

The retinal neurome is composed of multiple cell classes that communicate within and between each other to form the initial steps in neuronal processing of visual information. The vertical flow of information occurs in order of: photoreceptors, bipolar cells, and ganglion cells. In the vertical signaling pathway, light is converted into electrical and chemical signals, and sent to the brain through ganglion cell axons, which form the optic nerve. Increasing the complexity and modifying the vertical signaling of the retina are the laterally extending horizontal cells in the outer retina and amacrine cells in the inner retina. These cells serve to refine and filter by means of feedback, feed forward, and serial inhibition. In particular, there are ~30-40 different types of amacrine cells that form synaptic interactions in the inner plexiform layer to feedback onto bipolar cells, feed forward onto ganglion cells, or reciprocal interactions with other amacrine cell types (Masland, 1988, 2001a, b, 2012b, a). Some functions of amacrine cells have been described, such as their role in light adaptation, direction selectivity, and mediating signals from the rod and cone photoreceptor pathways (Masland, 1988, 2012b). However, the role of the majority of amacrine cell types in the processing of visual information remains unclear. Many amacrine cell types share similar neurotransmitter composition and stratification patterns, but these features only aid in broadly defining the amacrine cell type. Instead, in order to determine the synaptic partners, the input and output signaling pattern, and ultimately an amacrine cell’s influence on ganglion cells, it will require a combination of targeted labeling and anatomical and physiological studies.

The goal of this study was to describe properties of neuropeptide-releasing amacrine cells and their interactions with inner retinal microcircuits. One microcircuit discussed is composed of an inhibitory-excitatory reciprocal network between dopamine-releasing amacrine cells and melanopsin-containing intrinsically photosensitive retinal ganglion cells. This circuit is important for light adaptation and non-image forming vision. Specifically, this study was focused on examining somatostatin-releasing amacrine cell modulation of the aforementioned
microcircuit. This study also aimed to detail the intrinsic properties of a novel vasoactive intestinal polypeptide-containing amacrine cell, as well as characterize its synaptic partners in the inner retina. The plethora of neuronal interrogation of visual information begins at the retina, and the amacrine cell crosstalk and input to ganglion cells aid in the tuning of signals that are subsequently relayed to the brain for interpretation of the visual scene.

**Retinal anatomy and visual signaling**

The retina is a highly organized laminar structure that contains three cellular layers, and two synaptic layers. The five main neuronal classes of cells are photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. The distal or ‘outer’ retina is located furthest from the point of light entry, whereas the proximal layers are termed the ‘inner’ retina. Photoreceptor cell bodies are located in the outer nuclear layer (ONL). Phototransduction or the conversion of light into electrical and chemical signals occurs in photoreceptors, and that signal from the outer retina is transmitted to the inner retina via parallel signaling of bipolar cells. The outer plexiform layer (OPL) is comprised of photoreceptor axon terminals, horizontal cell axons and dendrites, and bipolar cell dendrites, and is also the site of the first synapse of the retina. The synaptic interactions of the OPL are important for antagonistic center-surround properties. The somata of horizontal cells are confined to the distal part of the inner nuclear layer (INL), whereas the somata of bipolar cells, amacrine cells, and displaced ganglion cells are located in the more proximal portion of the INL. The synaptic signaling between bipolar, amacrine and ganglion cells are made in the inner plexiform layer (IPL), which is further organized in a laminar fashion, such that the more distal layers – strata 1 and 2 -- carry “off” light responses or decrements in light, and the proximal layers – strata 3, 4, and 5 -- convey “on” light responses or increments in light. The synaptic interactions of the IPL is important for processing different aspects of visual stimuli including edges, motion, contrast, luminance, and colors. Finally,
neuronal signaling in the retina is sent to the brain via ganglion cell axons, which form the optic nerve.

In general, photoreceptors, bipolar cells, and ganglion cells are glutamatergic, whereas horizontal cells and amacrine cells are inhibitory, and signal using the inhibitory neurotransmitters GABA and glycine. Although we can make general classifications of these neurons (ie. five classes), there is an incredible amount of diversity of structure and function within each class that serve to convey integrated signaling of vision.

**Photoreceptors**

Photoreceptors are the primary light sensing neurons of the retina. Photons are transduced into chemical and electrical signal at the photoreceptor outer segments via light-sensitive photopigments and proteins. Each photoreceptor type is named according to their outer segment shapes. There are two types of photoreceptors: 1) rods, which are responsible for scotopic (dim light) visual processing, and 2) cones, which are involved in photopic vision and can be further subdivided into 3 subtypes that have opsins that are sensitive to short, medium, and long wavelengths (Nathans et al., 1986, Baylor et al., 1987, Jeon et al., 1998). Photoreceptors are hyperpolarized in the presence of light, thus reduces the amount of glutamate released (Copenhagen and Jahr, 1989, Kleinschmidt, 1991, Thoreson and Witkovsky, 1999).

**Horizontal cells**

Most mammalian retinas have two types of horizontal cells - type A and B – which can be identified based on their morphology (Kolb, 1970, 1974, Kolb and Normann, 1982). Type A horizontal cells are axonless, and have thick dendrites and larger dendritic field compared to type B horizontal cells (Kolb and Normann, 1982). Their dendritic processes contact and receive excitatory input from cones and make an inhibitory output back onto cones (Dacheux and
Type B horizontal cells, on the other hand, have an extended axon that allows them to make feedback onto rods, as well as a dendritic tree that is responsible for reciprocal communication with cones (Dacheux and Raviola, 1982, 1986, Raviola and Dacheux, 1987, Wässle and Boycott, 1991, Peichl and Gonzalez-Soriano, 1994). Some defining roles that horizontal cells play in vision include edge enhancement, gain adjustment, and local light adaptation and sensitivity (Thoreson and Mangel, 2012).

Bipolar cells

Bipolar cells relay light signals from photoreceptors and horizontal cells to amacrine and ganglion cells. Based on their light responses, bipolar cells can be divided into the ON and OFF types (Kolb and Nelson, 1983, Hartveit, 1997, DeVries, 2000). On the other hand, the bipolar cell class is anatomically subdivided into 11 types of cone bipolar cells and 1 type of rod bipolar cell (RBC) (Ghosh et al., 2004, Wässle et al., 2009, Helmstaedter et al., 2013). The RBC signaling is mostly mapped out, such that we know RBCs’ receive input only from rod photoreceptors, and RBC output is computed by an AII amacrine cell, which utilize the cone bipolar cell signaling pathway to then relay rod signaling (see section Microcircuits of the retina).

The cone bipolar cell signaling follows a principle of ‘parallel informational channels’ (Wässle et al., 2009). Evidence of this concept was confirmed in the mouse retina, which showed each cone makes synaptic inputs onto each cone bipolar cell type, and each cone bipolar cell contacts all cones within its dendritic arbor (Wässle, 2004, Wässle et al., 2009). Therefore, each cone bipolar cell will relay a unique feature from the cone it contacts to the inner retina. Based on where the cone bipolar cell axons stratify within the IPL, it will provide information to activate ON or OFF channels of ganglion cells. Thus the outputs of the two photoreceptor types are dependent on the parallel signaling of bipolar cells onto ganglion cells (Ghosh et al., 2004, Wässle et al., 2009).
Amacrine cells

There are ~30–40 morphological amacrine cell types, most of which are undefined with regards to their intrinsic electrophysiological properties, function, and connectivity (Masland, 2012b). A major obstacle in understanding these features of each amacrine cell type is a reliable method to consistently identify the cell type for anatomical mapping and physiological recordings. It is important to be able to detail amacrine cell properties because of their role in tuning bipolar cell and ganglion cell signaling, as well as other amacrine cell transmission. The pivotal development that has fundamentally changed the understanding of retinal circuitry, and will be vital in characterizing amacrine cell types, is transgenic mouse models. However, it is important to note that transgenic mouse lines cannot be simply accepted for targeted understanding of specific amacrine cell types without proper characterization of the transgene labeling prior to use for targeted studies. As reported here and in previous studies (see Chapter 2), transgenic mouse lines that are used for specific labeling of cell types can display ectopic transgene expression in the central nervous system including the retina, and must be carefully characterized prior to use for subsequent studies of amacrine cell function and connectivity.

Amacrine cells as a class of retinal neurons are unique in that they lack axons, but have processes that have axon-like properties. These axon-like processes are long and largely functions to serve as the output structures of the amacrine cells. Nevertheless, amacrine cells can receive and send information through their many processes, and stratify in different layers of the IPL. Based on several factors such as their neurotransmitter expression, IPL stratification pattern, size of their dendritic arbor, and their response to light stimuli, amacrine cells form the most diverse population of interneurons in the retina (Masland, 2012a, b). In general, amacrine cells form bi-directional interactions with bipolar cells, ganglion cells, and other amacrine cells. Most amacrine cells express one of two primary inhibitory neurotransmitters GABA and glycine (Zhang and McCall, 2012), however a recent study revealed the presence of a non-GABAergic non-glycinergic (nGnG) amacrine cell type (Kay et al., 2011). This general expression of
inhibitory neurotransmitters is insufficient for the selective transgenic isolation of specific amacrine cell types. Therefore, the difficulty in understanding the role of amacrine cells, or even how many amacrine cells exist, lies in identifying other specific marker(s) for amacrine cells such as synthesizing enzymes, calcium binding proteins, or transporters (Zhang and McCall, 2012). Some have identified individual amacrine cell types due to their co-expression of GABA or glycine with other neurotransmitters or neuropeptides such as, dopamine, somatostatin, indoleamine, or acetylcholine.

Transgenic identification of subsets of amacrine cells has allowed researchers to characterize multiple types of amacrine cell morphology and function. The most well-defined amacrine cells include the AII, A17, dopamine-containing and starburst amacrine cells. Their roles in visual processing are described in the ‘Microcircuits of the Retina’ section of this chapter. In brief, the AII amacrine cells are the most numerous amacrine cell type. They are classified as glycinergic narrow-field (<100 µm in dendritic arbor diameter) cells that have processes close to their somata and ramify across the IPL (MacNeil et al., 1999, Marc et al., 2014). On the other hand, A17 and starburst amacrine cells are wide-field (>500 µm) cells that release GABA. In addition to containing GABA, A17 amacrine cells are indolamine-accumulating, and starburst amacrine cells express acetylcholine (Ehinger and Floren, 1978, Vaney, 1986, Menger and Wässle, 2000, Wright and Vaney, 2000, Taylor and Smith, 2012).

More recently, studies have reported the presence of a small population of amacrine cells that express the vesicular glutamate transporter 3 (VGLUT3), which is indicative of a putative excitatory amacrine cell type. These cells also express glutamate and glycine (Fremeau et al., 2002, Haverkamp and Wässle, 2004, Johnson et al., 2004, Lee et al., 2014). The specificity of VGLUT3 to a subset of amacrine cells is particularly useful for studying their morphology and function, and has facilitated much progress in defining its role in visual processing (Grimes et al., 2011, Lee et al., 2014, Kim et al., 2015). With these examples in mind, this report will focus on utilizing specific transgenic mouse models to characterize
amacrine cell modulation of inner retinal microcircuitry as well as defining the intrinsic properties of a specific amacrine cell type.

**Wide-field amacrine cells**

Among the numerous amacrine cell types that remain unclearly defined are the rarely occurring wide-field amacrine cells. Wide-field amacrine cells typically have a dendritic arbor diameter that is greater than 500 µm and axon-like processes that can extend over 1 mm. The limitation to studying wide-field amacrine cells is having a specific marker. Despite such a limitation several studies have been able to characterize wide-field amacrine cells in retinas of salamander, mouse, rat, cat, rabbit, and non-human primates by an arduous technique of large scale intracellular injections of Neurobiotin or horseradish peroxidase (Dacey, 1989, Famiglietti, 1992a, b, Völgyi et al., 2001, Wright and Vaney, 2004, Greschner et al., 2014).

There are sixteen identified types of wide-field amacrine cells in the mouse retina (Badea and Nathans, 2004, Lin and Masland, 2006). The characterized wide-field amacrine cells can be differentiated into two groups based on the presence or lack of axons-like processes (polyaxonal amacrine cells). Another differentiating factor is their stratification pattern where some are either monostratified or bistratified. It is worth noting that there is at least one wide-field amacrine cell type that stratifies in each major strata of the IPL. Finally, the somata of wide-field amacrine cells vary in their location, some are located in the INL, whereas others are displaced into the GCL.

Due to its morphological features, wide-field amacrine cells must integrate many signals, and are presumably responsible for long range signaling (Manookin et al., 2015). Given that they are GABAergic, wide-field amacrine cells likely cause a global inhibition when they are stimulated, which is determined by its stratification in the IPL (Lin and Masland, 2006, Masland, 2012b).
**Dopamine-expressing amacrine cells**

One very well characterized wide-field amacrine cell type is the type 1 dopamine-expressing (DA) amacrine cell, which has been reported in retinas of multiple species including amphibians, rodents, rabbits, cats, and non-human primates (Brecha et al., 1984, Versaux-Botteri et al., 1984, Oyster et al., 1985, Mariani and Hokocz, 1988, Nguyen-Legros, 1988, Dacey, 1990, Tauchi et al., 1990, Zhu and Straznicky, 1990). DA amacrine cell somata are located in the INL and its processes are stratified in strata 1 and 3 of the IPL, and occasionally some processes reach out to the OPL. DA amacrine cells synthesize and release both dopamine and GABA to act directly on its neighboring cells or in a paracrine fashion by volume transmission (Puopolo et al., 2001, Hirasawa et al., 2009). Dopamine and GABA are packaged in the same vesicle and simultaneously released upon stimulation (Hirasawa et al., 2012). Dopamine is released upon flickering light and steady background stimulation, as well as prolonged darkness, and is implicated in establishing gain of retinal neurons during bright light vision (Bauer et al., 1980, Mangel and Dowling, 1985, Godley and Wurtman, 1988, Witkovsky, 2004). Light regulation of dopamine activity is dependent on rod and cone input (Cameron et al., 2009) and melanopsin containing ganglion cells (Zhang et al., 2008). Dopamine can regulate gap junctions, modulate light/dark adaptation, and serve as the output of the retinal circadian clock (Piccolino et al., 1984, Pozdeyev et al., 2008, Bloomfield and Volgyi, 2009, Hu et al., 2010, Yang et al., 2013, Jin et al., 2015). The characterization of DA amacrine cell properties and connectivity in the retina was greatly facilitated by the development of transgenic mouse lines that labeled these cells with reporter proteins (see Chapter 2). DA amacrine cells exhibit spontaneous spiking, which is a key factor regulating dopamine secretion (Feigenspan et al., 1998, Puopolo et al., 2001). In situ studies show DA amacrine cells have differential spontaneous burst spike patterns that are mediated by glycinergic and GABAergic inhibition, all of which are light dependent (Zhang et al., 2007). Finally, DA amacrine cell spiking in response to light occurs as ON-transient and ON-sustained (Zhang et al., 2007).
Neuropeptide-expressing amacrine cells

An amacrine cell subtype co-expresses neuropeptides and GABA or glycine. Since the 1980s there have been at least 20 different types of neuropeptides identified in the vertebrate retina (Karten and Brecha, 1980, Brecha et al., 1981a, Brecha et al., 1981b, Larsen, 1995, Gabriel, 2013, Szabadfi et al., 2014). However, not all neuropeptides in the retina are synthesized and released by neurons, rather some, such as angiotensin II and erythropoietin, are expressed in Müller cells (Gabriel, 2013, Szabadfi et al., 2014).

The precise role of each neuropeptide and neuropeptide-releasing cells in retinal processing is unclear, however, studies have began to elucidate some of their functions. Of interest to this report is the role of somatostatin (SRIF)- and vasoactive intestinal peptide (VIP)-containing amacrine cells in inner retinal microcircuits.

Somatostatin-containing amacrine cells: morphology and physiological properties

SRIF is expressed in rarely occurring wide-field amacrine cells that are localized to the INL and displaced into the GCL, and are preferentially located in the inferior retina. Their processes mainly ramify in S1, 3, and 5 of the IPL (Tornqvist et al., 1982, White et al., 1990, Larsen, 1995). Physiological studies on SRIF suggests that it acts on the retinal networks to play a role in adaptation mechanism, ganglion cell receptive fields types, and increases signal to noise ratio (Zalutsky and Miller, 1990, Cervia et al., 2008). The actions of SRIF are based on its specific receptor subtypes (sst1-5), which are localized to different cells within the retina (Epelbaum, 1986, Zalutsky and Miller, 1990, Johnson et al., 1998, Johnson et al., 1999, Johnson et al., 2000, Cristiani et al., 2002, Wu et al., 2012, Farrell et al., 2014). For example, sst2 receptors are localized to photoreceptors, which suggest SRIF can affect phototransduction and light adaptation processes. On the other hand, sst2 receptors are expressed in the inner retina by DA amacrine cells, which can affect light adaptation and circadian rhythms (Cervia et
In addition, in the inner retina retinal ganglion cells express sst4 receptors. Through the sst4 receptors SRIF potentiates K+ currents and suppresses Ca2+ channel currents in retinal ganglion cells (Farrell et al., 2014).

Vasoactive intestinal polypeptide-containing amacrine cells: localization within the retina and its intrinsic physiological properties

On the other hand, VIP amacrine cells were first identified 35 years ago in the rat retina (Loren et al., 1980). In rat retina there were four identified morphological types of VIP amacrine cells, which are named accordingly: 1) bistratified, 2) unistratified, 3) diffuse, and 3) stratified diffuse (Terubayashi et al., 1983). VIP immunoreactive cells were also GABA immunoreactive, and their somata are localized to the INL and displaced into the GCL (Casini and Brecha, 1992). Previous studies investigating VIP effects in the retina of lower vertebrates and mammals showed that VIP potentiated GABAergic and cholinergic transmission (Fukuda et al., 1987, Veruki and Yeh, 1992, Feigenspan and Bormann, 1994), possibly through increasing cAMP production (Schorderet et al., 1981, Watling and Dowling, 1983).

More recently, with the use of a transgenic mouse line that expresses Cre recombinase in VIP amacrine cells, our group and others have began detailing the intrinsic morphological and physiological properties, synaptic partners, and light responses of VIP amacrine cells (Park et al., 2015). The results of my findings will be detailed in Chapter 4. Briefly, the specificity of this mouse line was confirmed using VIP antibody labeling of retinas from VIP-Cre mice crossed with a tdTomato (Ai14) reporter mice (Park et al., 2015). Park et al. (2015) noted the presence of three different morphological types, each of which displayed differential amounts of inhibitory and excitatory synaptic inputs. They also found VIP amacrine cells receive ON and OFF bipolar cell inputs that shape their spatial receptive field properties (Park et al., 2015). Finally, the outputs of VIP amacrine cells are five types of ganglion cells, which include the OFF δ, ON-OFF DS, W3, ON α, and OFFα (Park et al., 2015).
Ganglion cells

Ganglion cells are the only output neurons of the retina. They signal to central brain regions through their axons, which form the optic nerve. There are ~20 morphological types of ganglion cells identified in mouse, rabbit, cat, and monkey retinas (Masland, 2012a, Sanes and Masland, 2015). These morphological types can be characterized into categories based on physiological functions including: 1) local edge detector, 2) ON- and OFF-tonic cells, 3) blue-ON and –OFF cells, 4) ON direction selective cell, 5) ON-OFF direction selective cell, 6) ON- and OFF-transient, 7) transient ON-OFF ganglion cell, 8) uniformity detector, and 9) intrinsically photosensitive (melanopsin) cells (Masland, 2012a).

Ganglion cells are differentially excited and inhibited by bipolar and amacrine cell inputs, respectively, depending on IPL stratification of their dendrites. However, recent reports suggest en passant synapses may occur, which deviate from conventional stratification dependent transmission (Dumitrescu et al., 2009). Each ganglion cell type contacts multiple bipolar cell types, these contacts are comprised of a unique combination of the 11 different bipolar cell types, and within these combinations there are groups of bipolar cells that make up the majority of the contacts, whereas there are groups of bipolar cells that make fewer contacts (Dunn and Wong, 2014).

Melanopsin-containing intrinsically photosensitive retinal ganglion cells

One unique ganglion cell subtype is the intrinsically photosensitive retinal ganglion cell (ipRGC). These cells represent only ~2-5% of the total RGC population (Robinson and Madison, 2004, Hattar et al., 2006, Lin et al., 2008, Ecker et al., 2010). Though they are few in number, they are unique because they contain the photopigment melanopsin, which signals gross changes in light intensity leading to non-image forming functions such as pineal melatonin release, circadian photoentrainment and pupil constriction (Ecker et al., 2010, Lucas et al., 2014).
ipRGC phototransduction cascade is highly amplified, however, they are much less sensitive in the detection of photons compared to the conventional photoreceptors (rods and cones) due to the lack of specialized photopigment-concentrating organelles, such as the rod/cone outer segments. Also, unlike conventional photoreceptors, ipRGC light response results in cellular depolarization and leads to an irradiance-dependent increase in firing (Lucas et al., 2014). The intrinsic light response of ipRGCs is sustained over a long duration of constant illumination (Berson et al., 2002, Hattar et al., 2002, Berson, 2007, Ecker et al., 2010). ipRGCs firing is dependent on a combination of outer retinal input, where their dendrites receive synaptic input from bipolar and amacrine cells, as well as melanopsin-mediated phototransduction (Weng et al., 2013), which can be independent of ON-bipolar cell inputs. The spectral sensitivity of melanopsin is at $\lambda_{\text{max}}$ at 480 nm.

There are five morphological types of ipRGCs in the mouse and rat retinas (Viney et al., 2007, Berson et al., 2010, Ecker et al., 2010, Estevez et al., 2012) that were identified based on a combination of melanopsin immunoreactivity, retrograde labeling, and a transgenic mouse model (OPN4-EGFP). Related to this report are the type 1 ipRGCs (M1 ipRGCs). M1 ipRGC somata are located in the GCL, their dendrites pass through the ON sublaminae and are exclusively stratified in stratum 1 of the OFF sublamina of the IPL (Schmidt and Kofuji, 2009, Schmidt et al., 2011). These cells receive ectopic synapses from type 6-cone bipolar cell in stratum 1 of the IPL, which is where its dendrites co-stratify with the DA amacrine cell processes (Østergaard et al., 2007, Dumitrescu et al., 2009). The M1 ipRGC axons project to the olivary pretectal nucleus and suprachiasmatic nucleus, which are the sites for pupillary light reflex and the circadian clock, respectively (Berson et al., 2002). Types M2, M4, and M5 have dendrites that ramify in the ON sublamina of the IPL (Hattar et al., 2002, Viney et al., 2007, Schmidt et al., 2011). Although the ON ipRGCs have similar stratification patterns they have other distinguishing features. For example, M2 ipRGCs have the largest dendritic arbor, M4 ipRGCs have somata with large diameters and dense dendrites, and M5 ipRGCs have dendrites that are
bushy in appearance. Finally, the M3 ipRGC somata are located in the GCL and very few are displaced into INL. Their dendrites are stratified in both the ON and OFF sublaminae of the IPL (Schmidt and Kofuji, 2011).

In addition to their unique morphology, the different ipRGC types have distinct electrophysiological properties. Each ipRGC type shows a different spontaneous spike rate as well as varying amplitudes and temporal responses to light stimulation (Zhao et al., 2014). In order to isolate the melanopsin based light response (480 nm) rod/cone signaling was pharmacologically blocked. Of interest to this report are the distinguishing membrane properties and light responses of the M1 ipRGCs, which show an average resting potential of -62 mV, and are either slow spiking or lacked spontaneous spiking in the dark (Reifler et al., 2015). Compared to the other ipRGC types, M1 ipRGCs had the lowest intensity threshold, a shorter latency to reach peak amplitude, and the highest peak amplitude (Ecker et al., 2010).

Established microcircuits in the retina

The diverse populations of amacrine cells suggests that they are not only morphological distinct, but functionally unique. Each amacrine cell could form many microcircuits that mediate inhibitory feedback and feedforward signaling, refinement of bipolar and ganglion cell signals, and regulation of specific visual tasks.

The direction selectivity microcircuit in the inner retina is facilitated by starburst amacrine cell input. Starburst amacrine cells contain and release acetylcholine and GABA, and although the role of its GABAergic signaling is known, the role of its cholinergic transmission remains unclear (Hayden et al., 1980, Brecha et al., 1988, Vaney and Young, 1988, O'Malley and Masland, 1989, O'Malley et al., 1992, O'Malley and Masland, 1993). In short, starburst amacrine cells have at least two unique functions: 1) they are responsible for spontaneous waves of excitatory activity that are critical for formation of visual circuits during development (Feller et al., 1996) and 2) they generate signals for direction selectivity. With regard to its role in
direction selectivity, starburst amacrine cells are anatomically placed in the INL and displaced into the GCL, and their processes are strategically localized to the OFF and ON layers of the IPL, respectively (Famiglietti, 1987). This allows the cells in the INL to respond to decrements of light and those in the GCL to respond to increments of light. These processes co-stratify with dendrites of ON-OFF and ON direction selective ganglion cells. With these connections starburst amacrine cells send more GABA-mediated inhibition onto direction selective ganglion cells in the opposite direction of the stimuli (Wei et al., 2011, Yonehara et al., 2011, Taylor and Smith, 2012, Park et al., 2014).

Next, there are two microcircuits that define the rod bipolar cell pathway. Rods make synapses onto rod bipolar cells, which depolarize in response to light. Rod bipolar cells then relay a glutamatergic signal onto A17 and AII amacrine cells forming a dyad synapse that differentiates into two independent microcircuits (Kolb and Nelson, 1983, Dacheux and Raviola, 1986, Raviola and Dacheux, 1987). In the rod bipolar cell-A17 amacrine cell microcircuit, A17 amacrine cells return conventional vesicular GABAergic feedback signaling that acts on GABA_A and GABA_C receptors on the rod bipolar cell terminal (Raviola and Dacheux, 1987, Hartveit, 1999, Zhang et al., 2002, Chavez et al., 2010, Grimes et al., 2010, Grimes et al., 2015). On the other hand, the rod bipolar cell-AII amacrine cell microcircuit is responsible for converging the signals between the rod and cone photoreceptor pathways (Demb and Singer, 2012). Electron micrographs and electrophysiological recordings have aided in the understanding of how AII amacrine cells fit in the scheme of scotopic and photopic vision. The general network is described as rod bipolar cells form glutamatergic synapses onto AII amacrine cells, which then provides glycinergic synapses onto OFF cone bipolar cells and OFF ganglion cells, and form gap junctions between other AII amacrine cells and to terminals of ON cone bipolar cells (Strettoi et al., 1992, Demb and Singer, 2012, Hartveit and Veruki, 2012, Marc et al., 2014). These microcircuits are critical for night vision signaling in the retina.
In this study I was focused on delineating neuropeptide modulation of an established microcircuit in the inner retina, which is composed of a bi-directional communication between DA amacrine cells and M1 ipRGCs. In this microcircuit DA amacrine cells provide inhibitory input onto ipRGCs through the D1 receptor (Van Hook et al., 2012). On the other hand, light driven DA amacrine cell responses are determined by both ON-bipolar cell and ipRGC input. Therefore, ipRGCs provide excitatory drive for sustained DA amacrine cell light response (Zhang et al., 2008, Zhang et al., 2012).

In addition to functional electrophysiological data suggesting a reciprocal interaction between the two cell types, the two cell types share structural associations showing correlative anatomical evidence of interactions between the cells. DA amacrine cell processes and ipRGC dendrites are apposed in stratum 1 of the IPL (Belenky et al., 2003, Viney et al., 2007, Vugler et al., 2007, Dunitrescu et al., 2009). Furthermore, M1 ipRGCs receive dopamine modulation via D1 receptors, whereas the sustained light response of DA amacrine cells driven by M1 ipRGCs is dependent on AMPA/Kainate receptors, thus both cell types also have specific receptors that allow for synaptic interactions (Puopolo et al., 2001, Wong et al., 2007, Zhang et al., 2008, Van Hook et al., 2012). This circuit mediates functions responsible for light adaptation, pupillary light reflex, and circadian control of light response (Barnard et al., 2006, Zhang et al., 2008, Van Hook et al., 2012). The aim was to determine SRIF neuropeptide-mediated regulations of this microcircuit, and to what extent this changes the membrane properties and light responses of the cells in question (see Chapter 3).

Additionally, chapter 4 of this report begins the characterization of the VIP amacrine cells. I examined intrinsic electrophysiological properties of VIP amacrine cells including the presence of different ion channels on VIP amacrine cells, pharmacological isolation of inhibitory synaptic inputs and anatomical localization of excitatory inputs.
AIMS:
The computation of visual stimuli by ganglion cells prior to relaying that signal to the brain is critically determined by amacrine cell inputs. Amacrine cells are the most diverse, yet least understood retinal cell class, and most remain undefined in its circuit function. The objective of this study is to define the means by which neuropeptidergic amacrine cell inputs may impact inner retinal microcircuits. In order to do so, I described specific transgenic mouse lines, tested neuropeptide influences on an established inner retinal circuit using anatomical, electrophysiological, and pharmacological studies, and defined intrinsic electrophysiological properties of a neuropeptide releasing amacrine cell.

Aim 1. Characterization of specific transgenic mouse lines to study dopamine amacrine cells.
The goal of Aim 1 is to characterize the retinas of four different transgenic mouse lines that can be used to study dopamine-containing neurons and their relationship with SRIF-containing amacrine cells. This present study highlights the importance of careful characterization of transgenic mouse lines that are used in studies aimed at targeted electrophysiological recordings and description of cell-type specific anatomical features such as synaptic partners and receptor localization. The results are detailed accounts of transgene labeling compared to classical neurotransmitter markers. In addition, we performed Neurobiotin cell fills and quantitative analysis of cell numbers and soma/dendritic/axonal sizes. These findings are outlined in Vuong et al., 2015 (In press).

Aim 2. Somatostatin-mediated parallel inhibition of dopamine amacrine cells and type 1 intrinsically photosensitive retinal ganglion cells.
Aim 2 tested the hypothesis that somatostatin modulates the cellular properties of dopamine amacrine cells and M1 ipRGCs. These two cell types form a bi-directional inner retinal microcircuit that regulates retinal mediated functions such as light adaptation, circadian
photoentrainment, and signaling of non-visual information. Transgenic mouse models, anatomical modeling, and patch clamp electrophysiology were used to determine somatostatin-mediated inhibition of the microcircuit through specific somatostatin receptor subtypes. These findings are reported in Vuong et al., 2015 (Submitted).

Aim 3. *Intrinsic properties and synaptic partners of vasoactive intestinal polypeptide amacrine cells.*

The goal of Aim 3 is to identify the intrinsic channel properties of vasoactive intestinal polypeptide (VIP) amacrine cells. With the use of a transgenic mouse line that expresses Cre recombinase in VIP immunoreactive amacrine cells and patch clamp electrophysiology I am able to define key intrinsic properties of this cell type including specific ion channel expressions and synaptic partners. In addition, using this same VIP-Cre line crossed with a Cre-dependent channelrhodopsin mouse line, as a proof of concept, I could selectively activate these cells and determine the effects of VIP on ganglion cells, bipolar cells, and other amacrine cell types. These findings will greatly aid in understanding the role of VIP amacrine cells in visual processing.
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Heterogeneous transgene expression in the retinas of the TH-RFP, TH-Cre, TH-BAC-Cre and DAT-Cre mouse lines*

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Abstract

Transgenic mouse lines are essential tools for understanding the connectivity, physiology and function of neuronal circuits, including those in the retina. This report compares transgene expression in the retina of a tyrosine hydroxylase (TH)-red fluorescent protein (RFP) line with three catecholamine-related Cre recombinase lines [dopamine transporter (DAT)-, TH-bacterial artificial chromosome (BAC)-, and TH-Cre] that were crossed with a ROSA26-tdTomato reporter line. Retinas were evaluated and immunostained with commonly used antibodies including those directed to TH, GABA and glycine to characterize the RFP or tdTomato fluorescent-labeled amacrine cells, and an antibody directed to RNA-binding protein with multiple splicing to identify ganglion cells. In TH-RFP retinas, labeled type 1 and 2 dopamine (DA) amacrine cells were identified by their characteristic cellular morphology and, type 1 DA cells by their expression of TH immunoreactivity. In the TH-BAC-tdTomato retinas, labeled AII amacrine cells were predominant and there were also medium-diameter ganglion cells. In TH-tdTomato retinas, all tdTomato fluorescence was localized to amacrine cells; intracellular injections of Neurobiotin into these cells revealed four types of polyaxonal amacrine cells. In the TH-BAC- and TH-tdTomato retinas, less than 1% and ~6%, respectively, of the fluorescent cells were the expected type 1 DA amacrine cells. Retinas from two DAT-tdTomato founder lines had different patterns of tdTomato fluorescent cell expression. Founder line 1 had monostratified amacrine cells that ramified in stratum 2/3 of the inner plexiform layer. Neurobiotin injections into founder line 2 DAT-tdTomato retinas showed two types of bistratified and two types of monostratified amacrine cells. Furthermore, neither DAT-tdTomato founder line contained type 1 DA amacrine cells. Although each of the Cre lines were generated with the intent to specifically label DA cells, our findings show a cellular diversity in Cre expression in the adult retina, and indicate the importance of careful characterization of transgene labeling patterns. These mouse lines with their distinctive cellular labeling patterns will be useful tools for future studies of retinal function and visual processing.
Introduction

Transgenic mouse models are one of the major tools currently used for investigations of the intricate anatomical, biophysical and functional properties of neuronal cell populations and their networks. For instance, studies using mouse models have contributed to the identification and manipulation of the connectivity, interactions, and activity of neurons within a network, which has advanced our understanding of their role in neuronal processing in multiple structures including the retina, cortex, hippocampus, olfactory bulb and cerebellum (Ramirez et al., 2013, Hammen et al., 2014, Pohlkamp et al., 2014, Robinson et al., 2014, Sternson and Roth, 2014, Zhu et al., 2014a, Zhu and Roth, 2014). The use of transgenic mouse models has been especially powerful for studies of intrinsic retinal circuitry and function, including amacrine and ganglion cell properties and connectivity, as well as furthering the understanding of the functional connectivity of retinal ganglion cells with central visual nuclei (Kim et al., 2010, Kay et al., 2011a, Rivlin-Etzion et al., 2011, Yamagata and Sanes, 2012, Zhang et al., 2012, Dhande et al., 2013, Farrow et al., 2013, Yonehara et al., 2013, Vlasits et al., 2014, Zhu et al., 2014b).

Amacrine cells are classified based on their general morphology, including somal size, the stratification pattern of their processes in the inner plexiform layer (IPL), and their neurochemical expression (Ramón y Cajal, 1893, Boycott and Wässle, 1974, Brecha et al., 1979, Karten and Brecha, 1980, Kolb et al., 1981, Vaughn et al., 1981, Masland, 1988, Casini and Brecha, 1991). Based on these criteria, 30 to 40 different amacrine cell types have been identified in the mammalian retina (Kolb and Nelson, 1981, Xin and Bloomfield, 1997, MacNeil and Masland, 1998, MacNeil et al., 1999, Masland, 2001, Badea and Nathans, 2004) and they are likely to mediate different functions in visual processing (Masland, 2001, Wässle, 2004).

One well-studied wide-field amacrine cell type is the tyrosine hydroxylase (TH) or dopamine (DA) amacrine cell, which is also known as a type 1 DA amacrine cell. This amacrine cell synthesizes and releases dopamine, in addition to the inhibitory neurotransmitter GABA (Wulle and Wagner, 1990, Hirasawa et al., 2009, Contini et al., 2010, Hirasawa et al., 2012).
The morphology of type 1 DA amacrine cells have been thoroughly characterized in retinas of multiple species, including amphibians, rodents, lagomorphs, felines and primates using antibodies against TH, the rate-limiting enzyme for the synthesis of dopamine (Brecha et al., 1984, Versaux-Botteri et al., 1984, Oyster et al., 1985, Mariani and Hokoç, 1988, Nguyen-Legros, 1988, Dacey, 1990, Tauchi et al., 1990, Zhu and Straznicky, 1990). Physiological studies indicate multiple retinal functions for dopamine, including a role in light/dark adaptation of retinal circuits and serving as the output of the retinal circadian clock. Moreover, low levels of dopamine are often concomitant with retinal disease including diabetic retinopathy (Teakle et al., 1993, Witkovsky, 2004, Aung et al., 2014).

The identification of type 1 DA amacrine cells in transgenic lines have been of great value in advancing the understanding of their functional connectivity in the inner retina. For example, labeled type 1 DA amacrine cells have aided in defining the connectivity between these cells and GABA-containing and AII amacrine cells, ON-cone bipolar cells, and melanopsin-containing ganglion cells (Gustincich et al., 1997, Feigenspan et al., 2000, Zhang et al., 2007, Contini et al., 2010, Van Hook et al., 2012, Zhang et al., 2012, Newkirk et al., 2013). Other studies used isolated type 1 DA amacrine cells, labeled by human placental alkaline phosphatase (hPLAP), to characterize their intrinsic biophysical properties, including the presence of transient A-type K\(^+\), Ca\(^{2+}\) and TTX-sensitive Na\(^+\) currents, and rhythmic spontaneous action potentials (Feigenspan et al., 1998, Xiao et al., 2004). More recently, type 1 DA amacrine cells identified by green fluorescent protein (GFP) in retinal whole mounts of a dopamine receptor 2 transgenic mouse line were used to characterize their resting spontaneous spike properties and light responses (Newkirk et al., 2013).

Although there have been many advances in understanding type 1 DA amacrine cell function, their connectivity, functional properties and influence on other cells in the retinal network has not been completely established. Studies addressing these topics would be greatly facilitated by using a mutant mouse line with Cre recombinase activity exclusively in type 1 DA
amacrine cells to manipulate these cells. In this study, we have evaluated three transgenic mouse lines expressing Cre, and compared them to a previously published TH-RFP mouse line with DA amacrine cells expressing red fluorescent protein (RFP) (Zhang et al., 2004). Cre expression under the control of TH or dopamine transporter (DAT) regulatory elements, is expected to provide specific labeling of the type 1 DA amacrine cells in the retina, as in the central nervous system (Gelman et al., 2003, Lindeberg et al., 2004, Zhuang et al., 2005, Bäckman et al., 2006). However, our studies of the cellular expression of Cre reporter expression in the retinas of these mouse lines show that there is a surprisingly small percentage or a lack of type 1 DA amacrine cells labeled in these retinas. In contrast, these retinas did contain ectopic yet identifiable Cre-dependent fluorescently labeled cell types in the inner retina, including AII, polyaxonal amacrine cells (PAC), monostratified and bistratified amacrine cells, displaced amacrine cells, and a few ganglion cells. These Cre-expressing mouse lines will be of value to other investigations of the connectivity and physiology of the aforementioned amacrine cells.

Materials and Methods

Animal care and all experiments were carried out in accordance with the guidelines for the welfare of experimental animals issued by the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals and the University of California Los Angeles (UCLA) Animal Research Committee.

Mouse lines

Tyrosine hydroxylase-Cre recombinase (TH-Cre) (B6.Cg-Tg (TH-Cre)1Tmd/J; #008601) and dopamine transporter-Cre (DAT-Cre) (B6.SJL-Slc6a3tm1.1(cre)Bkmn/J; #006660) transgenic mouse lines were obtained from The Jackson Laboratory (Bar Harbor, ME). The TH-bacterial artificial chromosome-Cre (TH-BAC Cre) (Tg (TH-Cre) fl172Gsat/Mmucd; #029177-UCD)
transgenic mouse line was obtained from the Mutant Mouse Regional Resource Centers (MMRRC at University of California, Davis, California). The Cre-dependent tdTomato (B6.Cg-Gt (ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J.; #007908) and the Brainbow 2.1 (Gt(ROSA)26Sor^{tm1(CAG-Brainbow2.1)Cle}/J; JAX #13731) reporter lines were obtained from The Jackson Laboratory. The TH-red fluorescent protein (TH-RFP) mouse line was developed by Dr. Douglas G. McMahon and bred at UCLA for these studies (Zhang et al., 2004).

Mouse lines (DAT-tdTomato, TH-tdTomato, and TH-BAC-tdTomato) were generated by crossing the Cre-recombinase transgenic mouse lines: TH-Cre (Savitt et al., 2005), DAT-Cre (Bäckman et al., 2006) and TH-BAC Cre (Gong et al., 2007) to a tdTomato reporter line (Ai14) (Madisen et al., 2010). The DAT-Brainbow mouse line was generated by crossing the DAT-Cre line with the Brainbow2.1 reporter line (Livet, 2007). Breeding was carried out in the UCLA Division of Laboratory Animal Medicine facility, and offspring were genotyped to check for Cre and reporter expression. Primers used for detecting Cre were oIMR1084 (5'-GCG GTC TGG CAG TAA AAA CTA TC-3') and oIMR1085 (5'-GTG AAA CAG CAT TGC TGT CAC TT-3'). A predicted band at ~100 bp indicated the presence of the Cre transgene. Detection of tdTomato required two primer sets: 1) oIMR9020 (5'-AAG GGA GCT GCA GTG GAG TA-3') and oIMR9021 (5'-CCG AAA ATC TGT GGG AAG TC-3') for wild-type forward and reverse primers, and 2) oIMR9103 (5'GGC ATT AAA GCA GCG TAT CC-3') and oIMR9105 (5'-CTG TTC CTG TAC GGC ATG G-3') for the presence of tdTomato. A predicted band at 196 bp indicated the transgene for tdTomato, and heterozygote mice had predicted bands at 297 and 196 bp. The TH-RFP transgenic mouse line contained a TH-RFP transgene consisting of a 4.5 kb fragment of the rat tyrosine hydroxylase promoter ligated to DsRed2-1 (Zhang et al., 2004). Primers used for detecting RFP were 5'-GCA CCT TGA AGC GCA TGA A-3' and 5'-CAC TTT GTT ACA TGG GCT GGG-3'. A predicted band at ~590 bp indicated the presence of the transgene.

Tissue preparation
Male or female adult mice (4-6 weeks old) were deeply anesthetized using 1-3% isofluorane (IsoFlo, Abbott Laboratories, North Chicago, IL) and killed by decapitation or cervical dislocation. Eyes were enucleated, and the cornea and lens were removed.

*Vertical retinal sections:* Eyecups were immersion fixed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 for 15-60 minutes. Eyecups were subsequently washed in 0.1 M PB for 30 minutes and stored in 30% sucrose overnight at 4°C, and then embedded in optimal cutting temperature medium (Sakura Finetek Inc., Torrance, CA) and sectioned vertically at 12 µm. Retinal sections were placed onto gelatin-coated slides and stored at -20°C until used for immunohistochemistry.

*Whole-mount retinal preparation:* The retinas were removed from the eyecups, and four small incisions were made to lay the retina flat. Retinas were mounted, ganglion cell layer (GCL) up, onto nitrocellulose membrane filters (cat #HABP04700; Millipore Corporation, Billerica, MA), and fixed for 30-60 minutes in 4% PFA in 0.1M PB at room temperature. Whole-mounted retinas were then processed for immunohistochemistry.

**Immunohistochemistry**

*Vertical retinal sections:* Retinal sections were processed for immunohistochemical labeling using an indirect immunofluorescence method (Hirano et al., 2005). Frozen retinal sections were thawed for 10 minutes at 37°C on a warming plate, then washed three times for 10 minutes with 0.1 M PB (pH 7.4). Sections were then incubated in a blocking solution of 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X-100 in 0.1 M PB for 1 hour at room temperature. Following removal of the blocking solution, the primary antibody solution was immediately added to the sections and incubated for 12-16 hours at 4°C. Primary antibody solution contained 3% NGS, 1% BSA, 0.05% sodium azide and 0.5% Triton X-100 in
0.1M PB. Retinal sections were then washed three times for 10 minutes in 0.1 M PB. The sections were then incubated in their corresponding secondary antibodies goat anti-rabbit, -rat or -mouse conjugated to Alexa 568 or 488 (1:1000; Invitrogen, Grand Island, NY) for two hours at room temperature. The secondary antibody was removed and sections were washed three times in 0.1 M PB for 10 minutes per wash. Sections were air-dried and mounted using Aqua Poly/Mount (Polysciences, Warrington, PA). To control for nonspecific binding of the secondary antibody, the primary antibodies were omitted in the single-labeling studies.

Whole-mounted retina: Whole-mounted retinas were processed for immunohistochemical labeling with a protocol similar to that used for vertical sections. Whole-mounted retinas were fixed, washed in 0.1 M PB and then incubated in blocking solution overnight at 4°C. The retinas were then transferred to primary antibody solution and incubated for 5 days at 4°C. Retinas were washed in 0.1 M PB for 3 times for 20 minutes each time, and then incubated in secondary antibodies for 2 days at 4°C. Following removal from secondary antibody solution, retinas were washed three times in 0.1 M PB for 20 minutes for each wash. Similar to the retinal sections, whole-mounted retinas were briefly air-dried and mounted using Aqua Poly/Mount (Polysciences).

Antibodies

Retinal sections and whole mounts were processed with the following primary antibodies and dilutions: mouse monoclonal antibody against tyrosine hydroxylase (TH) (1:2000; MAB5280 clone 2/40/15; Millipore, Billerica, MA), mouse polyclonal antibody against calretinin (1:2000; 010399 clone 6B3; Swant, Bellinzona, Switzerland), rabbit polyclonal antibody against GABA [1:2000 (transverse sections) and 1:500 (whole mount); A2052; Sigma-Aldrich, St Louis, MO, USA], rat polyclonal antibody against glycine [1:3000 (transverse sections) and 1:1000 (whole mount); IG1002; ImmunoSolution, Everton Park, Queensland, Australia], goat polyclonal against choline acetyltransferase (ChAT) (1:250; No. AB144P; EMD Millipore), and guinea pig
polyclonal antibody against RNA-binding protein with multiple splicing (RBPMS) (1:20000) ((Rodriguez et al.)). The antibodies used in this study are listed in Table 1.

**Confocal image acquisition**

The immunostaining images were acquired with a Zeiss 510 Meta or 710 confocal laser scanning microscope (LSM Carl Zeiss, Thornwood, NY) equipped with 488, 543, and 633 nm laser lines. Confocal scans were captured using a Plan Neofluar 25X 0.8 NA corrected water objective and a C-APOCHROMAT 40X 1.2 NA corrected water objective. Projections of three images (1024 X 1024 pixels or 2048 X 2048 pixels) with a total of 0.9-1.0 µm thickness (z-axis step between 0.3-0.5 µm) were collected and adjusted for brightness and contrast in Adobe Photoshop CS2 v.9.02 (Adobe Systems, San Jose, CA).

**Quantification of somal number, somal size, and field size**

Digital images for cell counting were collected at 0.5 mm intervals from the optic nerve head to the peripheral retina in the superior, inferior, temporal and nasal retinal quadrants. Four retinal fields (425 x 425 µm²) per quadrant were collected for each retina using a Plan Apochromat 20X/0.8 na corrected air objective with a 0.7-1.0 magnification factor or a Plan Apochromat 40X/1.2 na corrected water objective. Cells were manually counted from the digital images using cell counter in ImageJ (http://rsb.info.nih.gov/ij/index.html) to determine cell number and density (cells/mm²).

Somasal diameter sizes and dendritic field sizes were measured from whole mounted retinas using Zeiss LSM image browser 510 proprietary software (version 3.2; Carl Zeiss, Thornwood, NY). Diameter sizes measurements were calculated as the longest distance between any two points on an object’s perimeter, or the maximum caliper. Field size measurements were made for five cells in each quadrant of a retina. These values were then averaged to determine the mean field size.
To characterize the cells expressing the transgenic reporter, whole-mounted retinal preparations of the TH-RFP, and the DAT-, TH-BAC-, and TH-tdTomato retinas were immunostained using multiple antibodies (Table 1), imaged and analyzed for co-expression. To determine the percent of co-localization, the number of RFP or tdTomato fluorescent cells and the number of immunoreactive cells that showed co-labeling were counted. Counts were made from 2-4 retinas from 2-4 different animals from each mouse line, and 16 retinal fields per retina.

**Intracellular injections**

Intracellular injections were performed as described previously (Pérez de Sevilla Müller et al., 2007, Müller et al., 2010a, Müller et al., 2010b). TdTomato-expressing cells were visualized with a Zeiss 40X water-immersion objective. Borosilicate glass electrodes (#60200; A-M Systems; Sequim, WA) were pulled and filled at their tips with 0.5% Lucifer Yellow (Sigma-Aldrich) 4% N-(2-aminoethyl)-biotinamide hydrochloride (Neurobiotin; Vector Laboratories, Burlingame, CA), and back-filled with 0.1 M Tris buffer, pH 7.4. Under visual guidance provided by the tdTomato fluorescence, cells were targeted for injection. First, Lucifer Yellow was iontophoresed (−1 nA) into the labeled cells and when its morphology could be visualized, the polarity of the current was reversed (+1 nA) and Neurobiotin injected for 3 minutes. After the final injection, the retina was kept in the bath solution for at least 30 minutes to allow diffusion of the Neurobiotin. The retinas were fixed in 4% PFA for 10 minutes. Neurobiotin was visualized by incubating injected retinas overnight at 4°C with streptavidin–indocarbocyanine (1:500; FITC; Jackson ImmunoResearch, West Grove, PA) in 0.1 M PB containing 0.3% Triton X-100 (Sigma-Aldrich). Retinas were washed in PB 3 times for a total of 30 minutes and mounted in Vectashield (Vector Laboratories).

**Results**
General cellular labeling patterns in the TH-RFP, TH-BAC-tdTomato, TH-tdTomato, and DAT-tdTomato retinas

TH-RFP: As reported previously, TH-RFP retinas express RFP in sparsely distributed somata located in the proximal inner nuclear layer (INL) with numerous processes that ramify in the OFF layer of the inner plexiform layer (IPL) (Fig. 1A) (Zhang et al., 2004). A few varicose processes ramified in the middle of the IPL (not shown) and in the outer plexiform layer (OPL) (Fig. 1A, arrows). A lower level of RFP expression was in small caliber processes (Fig. 1A, arrowheads) and small somal diameter amacrine cells (not shown). Somal diameter and dendritic field size, as well as the cell’s morphology were analyzed in whole-mount retinas. The average diameter of the large RFP fluorescent somata was 12.43 ± 2.33 μm (n=178 cells; 4 retinas; Table 2) and their average dendritic field size was about 850 μm (n=8 cells; 3 retinas) (Zhang et al., 2004). The morphology and distribution of the large RFP fluorescent cells in TH-RFP retinas are consistent with earlier descriptions of type 1 DA amacrine cells in the mouse retina (Fig. 2A) (Ballesta et al., 1984, Versaux-Botteri et al., 1984, Voigt and Wässle, 1987, Nguyen-Legros, 1988, Wulle and Schnitzer, 1989, Wulle and Wagner, 1990). The density of the type 1 DA amacrine cells was uniform throughout the retina with an average of 37 ± 17 cells/mm² (n=3 retinas; Table 2). TH-RFP retinas also contained another amacrine cell type (Fig. 2A, arrow) (Zhang et al., 2004). These cells had a lower level of fluorescence and a somal diameter of 9.89 ± 1.24 μm (n=136 cells; 2 retinas; Table 2). Their average density was 181 ± 54 cells/mm² (n= 2 retinas; Table 2). These observations are also consistent with an earlier report of type 2 DA amacrine cells in the TH-RFP retinas (Zhang et al., 2004).
Figure 1. Transgene reporter expression in TH transgenic mouse lines.

RFP and tdTomato expression driven by dopamine cell type specific promoter displays a heterogeneous labeling of amacrine cells in the retina. (A) RFP expression is limited to a subset of amacrine cells localized to the INL and primarily stratifies in S1 (arrowheads) and into the OPL (arrows). (B) TH-BAC-tdTomato line has transgene expression in narrow-field amacrine cells (arrowhead), medium diameter amacrine cells, and putative ganglion cells and their axons in the NFL (arrows). tdTomato expression in the processes is distributed throughout the IPL. (C) TH-tdTomato reporter expression is localized to small- and medium-sized somata in the INL and GCL. There are three primary bands of labeling in the IPL. S1: strata 1 of IPL. IPL: inner plexiform layer. INL: inner plexiform layer. GCL: ganglion cell layer. RFP: red fluorescent protein. Scale bar: 20 µm.
Figure 2. Reporter expression in whole mount retinas of the TH transgenic lines.

Reporter expression in whole mounted retinas were found in soma and process of cells that were distributed throughout the entire retina. (A) RFP expression in amacrine cells bodies localized to the INL and processes in S1 and 2/3. Lower levels of RFP were found in smaller soma sized cells (Arrow). (B) tdTomato expression in the TH-BAC-tdTomato line shows extensive labeling of narrow-field amacrine cells, medium-sized amacrine cells, and putative ganglion cells and their axons (arrowheads). (C) tdTomato expression under the TH promoter was found in cell somata primarily localized in the INL and a few in the GCL. Bottom panels are the z-plane rotation of the whole mount image. Scale bar: 50 µm.
**TH-BAC-tdTomato:** The TH-BAC-tdTomato retinas contained multiple small and medium diameter fluorescent cells that differed based on 1) their somal location, in the proximal INL and GCL, and 2) the morphology and sparse distribution of their processes in multiple strata of the IPL (Fig. 1B and 2B). The most prominent type was a narrow-field amacrine cell in the proximal INL with processes that formed lobular processes in the OFF sublamina, and varicose arborizations in the ON sublamina of the IPL (Fig. 1B, arrowhead). These cells had a small somal diameter that measured 6.48 ± 1.04 μm, (n=300 cells; 4 retinas; Table 2). The morphological features of this amacrine cell type are similar to descriptions of AII amacrine cells (Casini et al., 1995, Wässle et al., 1995, Massey and Mills, 1999, Wässle et al., 2009, Pang et al., 2012). A group of medium diameter cells (9.10 ± 0.57 μm; n=71 cells; 4 retinas; Table 2) was also in the INL. In the GCL, there were small diameter ganglion cells (10.54 ± 0.01 μm, n=676 cells; 3 retinas; Table 2) with axonal processes in the nerve fiber layer (NFL) (Fig.1B, arrows and 2B arrowheads to ganglion cell somata and their axons), and small diameter (6.86 ± 1.06 μm; n=139 cells; 2 retinas; Table 2) putative displaced amacrine cells based on the lack of observable axonal processes. The different cell types did not show regional differences in their density.

**TH-tdTomato:** The TH-tdTomato retinas also had multiple fluorescent cell types in the INL and GCL based on the stratification patterns of their processes in the IPL. Their processes mainly arborized in the OFF and ON-OFF sublaminae, and few processes arborized in the ON sublamina of the IPL (Fig. 1C and 2C). Fluorescent somata in the INL were found distal (arrowhead) and proximal (arrow) to the IPL (Fig. 1C). In the INL, cells were grouped into small (6.76 ± 0.99 μm; n=236 cells; 2 retinas; Table 2) and medium (10.11 ± 1.49 μm; n=84 cells; 2 retinas; Table 2) somal diameters. In the GCL, there were also two somal diameter groups, that measured 7.25 ± 1.02 μm and 10.34 ± 1.40 μm (n=36 and n=44 cells, respectively; 2 retinas; Table 2). The different sized cells in the INL and GCL occurred with a similar frequency in all
retinal regions. Medium diameter cells in both the INL and GCL had thick lateral processes (Fig. 2C) that extended over 500 µm in diameter (n=10 cells; 2 retinas). These cells were wide-field amacrine cells located in the INL or displaced into the GCL (Fig. 2C) (MacNeil and Masland, 1998, Völgyi et al., 2001, Völgyi et al., 2009).

**DAT-tdTomato:** Two founder lines of the same DAT-Cre line received from Jackson Laboratories about one year apart showed two consistently distinct patterns of tdTomato expression. In vertical sections of DAT-tdTomato retinas from founder line 1, fluorescent amacrine cells in the proximal INL and in the GCL gave rise to a single primary process that entered the IPL and arborized in a distinct band in the middle of the IPL (Fig. 3A). The average diameter of the fluorescent somata in the INL and GCL was 8.78 ± 1.42 µm (n=80 cells; 2 retinas; Table 2) and they were smaller than the type 1 and type 2 DA amacrine cells described previously in mouse retina (Wulle and Schnitzer, 1989, Gustincich et al., 1997, Zhang et al., 2004, Knop et al., 2011). The tdTomato fluorescent somata were distributed across the retina (43 ± 3 cells/mm²; n=2 retinas; Table 2) in both the proximal INL (Fig. 3A and 4A) and GCL (Fig. 3A) with more than 90% of these cells in the INL compared to ~8% in the GCL in whole-mounted retinas. They showed no obvious regional differences in their density in central, mid-peripheral and peripheral retinal regions.
Figure 3. Transgene reporter expression in the DAT transgenic mouse line.

The tdTomato expression driven by DAT specific promoter displays a heterogeneous labeling of amacrine cells in the retina. (A) Founder line 1 of the DAT-tdTomato line had tdTomato expression in a subset of amacrine cells localized to the INL and displaced into the GCL. There is a single band of processes in the middle of the IPL. (B) Founder line 2 of the DAT-tdTomato line had tdTomato expression, driven by the same DAT promoter, in medium diameter amacrine cells localized to the INL. tdTomato expression in the processes is distributed in S1, 3, 4, and 5 of the IPL. S: strata of IPL. IPL: inner plexiform layer. INL: inner plexiform layer. GCL: ganglion cell layer. RFP: red fluorescent protein. DAT: dopamine transporter. Scale bar: 20 µm.
Figure 4. Reporter expression in whole mount retinas of the DAT transgenic line.

Reporter expression in whole mounted retinas were found in soma and process of cells that were distributed throughout the entire retina. (A) tdTomato expression in the DAT-tdTomato (Founder 1) line is found in amacrine cells with similar somal size and they were distributed throughout the entire retina. (B) tdTomato expression in the DAT-tdTomato line (Founder 2) shows extensive labeling of medium-sized amacrine cells. tdTomato expression under the DAT promoter was found in cell somata primarily localized in the INL and a few in the GCL. Bottom panels are the z-plane rotation of the whole mount image. Scale bar: 50 µm.
On the other hand, vertical sections of DAT-tdTomato retinas from founder line 2 showed numerous somata in the proximal INL (Fig. 3B) and GCL (Fig. 4B). Their processes were localized in a narrow band in the OFF sublamina proximal to the INL, and broadly in the ON sublamina of the IPL (Fig. 3B), suggesting that there may be multiple types of cells labeled in this line. Moreover, there were several other cells in the INL that had robust tdTomato processes, which extended from the OFF sublamina into the ON sublamina of the IPL (Fig. 3B, arrows). The average somal diameter of these cells in the INL and GCL was $9.26 \pm 1.11$ (n=132 cells; 3 retinas; Table 2). The fluorescent tdTomato cells were spread evenly across the retina, and there were no observable regional differences ($646 \pm 160$ cells/mm$^2$ of retina; n=3 retinas; Table 2). Comparison of the tdTomato fluorescent somata counts in the GCL versus INL revealed fewer cells in the GCL (~13%) compared to the INL (~87%) (Fig. 4B).

**Characterization of the fluorescent cells in the transgenic retinas using specific amacrine and ganglion cell immunohistochemical markers**

*TH-RFP retina:* Previous studies have established that type 1 DA amacrine cells form a population of wide-field amacrine cells that robustly express TH-immunoreactivity in their somata and processes that mainly ramify in stratum 1 of the IPL in the mouse retina (Fig. 5A) (Ballesta et al., 1984, Versaux-Botteri et al., 1984, Wulle and Wagner, 1990, Witkovsky, 2004). In the TH-RFP retinas, large diameter fluorescent amacrine cells in the INL and their processes contained TH immunoreactivity (Fig. 5A). TH immunostaining in finer caliber processes was more prominent compared to RFP fluorescence (Fig. 5A, arrowheads). All large diameter RFP fluorescent somata contained TH immunoreactivity and conversely all TH immunoreactive somata contained RFP fluorescence (Table 3) in all retinal regions of whole-mount preparations. Consistent with previous studies, the small diameter and weakly fluorescent somata did not contain TH immunoreactivity (not shown) (Gustincich et al., 1997, Zhang et al., 2004, Knop et al., 2011).
Figure 5. RFP fluorescence compared to TH, calretinin, GABA, and glycine immunoreactivity.

Defined co-localization of TH, calretinin, and GABA immunoreactivity with RFP fluorescence shows the TH-RFP line contains labeled type 1 and 2 DA amacrine cells. (A) Large soma sized RFP fluorescent cell (type 1 DA amacrine cells) is co-localized with TH immunoreactivity. Arrowhead: TH immunoreactivity is stronger in fine caliber processes compared to RFP fluorescence. (B) Calretinin immunoreactivity is not co-localized with large soma-sized RFP fluorescent cells, but does co-localize with small soma-sized RFP fluorescent cells; RFP labeled processes are in S1, 2/3, and weakly in 3. (C) All RFP expressing amacrine cells contain GABA immunoreactivity. Arrowhead shows co-localization. (D) RFP expressing cells do not contain glycine immunoreactivity. Arrow indicates lack of co-localization between RFP fluorescence and glycine immunoreactivity. Scale bar: 20 µm.
To aid in determining the level of stratification of the RFP fluorescent processes, we used a calretinin antibody, which labels amacrine cells and ganglion cells in the INL and GCL, and their processes in strata 1/2, 2/3 and 3/4 of the IPL (Fig. 3B) (Haverkamp and Wässle, 2000, Ghosh et al., 2004). RFP fluorescent processes were in strata 1, 2/3, and 3, and in the OPL. RFP fluorescent processes in strata 2/3 and 3 were fainter in comparison to calretinin fluorescent processes. The small diameter RFP fluorescent cells and their processes in stratum 2/3 were calretinin immunoreactive (Fig. 5B).

Next, we tested for GABA and glycine expression in the RFP fluorescent cells. All RFP fluorescent cells contained GABA immunoreactivity (Fig. 5C, arrowhead), but they did not contain glycine immunoreactivity (Fig. 5D, arrow). These findings are consistent with previous reports on type 1 and 2 DA amacrine cells (Wulle and Wagner, 1990, Nguyen-Legros et al., 1997, Völgyi et al., 1997).

TH-BAC-tdTomato retina: In the TH-BAC-tdTomato retinas there were very few medium to large diameter fluorescent cells with TH immunoreactivity (Fig. 6A, inset). In retinal whole-mount preparations, 0.32% (n=50/15415 cells; 4 retinas; Table 3) of the fluorescent cells contained TH immunoreactivity. Conversely, about 11% (n=50/449 cells; 4 retinas) of the TH-immunoreactive cells expressed tdTomato fluorescence.
Figure 6. Expression of tdTomato fluorescence and TH, calretinin, GABA, and glycine immunoreactivity in vertical sections of TH-BAC-tdTomato retinas.

(A) TH-BAC-tdTomato cells rarely co-localize with TH immunoreactive amacrine cells. Inset shows a rare case of co-labeling of a cell with tdTomato fluorescence and TH immunoreactivity. (B) A subset of TH-BAC-tdTomato expressing cells co-localized with calretinin immunoreactivity in the GCL. (C) TH-BAC-tdTomato expression is found in some GABA immunoreactive cells. (D) There is tdTomato expression in some glycine immunoreactive cells. Arrowheads indicate co-localization. Arrows highlight cells that do not co-localize. Scale bar: 20 µm.
Varicose tdTomato fluorescent processes ramified extensively in strata 1, 2/3, 4 and 5 of the IPL, and there were also thin, smooth fluorescent processes in the NFL (Fig. 1B and 6A, bottom). There were fewer fluorescent processes in strata 1 and 2/3 compared to strata 4 and 5 of the IPL (Fig. 1B and 6). TdTomato fluorescent cells with calretinin immunoreactivity were in the INL (not shown) and in the GCL (Fig. 6B) and their co-localized processes were in the OFF sublamina of the IPL.

Calretinin and GABA immunoreactivity was co-localized to about 9% and 8% (n=1587/17303 and 930/12576 cells; 3 retinas) of the tdTomato fluorescent cells, respectively (Fig. 6B and C;Table 3). In the INL and GCL, about 9% and 74% of the fluorescent cells, respectively, were GABA immunoreactive (Table 3). The small diameter cells found in the GCL (6.86 ± 1.06 µm; n=139 cells; 2 retinas; Table 2) were identified as displaced amacrine cells based on their expression of GABA immunoreactivity (Fig. 6C). The medium diameter (9.10 ± 0.57 µm; n=20 cells; 2 retinas; Table 2) cells in the INL (Fig. 7A, left panel, arrows) were GABA immunoreactive, and had processes that ramified in the OFF sublamina of the IPL. These wide-field amacrine cells had field sizes that were greater than 300 µm (n=10 cells; 2 retinas) in diameter and were found throughout the retina, but were infrequent overall (Fig. 7A, left panel, arrows).
Figure 7. Characterizations of TH-BAC-tdTomato whole mount retinas reveal several distinct types of amacrine cells.

Several amacrine cell types are labeled in the TH-BAC-tdTomato line. (A) In the INL and OFF sublamina there are the infrequently occurring wide-field amacrine cells (left panel, arrows). They have polyaxonal-like properties, and their processes extend more than 200 µm laterally across the retina. Also localized to the INL are clusters of glycine immunoreactive amacrine cells. Defined by a narrow-field morphology, these cells are AII amacrine cells (right panel). (B)
In the GCL there is tdTomato expression found in displaced amacrine cells, and ganglion cells and their axons (left panel). Arrowheads point to cells co-localized with RBPMS immunoreactivity, a retinal ganglion cell marker, and shows this line also contains tdTomato fluorescent ganglion cells (right panel). Scale bar: 50 µm.
About 85% (n=40/47 cells; 2 retinas; Table 3) of the tdTomato-expressing cells in the INL contained glycine immunoreactivity (Fig. 6D, arrowhead), and displayed a stratification pattern in the IPL similar to that reported for AII amacrine cells (Fig. 6D and 1B) (Wässle et al., 1995, Menger et al., 1998, Massey and Mills, 1999). In the proximal INL, small diameter (6.48 ± 1.04 µm; n=300 cells; 2 retinas; Table 2) cells were characterized by lobular appendages in the OFF sublamina, and varicose arborizations in the ON sublamina of the IPL (Fig. 6 and 7A, right panel). These findings support the suggestion that AII amacrine cells are labeled in this line. AII amacrine cells in whole mounts were found in clusters in all retinal regions (Fig. 7A, right panel). The medium diameter cells in the GCL measured 10.54 ± 1.53 µm (n=181 cells; 2 retinas; Table 2). About 25% of the tdTomato fluorescent cells were not immunoreactive for GABA or glycine, and had smooth axon-like processes that extended along the NFL, suggesting they are ganglion cells (Fig. 7B, left). To test this possibility, whole-mounted retinas were immunostained with an antibody to RBPMS, a specific marker for retinal ganglion cells (Rodriguez et al., 2014). About 24% (n=947/3911 cells; 3 retinas) in the GCL, contained RBPMS (Table 3) and their axons contained RBPMS immunoreactivity (Fig. 7B, right panel, arrowheads). Less than 0.5% (n=50/10802 cells; 3 retinas) of the tdTomato cells in the INL contained RBPMS immunoreactivity (Table 3). The tdTomato cells that did not co-localize with GABA, glycine, or RBPMS are less than 5% and 1% of the tdTomato cells in the INL and GCL, respectively (see Discussion). The tdTomato cells that were co-localized to RBPMS were few and sparsely distributed, some were close in proximity and others were further apart (Fig. 7B, right panel, arrowheads). The soma size of the tdTomato cells that co-localized with RBPMS immunoreactivity in the GCL had soma sizes that ranged from 7.44 to 19.27, and averaged 10.98 ± 0.08 µm (n=719 cells; 3 retinas). Those in the INL ranged from 7.92 to 15.29 µm, and averaged 10.02 ± 0.32 µm (n=50 cells; 3 retinas). Collectively these findings indicate that multiple ganglion cell subtypes are labeled in this line (Sun et al., 2002, Völgyi et al., 2009).
*TH-tdTomato retina:* In vertical sections of TH-tdTomato retinas there were few medium to large diameter fluorescent cells with TH immunoreactivity (Fig. 8A inset). Numerous fluorescent cells also contained calretinin in both the INL and GCL, and their processes ramified in a distinct band in stratum 2/3 of the IPL, and weaker bands in strata 1 and 4 of the IPL (Fig. 8B). TdTomato cells were positive for GABA immunoreactivity (Fig. 8C) in the INL and GCL, but lacked glycine immunoreactivity (Fig. 8D).
Figure 8. Expression of tdTomato fluorescence and TH, calretinin, GABA, and glycine immunoreactivity in vertical sections of TH-tdTomato retinas.

(A) Most tdTomato fluorescence amacrine cells in the INL do not contain TH immunoreactivity. However, there are subsets of amacrine cells localized to the INL (inset) that co-label with TH immunoreactivity. (B) Several populations of cells labeled in the TH-tdTomato line co-express calretinin immunoreactivity. These cells are localized to the INL and GCL, and their processes correspond with strata 1, 2/3, and 4. (C) TH-tdTomato expressing cells in transverse sections is primarily GABA immunoreactive, in both the INL (arrowhead) and GCL (inset). (D) In vertical sections tdTomato fluorescence does not co-localize with glycine immunoreactivity (arrows). Scale bar: 20 µm.
The small diameter cells (6.76 ± 0.99 µm; n=236 cells; 2 retinas; Table 2) in the INL were monostratified cells with processes in strata 1 or 2/3 (Fig. 8B). The small diameter cells in the GCL (7.25 ± 1.02 µm; n=36; 2 retinas; Table 2) had processes that primarily ramified in stratum 2/3 (not shown), similar to type 2 DA amacrine cells or the monostratified cells in the DAT-tdTomato-founder 1 line. The medium diameter somata (10.11 ± 1.49 µm; n=84 cells; 2 retinas; Table 2) in the proximal INL had multiple primary processes that formed a thick band in the ON-OFF sublamina and other processes that were distributed throughout the IPL (Fig. 1C and Fig. 8). The medium diameter somata (10.34 ± 1.40 µm; n=44 cells; 2 retinas; Table 2) in the GCL primarily ramified in stratum 4 of the IPL (Fig. 1C and Fig. 8).

In retinal whole mounts, about 81% (n=1483/1829 cells; 3 retinas; Table 3) of the fluorescent somata contained calretinin immunoreactivity. Although in whole-mount retinas a few cells in the INL (1.5%; n=16/1062 cells; 2 retinas; Table 3) contained glycine immunoreactivity (Fig. 9A, arrows), the majority (89%) of the tdTomato fluorescent somata in the INL (Fig. 9B) and GCL (Fig. 9C) were GABA immunoreactive (n=221/248 cells; 2 retinas; Table 3).
Figure 9. TH-tdTomato expression is limited to amacrine cells.

In whole mount TH-tdTomato retinas, tdTomato fluorescent expression is found in GABA immunoreactive amacrine cells and very few and glycine immunoreactive amacrine cells. (A) Arrows point to the few tdTomato expressing cells that co-label with glycine immunoreactivity in the INL. (B) Most GABA immunoreactivity robustly co-localizes with tdTomato expression in the INL. Some tdTomato expressing cells have low levels of GABA immunoreactivity. (C) All tdTomato expression in the GCL co-labels with GABA immunoreactivity. Scale bar: 50 µm
Figure 10. tdTomato fluorescence is expressed in some starburst and type 1 DA amacrine cells in whole mount TH-tdTomato retinas.

tdTomato fluorescence in the TH-tdTomato line is localized to a subset of starburst and type 1 DA amacrine cells. (A) ChAT immunoreactivity co-localizes with a few tdTomato expressing cells in the INL. (B) ChAT immunoreactivity is found in a subset of tdTomato expressing cells in the GCL. (C) TH immunoreactivity co-labels with some tdTomato expressing cells in the INL (arrowheads), but tdTomato expression is not found in all type 1 DA amacrine cells, as indicated by TH immunoreactive cells that lack tdTomato fluorescence (arrows). Scale bar: 50 µm.
There were several types of fluorescently labeled GABA-containing cells, including a few that contained ChAT (Fig. 10A and B) or TH immunoreactivity (Fig. 10C). ChAT immunoreactivity was found in about 6% (n=63/1038 cells; 2 retinas; Table 3) of the tdTomato fluorescent cells. About 6% (n=141/2238 cells; 4 retinas; Table 3) of the fluorescent cells contained TH immunoreactivity (Fig. 10C arrow vs. arrowheads). Conversely, about ~28% (n=141/507 cells; 4 retinas, Table 3) of the TH-immunoreactive cells expressed tdTomato fluorescence. In addition, another group of GABA immunoreactive cells in the TH-tdTomato retinas were wide-field amacrine cells. In order to illustrate the individual morphological attributes of the wide-field amacrine cell types labeled in this mouse line, we performed intracellular injections of Neurobiotin into tdTomato-expressing cells in whole mount preparations. At least four types of polyaxonal amacrine cells contained tdTomato fluorescence. Classification of the PAC was determined based on their cellular localization, dendritic branching, and axon-like processes (Famiglietti, 1992a, b, Völgyi et al., 2001, Wright and Vaney, 2004, Völgyi et al., 2009, Greschner et al., 2014). In the INL, there were two types of polyaxonal amacrine cells: one that had radially oriented processes with prominent varicosities, which stratified in S2 of the IPL (Fig. 11A), and the second type corresponded to type 1 DA amacrine cells, which stratified in S1 of the IPL (Fig. 11A'). Cellular reconstructions of the Neurobiotin injected cells highlighted the morphological differences between the two types, in which the radially oriented polyaxonal amacrine cells have prominent thin and smooth, axon-like processes that extended 1-2 mm from the cell body (Fig. 11A") and the type 1 DA amacrine cells with wide-field processes that extended about 1 mm in diameter from the cell body (Fig. 11A"'). The GCL also had two types of polyaxonal amacrine cells, which are differentiated by asymmetrical (Fig. 11B) and radially oriented (Fig. 11B’) processes. The processes of the asymmetrical polyaxonal amacrine cells had numerous spines (Fig. 11B) and tended to have long axon-like processes that spanned the retina, and often they were over 1 mm in length (Fig. 11B”). In contrast, the processes of the radial polyaxonal amacrine cells had numerous
varicosities (Fig. 11B’') and they extended about 1 mm in diameter from the cell body (Fig. 11B’’’). The processes of both types of displaced polyaxonal amacrine cells stratified in S5 of the IPL (Fig. 11B and B’).
Figure 11. Intracellular injections of neurobiotin reveals multiple populations of polyaxonal wide-field amacrine cells.

tdTomato fluorescence in the TH-tomato line labels different morphological types of polyaxonal wide-field amacrine cells. (A) An example of a polyaxonal amacrine cell localized to the INL with radial and varicose processes that stratifies in S2 and 3 of the IPL. (A') Intracellular fill of a type 1 DA amacrine cell and its processes are found in S1 of the IPL. (A'') Cellular reconstruction of a wide-field polyaxonal amacrine cell. It has axon-like processes that extend the entire retina. (A''') Cellular reconstruction of a type 1 DA amacrine cells localized to the INL. (B) Neurobiotin intracellular injection of a polyaxonal amacrine localized to the GCL. Processes show many dendritic spines in S5 of the IPL. (B') An example of a polyaxonal amacrine in the GCL that has varicose processes. (B'') Cellular reconstruction of polyaxonal amacrine cell found in B, which is defined by an asymmetrical dendritic arborization. (B''') Cellular reconstruction of polyaxonal amacrine cell found in B', which is an example of a polyaxonal amacrine cell with varicose and radial dendritic arborizations. Scale bar: A, A', B, and B' = 50 µm, A'' = 300 µm, A'''' = 100 µm, B'' and B'''' = 300 µm
Finally, TH-tdTomato fluorescent somata did not contain RBPMS immunoreactivity (not shown), indicating that retinal ganglion cells in the TH-tdTomato retina do not express Cre activity.

*DAT-tdTomato retina – founder line 1:* DAT-tdTomato fluorescent cells in both the INL and GCL have a single primary process that entered the IPL and arborized in stratum 2/3 of the IPL (Fig. 12). The fluorescent cells were not TH immunoreactive (Fig. 12A), and their processes colocalized with the calretinin immunoreactive processes in stratum 2/3 of the IPL (Fig. 12B).
Figure 12. Expression of tdTomato fluorescence compared to TH, calretinin, GABA, and glycine immunoreactivity in vertical sections of DAT-tdTomato retinas (Founder line 1).

(A) tdTomato fluorescence does not co-localize with TH immunoreactivity. (B) TdTomato expression in the DAT-tdTomato line is co-localized with calretinin immunoreactivity in somata located in INL and GCL and processes that are and distributed to S2/3. Inset shows a tdTomato cell that does not co-express calretinin immunoreactivity. (C) All tdTomato fluorescent cells express GABA immunoreactivity, (D) but they are not glycine immunoreactive. Scale bar: 20 µm.
All fluorescent cells contained GABA immunoreactivity (Fig. 12C, arrowheads) and they lacked glycine immunoreactivity (Fig. 12D, arrows). About 67% (n=168/251 cells; 2 retinas) of the tdTomato fluorescent cells in the INL and GCL contained calretinin immunoreactivity (Table 3). This monostratified amacrine cell is similar to descriptions of a “bright” monostratified amacrine cell in a CD44-EGFP line (Sarthy et al., 2007).

The processes of the tdTomato fluorescent cells in the INL and GCL (Fig. 13A, B) formed a symmetrical field averaging at least 189.7 ± 23.8 µm (n=40 cells; 2 retinas) in diameter (Fig. 5A’, B’). In mid-peripheral retina, ~1 mm from the optic nerve, the nearest neighbor distance was 42.83 ± 34.7 µm (n=60 cells; 3 retinas). The fluorescent cells in the INL and GCL evaluated showed no differences in their somal diameters, stratification level in the IPL, and field size (Fig. 13). Together, their distinct morphology, including the narrow stratification of their processes in stratum 2/3 of the IPL and their expression of GABA immunoreactivity (Fig. 12 and 13) is consistent with their identity as a single cell type. Their morphological features, such as somal size, dendritic field size, and calretinin immunoreactivity are similar, but not identical to earlier descriptions of other monostratified amacrine cell types that ramify in the middle of IPL (Versaux-Botteri et al., 1986, Tauchi et al., 1990, Gustincich et al., 1997, Oh et al., 1999, Badea and Nathans, 2004, Sarthy et al., 2007, Knop et al., 2011, Knop et al., 2014).
DAT-tdTomato expressing cells are a single population of amacrine cells. (A) tdTomato expression is found in cell somata localized to the INL, and has a primary process that emanates from the cell soma and stratifies in S2/3 of the IPL. (A’) Cellular reconstructions of two tdTomato expressing cells in the INL show that both cells exhibit similar morphology. (B) tdTomato expression is localized to amacrine cell somata displaced into the GCL. These cells have the same morphology as those found in the INL, with a primary process leaving the cell soma and stratifying in S2/3 of the IPL. (B’) Cellular reconstructions of cells localized to the GCL show uniform type of monostratified amacrine cells. Note that the reconstructions of the amacrine cells are incomplete due to the difficulty of differentiating processes of individual cells. Scale bar: 50 µm.
DAT-tdTomato retina – founder line 2: Founder line 2 of the DAT-tdTomato line had fluorescent cells primarily in the INL (Fig. 3B, 4B, and 14). Similar to founder line 1, tdTomato cells in founder line 2 of the DAT-tdTomato line were not TH or glycine immunoreactive, but were all GABA immunoreactive (Fig. 14A, C, and D, and Table 3). Unlike founder line 1, the tdTomato fluorescent cells in founder line 2 did not co-localize with calretinin, and their processes were found in S1, 3, 4, and 5 (Fig. 14B). Thus, like the first founder line, the second founder line only labeled GABA expressing amacrine cells.
Figure 14. Expression of tdTomato fluorescence compared to TH, calretinin, GABA, and glycine immunoreactivity in vertical sections of DAT-tdTomato retinas (Founder line 2).

(A) tdTomato fluorescence does not co-localize with TH immunoreactivity. (B) TdTomato expression in the DAT-tdTomato line does not co-localize with calretinin immunoreactivity. TdTomato processes are distributed between the calretinin immunoreactive plexus, which corresponds to S1, 3, 4, and 5. (C) All tdTomato fluorescent cells express GABA immunoreactivity, (D) but they are not glycine immunoreactive. Scale bar: 20 µm.
To test whether there are multiple types of amacrine cells we made intracellular injections of Neurobiotin into tdTomato-expressing cells in whole mount preparations. The tdTomato fluorescent cells in the INL are predominantly composed of two types of bistratified amacrine cells and one type of monostratified amacrine cell (Fig. 15 A, B, and C). There were also a few large bistratified amacrine cells. They had an average somal diameter of 9.55 ± 0.21 µm, and their average dendritic field size was 670 ± 26.94 µm in the OFF sublamina and 665 ± 33.80 µm in the ON sublamina of the IPL (Fig. 15A, A’). These bistratified amacrine cells can be categorized as wide-field. On the other hand, the more frequent occurring small bistratified amacrine cells had an average somal diameter of 11 ± 1.55 µm, and an average dendritic field size of 388 ± 84.24 µm in the OFF sublamina and 297 ± 62.65 µm in the ON sublamina of the IPL (Fig. 15 B, B’). This group of bistratified amacrine cells can be classified as medium-field. In addition, the INL had wide-field amacrine cells that stratified in S1 of the IPL (Fig. 15C, C’). This cell had a soma size of 10.6 µm and a dendritic field size of 885.2 µm. Finally, in the GCL we found displaced amacrine cells with an average soma size of 10.0 ± 0.28 µm and a smaller dendritic field size (229 ± 48.41 µm) compared to the monostratified cells in the INL (Fig. 15 D, D’).
Figure 15. Neurobiotin intracellular injections of DAT-tdTomato fluorescent cells shows multiple populations of bistratified and monostratified amacrine cells.

DAT-tdTomato expressing cells are multiple populations of amacrine cells. (A) An example of a wide-field bistratified amacrine cell localized to the INL with symmetrical and varicose processes that stratifies in S1, 4 and 5 of the IPL. (A’) Cellular reconstruction of a wide-field bistratified amacrine cell. It has long processes in the OFF sublamina (black) and ON sublamina (red), and extends over 500 µm in diameter. (B) Neurobiotin intracellular injection of a medium-field bistratified amacrine cell localized to the INL. Processes in S1, 3, 4, and 5 of the IPL. (B’) Cellular reconstruction of medium-field bistratified amacrine cell found in B, which is defined by a radial dendritic arborization. (C) Neurobiotin intracellular injection of a wide-field amacrine localized to the INL. There are multiple processes that stratify in S1 of the IPL. (C’) Cellular reconstruction of the wide-field amacrine cell found in C, which is defined by long and radial dendritic arborization. (D) Neurobiotin intracellular injection of a displaced amacrine localized to the GCL. There are multiple processes that stratify in S4 of the IPL. (D’) Cellular reconstruction of the displaced amacrine cell found in B’. Scale bar: 100 µm
Together, these two founder lines produced two unique patterns of tdTomato expression. In particular, the analysis of Neurobiotin injected tdTomato fluorescent cells in whole mount retinas and GABA immunoreactivity in the transverse sections suggests that there are multiple amacrine types. An explanation for the differences in tdTomato expression in these DAT-Cre lines is a ‘faulty’ tdTomato reporter line. To test this possibility we generated the DAT-Brainbow line by breeding the DAT-Cre founder line 2 with the Brainbow2.1 mouse line. We found reporter expression in the same bistratified and monostratified amacrine cells (not shown). Thus, these differences were not from reporter line contamination, rather another example of ectopic transgene expression.

Discussion

This study has evaluated Cre-mediated fluorescence in the retinas of three Cre transgenic mouse lines crossed with a tdTomato reporter line (Ai14) (Savitt et al., 2005, Bäckman et al., 2006, Gong et al., 2007, Madisen et al., 2010). Their retinas had a low percentage or no type 1 DA amacrine cells with Cre activity in comparison to the previously characterized TH-RFP, TH-PLAP and TH-GFP mouse lines, which only had labeled type 1 and 2 DA amacrine cells (Gustincich et al., 1997, Matsushita et al., 2002, Zhang et al., 2004, Knop et al., 2011).

The retinas of many transgenic lines, including both Cre and BAC-Cre lines have reporter expression in specific retinal cell types (Rowan and Cepko, 2004, Haverkamp et al., 2009, Lu et al., 2009, Siegert et al., 2009, Ivanova et al., 2010). The cellular expression of the transgene in these lines includes: 1) the ectopic expression of the transgene in cell types that do not endogenously express the gene, 2) the incomplete expression of the transgene in its endogenous cell types, or 3) a combination of both (Gong et al., 2007, Haverkamp et al., 2009, Ivanova et al., 2010). These patterns of Cre-induced tdTomato fluorescence are also seen in our screen of the dopamine transmitter-related Cre lines: the TH-BAC- and TH-tdTomato retinas had incomplete labeling of type 1 DA amacrine cells as well as numerous other ectopically
labeled amacrine cells, and the DAT-tdTomato retina, founder line 1 had a single monostratified amacrine cell type, where as founder line 2 had predominantly bistratified and few monostratified amacrine cells, but no labeling of the type 1 DA amacrine cells.

**Transgenic expression of fluorescent reporters**

*TH-RFP:* The fluorescent labeling pattern in the TH-RFP transgenic mouse line is similar to previous findings by Zhang et al. (2004). The TH-RFP line contains type 1 DA amacrine cells, which were defined by their characteristic morphology, including a medium to large soma, extensive wide-ranging arborizations in stratum 1 of the IPL, and TH (Fig. 3A) and GABA (Fig. 3C) immunoreactivity. In addition, TH-RFP retinas contain type 2 DA amacrine cells. However, type 2 DA amacrine cells in mouse retina, have not been reported to contain TH immunoreactivity (Zhang et al., 2004) suggesting an absence or a very low level of TH protein (Stuber et al., 2015). The lack of TH immunostaining in the fluorescent type 2 DA amacrine cell types was attributed to high sensitivity of the transgenic reporters compared to that of immunohistochemical detection of TH immunoreactivity (Zhang et al., 2004). Type 2 DA amacrine cells do not express TH immunoreactivity in the different lines that they have reported their presence.

**TH-BAC-, TH-, and DAT-Cre lines:**

*TH-BAC-tdTomato:* The TH-BAC-Cre line was generated by inserting an intron containing a Cre gene at the start of a Th gene coding region (ATG) (<150kb) into a BAC vector (Gong et al., 2007). Cre expression was in TH-immunoreactive neurons in all of the catecholamine cell groups (Gong et al., 2007). In addition, Cre-induced EGFP fluorescence was present in some striatal and hypothalamic neurons that were not TH-immunoreactive (Komori et al., 1991, Marin et al., 2005, Gong et al., 2007). Interestingly, other lines in this series, including the ChAT, Slc6a4, Drd1a and Drd2, which were produced by the same BAC-Cre strategy, also showed
ectopic adult expression in the central nervous system (Gong et al., 2007). Ectopic Cre activity was also common in our study of the TH-BAC-tdTomato retinas. In contrast to the rest of the central nervous system, TH-BAC-tdTomato retinas had a very low percentage of Cre-expressing TH-immunoreactive cells. However, there were numerous other amacrine cells, and a small number of ganglion cells with Cre-induced tdTomato fluorescence.

Fluorescent cells in the TH-BAC-tdTomato retinas were immunoreactive for the amacrine cell markers, GABA and glycine, and the ganglion cell marker, RBPMS (Rodriguez et al., 2014). The remaining cells that do not co-localize with GABA, glycine, or RBPMS, which make up less than 6% of the total tdTomato cells, possibly have a low level of GABA, glycine, or RBPMS, and the strong tdTomato signal possibly masked the antibody fluorescence in the cells. Another possibility is that these cells may be glutamate expressing amacrine cells in the INL or displaced into the GCL, or Neurod6 amacrine cells (Haverkamp and Wässle, 2004, Johnson et al., 2004, Kay et al., 2011b).

Fluorescent processes ramified in strata 1, 2/3, 4 and 5 of the IPL, and few fibers were found in the NFL. About 85% of the fluorescent amacrine cells in the INL were the glycine-immunoreactive AII amacrine cells (Table 3) based on their morphological features (Wässle et al., 1995, MacNeil and Masland, 1998, Menger et al., 1998, Massey and Mills, 1999). In addition, tdTomato fluorescent axons in the NFL and optic nerve head, and co-expression of tdTomato fluorescence and RBPMS-immunoreactivity indicate the presence of retinal ganglion cells in the TH-BAC-tdTomato line. However, these cells comprise a low percentage (less than 0.5% in the INL and ~24% in the GCL, Table 3) of the total number of Cre-expressing retinal cells. Their average somal diameter (10.98 ± 0.08 µm; Table 2) in the GCL is similar to many wide-field displaced amacrine cells (Pérez de Sevilla Müller et al., 2007). Moreover, the wide range of their soma diameters (7.44 to 19.27; n=719 cells; 3 retinas) in the GCL and heterogeneous dendritic stratification pattern suggests that there are multiple ganglion cell types (Sun et al., 2002, Völgyi et al., 2009). Based on these findings, ganglion cells will be difficult to
be reliably identified, and this line would be more useful for studying AII amacrine cells, due to their robust labeling and defined morphology.

**TH-tdTomato**: The TH-Cre line used in this study consists of a Cre gene driven by a 9 kb genomic fragment of the rat TH promoter. This line was obtained from the JAX laboratory (Savitt et al., 2005). The initial characterization of this line showed Cre-induced hPLAP and GFP activity in TH-immunoreactive neurons in all of the catecholaminergic cell groups (Savitt et al., 2005). In addition, in the retina, Cre activity was reported in amacrine cells with processes that ramify extensively and broadly in the middle of the IPL. The authors also noted that the pattern of cellular expression of Cre activity in the retina varied in the five TH-Cre lines they generated for their studies. In another TH-Cre line, also generated using the same 9 kb TH rat genomic fragment, type 1 DA amacrine cells were shown to express Cre and TH immunoreactivity, and hPLAP activity (Gelman et al., 2003). In a TH-Cre line generated with an IRES-Cre sequence inserted into the 3’ untranslated region of the *TH* gene, Cre activity is found in some cells in the GCL (Lindeberg et al., 2004). In contrast to these earlier findings, we observed a more complex cellular labeling pattern than the initial descriptions of the TH-Cre line (see Fig. 4 (Gelman et al., 2003)).

In these TH-Cre lines, Cre activity was in many but not all TH immunoreactive neurons in the dopamine-containing cell groups, and in addition, Cre activity was in many neurons in regions that do not contain TH immunoreactive neurons in the adult central nervous system (Min et al., 1994, Gelman et al., 2003, Lindeberg et al., 2004, Savitt et al., 2005, Lammel et al., 2015, Stuber et al., 2015). Regions with ectopic expression of Cre activity include, the interpedeuncular nucleus, supramammillary nucleus, and midline VTA regions of the posterior and anterior ventral midbrain (Lindeberg et al., 2004, Savitt et al., 2005, Lammel et al., 2015). In the retina of the TH-Cre line evaluated in this study, Cre activity was in a small percent of the
TH-immunoreactive amacrine cells and most of the Cre activity was in non-TH immunoreactive amacrine cells.

In the TH-tdTomato retina, Cre-induced fluorescent cell bodies are in the INL and GCL, and their processes ramify in strata 1, 2/3 and 4 of the IPL. TH-tdTomato fluorescent cells contained GABA, TH, ChAT and calretinin immunoreactivity. These results suggest multiple cell types, with ~89% of the total number of tdTomato fluorescent cells containing GABA immunoreactivity. There are a small percentage of tdTomato fluorescent amacrine cells that do not contain GABA or glycine immunoreactivity. Possible explanations for the lack of GABA or glycine immunoreactivity in these amacrine cells could be that they have low levels of the neurotransmitters, which cannot be detected by antibodies, or these amacrine cells are members of the recently described non-GABA or -glycine immunoreactive amacrine cell group (Kay et al., 2011b). The most prominently labeled GABA-immunoreactive cell types were the polyaxonal amacrine cells (Fig. 11), which could be readily identified in retinal whole-mounts based on their somal size and the extensive arborization of their processes (Lin and Masland, 2006). The polyaxonal amacrine cell localized to the INL that stratifies in S2 of the IPL shown in this study is most similar to the WA2, where both cells share multiple features including the same somata localization (INL), IPL stratification (S2), and axonal field size (~2-3 mm) (Lin and Masland, 2006). The two types of displaced polyaxonal amacrine cells that stratify in S5 of the IPL have not been reported in previous studies. This TH-Cre line will be highly useful for future studies of polyaxonal amacrine cells with the aim to better define their biophysical properties, connectivity and functional relationships.

DAT-tdTomato: The DAT-Cre line used in this study was generated by inserting an IRES sequence followed by the Cre gene into the 3’ untranslated region of the Dat gene (Bäckman et al., 2006). Cre-mediated fluorescence, and Cre and DAT immunoreactivity were restricted to dopaminergic neurons in the ventral tegmental area (VTA), substantia nigra pars compacta.
(SNpc) and retrorubral field, and weak Cre activity was also reported in neurons in the periglomerular layer of the olfactory bulb, and to some weakly expressing neurons in the hypothalamus (Bäckman et al., 2006, Lammel et al., 2015). In a DAT-BAC-iCre line, Cre activity was also confined to TH immunoreactive neurons in all of the dopaminergic groups in the central nervous system (Turiault et al., 2007). In addition, in a third DAT-Cre line with the Cre gene inserted downstream of the DAT promoter region, Cre immunoreactivity and activity was mainly in TH immunoreactive neurons in the VTA and SNpc, and in a few neurons in the cortex, septum and hippocampus (Zhuang et al., 2005). TH immunoreactive cells are not reported in cortex, septum and hippocampus of the adult central nervous system (Chinta and Andersen, 2005, Prakash and Wurst, 2006) indicating sparse ectopic Cre activity in this particular line.

Unlike the findings in the central nervous system of the DAT-Cre lines, where Cre activity is reported to be predominantly or exclusively located to neurons in the dopamine-containing cell groups (Zhuang et al., 2005, Bäckman et al., 2006, Turiault et al., 2007, Lammel et al., 2015), in the retina, the predominant type 1 DA amacrine cell do not express detectable levels of the tdTomato reporter, suggesting Cre activity is absent in these cells. The lack of Cre expression in type 1 DA amacrine cells is surprising, since DAT immunoreactivity has been localized to type 1 DA amacrine cells in the rat and bullfrog retina (Cheng et al., 2006), and there is high affinity uptake of dopamine by type 1 DA amacrine cells in the cat and rabbit retina (Ehinger and Floren, 1978, Pourcho, 1982, Tauchi et al., 1990).

In contrast, the present study shows that in two different founder lines, the DAT-tdTomato retinas robustly express Cre-mediated fluorescence in two different patterns: 1) only in monostratified amacrine cells or 2) predominantly bistratified amacrine and some monostratified amacrine cells. Both founder lines have GABA immunoreactive fluorescent cells and they lack TH and glycine immunoreactive cells (Fig. 12 and 14). However, despite these similarities, the two founder lines greatly differ in the morphological types of amacrine cells that are labeled.
Fluorescent cells in founder line 1 retinas form a single morphological type, with the majority of their somata in the INL and a smaller number of somata in the GCL. These cells have a single primary process that directly enters the IPL and ramifies extensively in a narrow band corresponding to stratum 2/3 in the IPL (Fig. 3A and 12). These cells have a symmetrical field that averages 189.7 ± 23.8 µm (n=40 cells; 2 retinas) in diameter. These features are consistent with the classification of these cells as a medium-field amacrine cell (MacNeil and Masland, 1998). The DAT-Cre line (founder line 1) is the first example to our knowledge of a transgenic mouse line that only contains this amacrine cell type.

The DAT monostratified amacrine cell reported in this study is similar to three other monostratified amacrine cell types, which ramify in the middle of the IPL in the mouse retina: 1) the type 2 DA, 2) the WF-S2/3 (“brightly labeled”), and 3) the CD44 (“bright”) amacrine cells (Gustincich et al., 1997, Zhang et al., 2004, Sarthy et al., 2007, Knop et al., 2011, Brüggen et al., 2014, Knop et al., 2014).

The DAT monostratified amacrine cells differ from type 2 DA amacrine cells. The average somal diameter of the DAT monostratified amacrine cells in the INL was 8.78 ± 1.42 µm (n=80 cells; 2 retinas; Table 2), which is slightly smaller than the type 2 DA amacrine cells which average ~9.5 ± 1.1 µm in diameter (Gustincich et al., 1997, Zhang et al., 2004, Knop et al., 2011). The average DAT amacrine cell field size (189.7 ± 23.8 µm; n=40 cells; 2 retinas) is several-fold smaller than the average type 2 DA amacrine cell field size (695 ± 148 µm) (Zhang et al., 2004, Knop et al., 2011). An additional difference is the density of the DAT monostratified amacrine cells, which is 43 ± 3 cells/mm² (n=2 retinas; Table 2) whereas the density of type 2 DA amacrine cell is higher at ~ 181 cells/mm² or ~250 cells/mm² (Knop et al., 2011). These differences suggest they are two different amacrine cell types.

The morphological features of the ‘brightly’ labeled EGFP-positive WA-S2/3 amacrine cells identified in the ChAT-EGFP mouse line (von Engelhardt et al., 2007) do not match the DAT monostratified amacrine cells (Haverkamp et al., 2009, Knop et al., 2014). One difference
is that most DAT monostratified amacrine cells are calretinin immunoreactive, whereas none of the WA-S2/3 amacrine cells are calretinin immunoreactive and the WA-S2/3 amacrine cell processes only stratify between the calretinin immunoreactive bands (Haverkamp et al., 2009, Knop et al., 2014). A second difference is that the DAT monostratified amacrine cell bodies are smaller than the WA-S2/3 amacrine cell bodies, which are $13.1 \pm 1.8 \mu m$ in diameter (Knop et al., 2014). The DAT amacrine cell field size and density was also smaller compared to the WA-S2/3 amacrine cell (~400-800 µm and 75-140 cells/mm$^2$, respectively) (Knop et al., 2014). There is also a “weakly labeled” EGFP cell type that ramifies in the middle of the IPL and expresses calretinin immunoreactivity in the ChAT-EGFP mouse line, (see Fig. 2O of (Knop et al., 2014)). We cannot determine if this “weakly labeled” EGFP amacrine cell is the same as the DAT monostratified amacrine cell, since there is a lack of information regarding its somal diameter and field size, as well as its density.

The DAT monostratified amacrine cell is most similar to a prominent (“bright”), monostratified amacrine cell type in a CD44-EGFP transgenic line (Sarthy et al., 2007). The DAT and CD44 monostratified amacrine cells have small somal diameters and share similar average field sizes of $189.7 \pm 23.8 \mu m$ (n=40 cells; 2 retinas) compared to ~189 µm (Sarthy et al., 2007), respectively. In addition, the ramification pattern of their processes in the middle of the IPL appear to be similar, and their processes overlap with the middle calretinin band (see Fig. 2G-I of (Sarthy et al., 2007)). However, there remains some uncertainty if these are the same cell type based on differences in their nearest neighbor distances of 101.5 µm and cell density of 112 cells/mm$^2$.

There is a caveat in the comparison of the DAT, type 2 DA, WA-S2/3 and CD44 labeled amacrine cells because of the possibility that only a proportion of the cell population is labeled by the transgene due to incomplete penetrance (Zhuang et al., 2005, Bäckman et al., 2006). Incomplete penetrance of the transgene would alter estimates of cell number, density, nearest neighbor distances, as well as influence the distribution of labeled cells in different retinal
regions. Therefore in comparing different cell types in these transgenic lines, both their neurochemical and morphological attributes become important factors for determining identity.

On the other hand, retinal sections from founder line 2 of the DAT-tdTomato line revealed two predominant bands of stratifications (S1 and S3, 4, and 5) in the IPL (Fig. 3 and 14), and whole mounts had an average of 646 ± 160 transgenic cells/mm² of retina (Fig. 4), which is observably different from generation 1 of the DAT-tdTomato line. In addition, Neurobiotin intracellular injections mainly labeled two types of bistratified amacrine cells in the INL and two types of monostratified amacrine cells, one in the INL and the other in the GCL (Fig. 15). These bistratified amacrine cells are different from the previously reported A1 cells because A1 somata are localized to the GCL and their processes primarily stratify near the GCL and few reach out towards the INL (Badea and Nathans, 2004). However, our small bistratified amacrine cells (Fig. 15B) show similar morphology to earlier descriptions of medium-field bistratified amacrine cells, which have processes that stratify in two principal planes and have a dendritic field size of 150-200 µm (Badea and Nathans, 2004). Although the presence of a diversity of medium-field bistratified amacrine cells have been observed (MacNeil and Masland, 1998, Badea and Nathans, 2004, Haverkamp and Wässle, 2004, Lee et al., 2015), unique to this mouse line is tdTomato expression in wide-field bistratified amacrine cells (Fig. 15A). Our report of the wide-field bistratified amacrine cell is most similar in morphology to the asymmetrical bistratified amacrine cell (MacNeil and Masland, 1998), but the asymmetrical bistratified amacrine cell stratifies in S2 and 4, and has a noticeably smaller field size. This indicates the founder line 2 of the DAT-tdTomato mouse line contains a wide-field bistratified amacrine cell.

In addition to the observation of bistratified amacrine cells, founder line 2 of the DAT-tdTomato line showed at least two populations of monostratified amacrine cells. The first monostratified amacrine cell is localized to the INL, and its processes ramify in S1 of the IPL (Fig. 15C). Based on Neurobiotin intracellular injections, these cells have a large dendritic field
size and are considered wide-field amacrine cells. These cells are similar in morphology and stratification pattern to the S1 amacrine cell, which has only been identified in rabbit retina (Vaney, 1986, Xin and Bloomfield, 1997, Li et al., 2002). In addition, the S1 monostratified amacrine cell in this report is similar to the WA-1 amacrine cells in the mouse retina, but are not the same cells because the WA-1 are always displaced into the GCL (Lin and Masland, 2006).

The second type of monostratified amacrine cell we found was displaced into the GCL (Fig. 15D). These cells have a medium-field dendritic field diameter (229 ± 48.41 µm), and have a morphology and stratification pattern similar to the previously described DA1 cells in the guinea pig retinas (Kao and Sterling, 2006). However, this particular morphology and stratification pattern has not been shown in mouse retinas, thus making it difficult to make an accurate comparison. Therefore, this generation of the DAT-tdTomato line shows a few distinctive populations of bistratified and monostratified amacrine cells, uniquely different from generation 1, emphasizing the importance of careful characterization of transgenic labeling prior to performing functional assays.

A possible explanation for the different labeling patterns between the two founder lines of the DAT-tdTomato line is a contaminated reporter line. Analysis of reporter expression in the DAT-Brainbow line showed sporadic fluorescent labeling in bistratified amacrine cells in the INL, and some monostratified amacrine cells in the INL and displaced in the GCL. These cells had the same morphology as those seen in the founder line 2 of the DAT-tdTomato line. Thus, the labeling differences were not a result of reporter line contamination.

The DAT-tdTomato mouse line with its expression in specific classes of amacrine cells and generational differences it will be important to do characterization studies. In addition, either of these lines could then be important tools for understanding their pre- and post-synaptic partners, and the function of the labeled cells in visual information processing. For instance, studies using the DAT-cre line could include deletion of synaptic proteins or receptors on the DAT monostratified cells, cell ablation studies, or experimental approaches to activate or silence
these cells using optogenetic or chemogenetic technologies (Nichols and Roth, 2009, Madisen et al., 2010, Pei et al., 2010, Madisen et al., 2012, Pinol et al., 2012, Zariwala et al., 2012, Incontro et al., 2014, Lee et al., 2014). Furthermore, the DAT-tdTomato transgenic line could be used to identify the targets of the amacrine cells, via anterograde transsynaptic viral injections (Beier et al., 2011, Lo and Anderson, 2011, Beier et al., 2013) or dual cell recordings in which the DAT amacrine cells are stimulated and recordings are made from neighboring cells (Liets et al., 2003, Zheng et al., 2004, Hidaka et al., 2005, Akrouh and Kerschensteiner, 2013).

**Ectopic and incomplete expression of Cre-induced fluorescence in the retina:**

All three Cre lines were characterized by a low percentage or lack of type 1 DA amacrine cells with Cre-induced fluorescence (Fig. 4A, 6A, and 8A). In these retinas, the majority of Cre-expressing cells were glycine- or GABA-immunoreactive amacrine cells. In addition, in the TH-BAC-tdTomato line, some small ganglion cells expressed Cre-dependent fluorescence. The TH-BAC- and TH-Cre retinas also showed partial penetrance of Cre into type 1 DA amacrine cells. The limited Cre-mediated fluorescence in type 1 DA amacrine cells may also be due to a low number of copies of Cre constructs knocked into the genome compared to the labeling of a majority of TH immunoreactive cells in the TH-RFP, TH-PLAP and TH-GFP retinas (Heintz, 2001, Gelman et al., 2003, Lindeberg et al., 2004, Savitt et al., 2005, Zhuang et al., 2005, Bäckman et al., 2006, Gong et al., 2007, Lammel et al., 2015).

Ectopic expression of Cre activity in amacrine cells is a predominant feature of the DAT-,
TH-BAC- and TH-Cre lines. One possible reason is that there is a positional influence of the exogenous DAT or TH genomic DNA inserts, in which their integration into the genome changes endogenous expression patterns (Gong et al., 2007). Another factor that may influence ectopic Cre activity is the lack of regulatory regions to switch off the Th gene (Min et al., 1994, Gelman et al., 2003). In addition, perhaps there is a retina-specific regulatory element lacking in these constructs or epigenetic factors that are needed to properly direct DA amacrine cell expression.
Ectopic expression can also be a result of the transient expression of the *Dat* or *Th* gene during retinal development. For instance, in the central nervous system, transient, developmental expression of *Th* gene occurs in the same regions showing ectopically Cre-expressing cells in mature tissue (Komori et al., 1991, Gong et al., 2002, Marin et al., 2005, Gong et al., 2007). It is possible the transient expression of the *Th* gene in amacrine cell progenitors accounts for the multiple ectopic labeled amacrine cell types observed in the TH- and TH-BAC-Cre retinas.

**Conclusion:**

Retinas of the three Cre lines characterized in this report can serve as a platform for future investigations of amacrine cells. The generations of DAT-tdTomato retinas labels a single class of monostratified amacrine cells or classes of bistratified and monostratified amacrine cells. The TH-BAC-tdTomato retina can be better used to study AII and amacrine cells. Retinas of TH-tdTomato mouse line can be used for studying polyaxonal wide-field amacrine cells, including their synaptic partners, intrinsic properties, and specific functions in visual processing.
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Table 2. Summary of density and somal size

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<td>TH-RFP</td>
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<td></td>
<td>type 2: 181 ± 54</td>
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<td>founder line 1: 43 ± 3</td>
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<td>DAT-tdTomato</td>
<td>founder line 2: 646 ± 160</td>
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</tr>
<tr>
<td>TH-BAC-tdTomato</td>
<td>1793 ± 731</td>
<td></td>
</tr>
<tr>
<td></td>
<td>INL: 6.48 ± 1.04a</td>
<td>GCL: 6.86 ± 1.06c</td>
</tr>
<tr>
<td></td>
<td>9.10 ± 0.57b</td>
<td>10.54 ± 1.53d</td>
</tr>
<tr>
<td></td>
<td>(0.92%)</td>
<td>(30.51%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH-tdTomato</td>
<td>189 ± 37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>INL: 6.76 ± 0.99a</td>
<td>GCL: 7.25 ± 1.02e</td>
</tr>
<tr>
<td></td>
<td>10.10 ± 1.49b</td>
<td>10.34 ± 1.40f</td>
</tr>
<tr>
<td></td>
<td>(10.84%)</td>
<td>(10.22%)</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a glycine-immunoreactive amacrine cells with small somal sizes
b GABA-immunoreactive amacrine cells with large somal sizes (0.92% or 10.84% of transgenic cells in INL)
c displaced amacrine cells with small somal sizes
d displaced amacrine and ganglion cells with large somal sizes (30.51% of transgenic cells in GCL)
e displaced amacrine cells with small somal sizes
f displaced amacrine cells with large somal sizes (10.22% of transgenic cells in GCL)
Table 3. Summary of immunohistochemical labeling

<table>
<thead>
<tr>
<th>transgenic animal line</th>
<th>immunoreactivity</th>
<th>% colocalized</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TH-RFP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type 1</td>
<td>anti-TH</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>anti-GABA</td>
<td>100</td>
</tr>
<tr>
<td>type 2</td>
<td>anti-TH</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>anti-GABA</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>anti-GABA</td>
<td>100</td>
</tr>
<tr>
<td>founder line 1</td>
<td>anti-calretinin</td>
<td>66.99 ± 1.53</td>
</tr>
<tr>
<td></td>
<td>anti-TH</td>
<td>0</td>
</tr>
<tr>
<td><strong>DAT-tdTomato</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>founder line 2</td>
<td>anti-GABA</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>anti-calretinin</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>anti-TH</td>
<td>0</td>
</tr>
<tr>
<td><strong>TH-BAC-tdTomato</strong></td>
<td>anti-Th</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>anti-GABA</td>
<td>8.51 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>anti-GABA</td>
<td>74 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>anti-glycine</td>
<td>85.11 ± 1.51</td>
</tr>
<tr>
<td></td>
<td>anti-calretinin</td>
<td>9.06 ± 1.23</td>
</tr>
<tr>
<td></td>
<td>anti-RBPMS</td>
<td>0.46 ± 1.41</td>
</tr>
<tr>
<td></td>
<td>anti-Th</td>
<td>5.95 ± 1.53</td>
</tr>
<tr>
<td></td>
<td>anti-GABA</td>
<td>89.11 ± 1.9</td>
</tr>
<tr>
<td><strong>TH-tdTomato</strong></td>
<td>anti-glycine</td>
<td>1.50 ± 0.03</td>
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<tr>
<td></td>
<td>anti-calretinin</td>
<td>81.08 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>anti-ChAT</td>
<td>6.10 ± 1.99</td>
</tr>
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</table>
References


Parallel inhibition of dopamine amacrine cells and intrinsically photosensitive retinal ganglion cells in a non-image forming visual circuit of the mouse retina *

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*In Press
Abstract
An inner retinal microcircuit composed of dopamine-containing (DA) amacrine cells and melanopsin-containing, intrinsically photosensitive retinal ganglion cells (M1 ipRGCs) processes information about the duration and intensity of light exposures, mediating light adaptation, circadian entrainment, pupillary reflexes and other aspects of non-image forming vision. The neural interaction is reciprocal: M1 ipRGCs excite DA amacrine cells and these, in turn, feed inhibition back onto M1 ipRGCs. We found that the neuropeptide somatostatin (SRIF) also inhibits the intrinsic light response of M1 ipRGCs and postulated that to tune the bidirectional interaction of M1 ipRGCs and DA amacrine cells, SRIF amacrine cells would provide inhibitory modulation to both cell types. SRIF amacrine cells, DA amacrine cells, and M1 ipRGCs form numerous contacts. DA amacrine cells and M1 ipRGCs express the SRIF receptor subtypes, sst2A and sst4, respectively. SRIF modulation of the microcircuit was investigated with targeted patch-clamp recordings of DA amacrine cells in TH-RFP mice and M1 ipRGCs in OPN4-EGFP mice. SRIF increases K⁺ currents, decreases Ca²⁺ currents, and inhibits spike activity in both cell types, actions reproduced by the selective sst2A agonist L-054,264 in DA amacrine cells and the selective sst4 agonist L-803,087 in M1 ipRGCs. These parallel actions of SRIF may serve to counteract the disinhibition of M1 ipRGCs caused by SRIF’s inhibition of DA amacrine cells. This allows the actions of SRIF on DA amacrine cells to proceed with adjusting retinal dopamine levels without destabilizing light responses by M1 ipRGCs, which project to non-image forming targets in the brain.
Significance statement

Amacrine cells form multiple microcircuits in the inner retina to mediate visual processing although their organization and function remain incompletely understood. The somatostatin (SRIF)- and dopamine (DA)-releasing amacrine cells act globally, and in this study they are shown to interact and modulate the light response of intrinsically photosensitive retinal ganglion cells (ipRGCs). SRIF amacrine cells target both DA amacrine cells and M1 ipRGCs for inhibition. SRIF’s parallel actions may serve to compensate for the loss of dopamine-mediated inhibition of M1 ipRGCs. This inhibitory tuning is of particular importance since the dopamine system mediates a broad range of light adaptational actions in the retina and M1 ipRGCs project to brain areas that influence sleep, mood, cognition, circadian entrainment, and pupillary reflexes.
Introduction

Microcircuits in the retina take part in the simultaneous processing of different features of visual information to generate the multiple channels that mediate visual function. Over 30 different types of interneurons called amacrine cells modulate the output of ganglion cells, the projection neurons of the retina (Masland, 2012). Dopamine-releasing (DA) amacrine cells are a particularly important inhibitory interneuron in this regard, having widespread influence on virtually all retinal cells and the visual information channels carried by ganglion cells to higher brain regions (Dowling, 1991; Witkovsky, 2004).

This report investigates the regulation of a mammalian retinal microcircuit that mediates reciprocal signaling between DA amacrine cells and type 1 melanopsin-containing intrinsically photosensitive retinal ganglion cells (M1 ipRGCs) (Sakamoto et al., 2005; Zhang et al., 2008; Schmidt et al., 2011; Van Hook et al., 2012; Atkinson et al., 2013; Dkhissi-Benyahya et al., 2013). Previous studies have shown numerous appositions between DA amacrine cell processes and M1 ipRGC dendrites in the inner plexiform layer (IPL) that may be unconventional synapses (Belenky et al., 2003; Viney et al., 2007; Vugler et al., 2007; Dumitrescu et al., 2009; Zhang et al., 2012), and that DA, acting through D1 receptors, inhibits M1 ipRGCs (Zhang et al., 2008; Van Hook et al., 2012; Hu et al., 2013).

In mammalian and chick retinas, DA amacrine cells regulate integral retinal networks that mediate light adaptation, circadian rhythms, color vision, contrast sensitivity, and visual acuity (Dowling, 1991; Masland, 2001; Ko et al., 2003; Witkovsky, 2004). DA amacrine cell activity leading to dopamine release occurs in response to light, steady background illumination, and prolonged darkness (Zhang et al., 2007; Contini et al., 2010; Newkirk et al., 2013). M1 ipRGCs provide excitatory drive to DA amacrine cells (Zhang et al., 2008; Zhang et al., 2012; Atkinson et al., 2013). This was shown in the rd1 mouse retina, which lacks rod and cone photoreceptors, and wild type mouse retina treated with L-AP4 (an agonist of the mGluR6 receptor that blocks signaling between photoreceptors and ON-bipolar cells), in which DA
amacrine cells exhibit sustained light responses mediated by AMPA- and kainate-type glutamate receptors, which are activated by glutamate release from ipRGC dendrites.

The reciprocal connectivity between DA amacrine cells and M1 ipRGCs is poised to provide critical regulation of non-image forming visual functions. Thus it is important to determine how additional inputs from other retinal neurons could coordinate the light-dependent responses of this microcircuit (Gustincich et al., 1997; Zhang et al., 2007). Previous work has indicated that an additional, unidentified amacrine cell subtype inhibits light responses of DA amacrine cells and M1 ipRGCs (Pérez-León et al., 2006; Wong et al., 2007; Atkinson et al., 2013; Newkirk et al., 2013). We identify a wide-field amacrine cell expressing somatotropin release inhibiting factor (somatostatin or SRIF) that mediates inhibition of the DA amacrine cell - M1 ipRGC microcircuit.

SRIF is synthesized and released by a subset of amacrine cells that mainly ramify in stratum 1 of the IPL in mammalian retinas (Tornqvist et al., 1982; White et al., 1990; Rickman et al., 1996; Cristiani et al., 2002). The effects of SRIF are global and mediated by G-protein-coupled receptor subtypes (sst1-5) identified in rod bipolar cells, horizontal cells, glycine- and tyrosine hydroxylase (TH)-immunoreactive amacrine cells, and ganglion cells (Johnson et al., 1998; Johnson et al., 1999; Johnson et al., 2000; Cristiani et al., 2002). In vertebrate retinas SRIF signaling cascades are generally inhibitory, modulating multiple voltage-gated ion currents in retinal cells (Akopian et al., 2000; Petrucci et al., 2001; Jian et al., 2009; Farrell et al., 2010; Chen et al., 2014).

We mapped the unusually close relationships made by the wide-field SRIF and DA amacrine cell processes in the IPL, as well as their numerous contacts with M1 ipRGCs. By visualizing specific sst2A and sst4 receptors on DA amacrine cells and M1 ipRGCs, respectively, we also show potential sites of interaction with released SRIF. We investigated SRIF modulation of the intrinsic properties of DA amacrine cells and the intrinsic properties and photoresponsivity of M1 ipRGCs using targeted whole-cell patch-clamp recordings of M1
ipRGCs in OPN4-enhanced green fluorescent protein (EGFP) retinas and DA amacrine cells in TH-red fluorescent protein (RFP) retinas. Our results establish the anatomical and physiological substrates through which SRIF amacrine cells regulate in parallel both DA amacrine cell and M1 ipRGC signaling, revealing a novel model of dual wide-field amacrine cell regulation within a retinal microcircuit that mediates light adaptation and non-image forming vision.

**Materials and Methods**

**Animals:** Animal housing and all experiments were in accordance with the guidelines and policies for the welfare of experimental animals prescribed by the UCLA Animal Research Committee, UCLA Division of Laboratory Animal Medicine and the U.S. Public Health Service Policy on Humane Care and use of Laboratory Animals.

Retinas were collected from male and female C57 BL/6J or OPN4-EGFP mice (4-6 weeks old) for immunohistochemistry studies. C57 BL/6J mice were from either Jackson Laboratory (Bar Harbor, ME) or the UCLA Division of Laboratory Animal Medicine. Transgenic mice expressing RFP under the TH promoter (Zhang et al., 2004) or EGFP under the OPN4 promoter (Schmidt et al., 2008) labeled DA amacrine cells or ipRGCs, respectively. The OPN4-EGFP mouse line was generated using an FVB/Ncr genetic background. TH-RFP and OPN4-EGFP mice were used for patch clamp electrophysiology. The mice were maintained on a 12-hour light/dark cycle.

Primers used for detecting RFP were 5'-GCA CCT TGA AGC GCA TGA A-3' and 5'-CAC TTT GTT ACA TGG GCT GGG-3'. A predicted band at ~590 bp indicated the presence of the transgene. Primers used for detecting GFP were 5'-CACATGAAGCAGCACGACTTCT-3' and 5'-GGGTGTTTCTGCTGGTAGGTGGTC-3'. A predicted band 331 bp indicated the presence of the transgene.

**Tissue Preparation**
Mice were deeply anesthetized using 1-3% isofluorane (IsoFlo, Abbott Laboratories, North Chicago, IL) and killed by decapitation or cervical dislocation. Eyes were enucleated, and the cornea and lens were removed.

**Vertical retinal sections:** Eyecups were immersion fixed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 for 15-60 minutes. Eyecups were subsequently washed in 0.1 M PB for 30 minutes and stored in 30% sucrose overnight at 4°C, and then embedded in optimal cutting temperature medium (Sakura Finetek Inc., Torrance, CA) and sectioned vertically at 12 µm. Retinal sections were placed onto gelatin-coated slides and stored at -20°C until used for immunohistochemistry.

**Whole-mount retinal preparation:** The retinas were removed from the eyecups, and four small incisions were made to lay the retina flat. Retinas were mounted, ganglion cell layer (GCL) up, onto nitrocellulose membrane filters (cat #HABP04700; Millipore Corporation, Billerica, MA), and immersed for 30-60 minutes in 4% PFA in 0.1M PB at room temperature. Whole-mounted retinas were then washed and processed for immunohistochemistry.

**Immunohistochemistry**

**Vertical retinal sections:** Retinal sections were processed for immunohistochemical labeling using an indirect immunofluorescence method (Vuong et al., 2015). Frozen retinal sections were thawed for 10 minutes at 37°C on a warming plate, then washed three times for 10 minutes with 0.1 M PB (pH 7.4). Sections were then incubated in a blocking solution of 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X-100 in 0.1 M PB for 1 hour at room temperature. Following removal of the blocking solution, the primary antibody solution was immediately added to the sections and incubated for 12-16 hours at 4°C. Primary antibody solution contained 3% NGS, 1% BSA, 0.05% sodium azide and 0.5% Triton X-100 in 0.1M PB. Retinal sections were then washed three times for 10 minutes in 0.1 M PB. The sections were then incubated with their corresponding secondary antibodies goat anti-
rabbit, rat or mouse conjugated to Alexa 568 or 488 (1:1000; Invitrogen, Grand Island, NY) for two hours at room temperature. The secondary antibody was removed and sections were washed three times in 0.1 M PB for 10 minutes per wash. Sections were air-dried and mounted using Aqua Poly/Mount (Polysciences, Warrington, PA). To control for nonspecific binding of the secondary antibody, the primary antibodies were omitted in the single-labeling studies.

**Whole-mounted retina:** Whole-mounted retinas were processed for immunohistochemical labeling with a protocol similar to that used for vertical sections. Whole-mounted retinas were fixed, washed in 0.1 M PB and then incubated in blocking solution overnight at 4°C. The retinas were then transferred to primary antibody solution and incubated for 5 days at 4°C. Retinas were washed in 0.1 M PB for 3 times for 20 minutes each time, and then incubated in secondary antibodies for 2 days at 4°C. Following removal from the secondary antibody solution, retinas were washed three times in 0.1 M PB for 20 minutes for each wash. Similar to the retinal sections, whole-mounted retinas were briefly air-dried and mounted using Aqua Poly/Mount (Polysciences).

For immunohistochemistry controls, all antibodies were tested using mouse retina in single immunostaining experiments to confirm specificity and optimize concentration prior to performing any double labeling experiments. Experiments omitting primary antibodies eliminated specific immunostaining. For double labeling controls, one of the two primary antibodies used for double labeling was omitted during the primary incubation step. In this case, only the immunostaining by the remaining primary antibody was detected.

**Antibodies (Table 1):** The following antibodies were used in this study: 1) rat monoclonal antibody against somatostatin (SRIF) (1:100; Millipore; MAB354, clone YC7), 2) rabbit polyclonal antibody against melanopsin (1:250; ThermoScientific, Waltham, MA; PA1-781) 3) mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:2000; Millipore; MAB5280, clone 2/40/15), 4) rabbit polyclonal antibody against sst$_{2a}$ (1:2000; Gramsch, Schwabhausen, Germany; SS-800), 5) rabbit polyclonal antibody against sst$_{4}$ (1:3000), was developed using a
synthetic peptide directed to the C-terminus of the rat sst₄ receptor (amino acids 362-384) (Farrell et al., 2010).
Table 1. Antibodies Used in this Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Antigen</th>
<th>Source</th>
<th>Catalog No.</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatostatin</td>
<td>Rt</td>
<td>Synthetic peptide corresponding to amino acids 1-14 of cyclic somatostatin conjugated to bovine thyroglobulin using carbodiimide.</td>
<td>Millipore Corporation, Billerica, MA</td>
<td>MAB354</td>
<td>1:100</td>
<td>Cristiani et al., 2002</td>
</tr>
<tr>
<td>Melanopsin</td>
<td>Rb</td>
<td>Synthetic peptide corresponding to residues E(455) QKS KTP KTKRHLPSLD RRM(474) of rat melanopsin</td>
<td>Thermo Scientific, Waltham, MA</td>
<td>PA1-781</td>
<td>1:250</td>
<td>Pérez de Sevilla Müller et al., 2014</td>
</tr>
<tr>
<td>Tyrosine Hydroxylase</td>
<td>Ms</td>
<td>Purified tyrosine hydroxylase (EC 1.14.16.2) from a rat pheochromocytoma</td>
<td>Millipore Corporation, Billerica, MA</td>
<td>MAB5280</td>
<td>1:2000</td>
<td>Vuong et al., 2015</td>
</tr>
<tr>
<td>sst4</td>
<td>Rb</td>
<td>C-terminus, Peptide: amino acids 362-384</td>
<td>Brecha Lab, UCLA</td>
<td></td>
<td>1:3000</td>
<td>Farrell et al., 2010</td>
</tr>
</tbody>
</table>
Confocal image acquisition

The immunostaining images were acquired with a Zeiss LSM510 Meta or LSM710 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY) equipped with 488, 543, and 633 nm laser lines. Confocal scans were captured using a C-Apochromat 40X 1.2 NA corrected water objective or a Plan-Apochromat 63X 1.4 NA corrected oil objective. To make the 2D figure panels of retinal transverse sections and whole mounts, projections of two to three images (2048 X 2048 pixels) with a total of 0.9-1.0 µm thickness (overlapping z-axis steps between 0.3-0.5 µm) were collected and adjusted for brightness and contrast in Adobe Photoshop CS2 v.9.02 (Adobe Systems, San Jose, CA). To make the 3D reconstructions of retinal whole mounts image stacks (z-axis steps between 0.3-0.5 µm) of the immunostained somata in the INL, through the entire IPL, and immunostained somata in the GCL were further processed using Image J Software (ImageJ, NIH).

Quantification of contacts

The percent fluorescent density of contacts was determined by dividing the fluorescent density of the contacts mask from the green (TH or melanopsin immunostained somata and processes) mask or red (SRIF immunostained somata and processes) mask and then multiplied by 100. To determine the fluorescent density of SRIF amacrine cells, DA amacrine cells, and melanopsin ipRGCs, each of these cells was first masked in 3D using a custom macro in Image J. In the macro, images were resampled to isotropic proportions and features enhanced with a rolling ball background subtraction. The green signal (A) was auto thresholded with an isodata threshold and the red signal (B) was thresholded with an Otsu algorithm. Thresholded signals were then binarized, smoothed by a 3D-Gaussian filter with a radius of 165 nm (contact radius), and the pixel intensity normalized to a range of 0 to 1. The contacts mask was produced by (A * B) ^ C, with C serving as a roll-off function to ensure that the value of two pixels separated by the contacting radius would be equal to 0.5. Empirically, the roll-off function was determined to be
4. The resulting fluorescent density values are the sum of all intensities of all pixels in that mask.

To estimate the non-specific contacts between the labeled cells, we calculated the percentage of fluorescent density of contacts upon rotating the red mask 90°, 180°, and 270°, compared to its original orientation (0°). The % fluorescent density of contacts is reported as mean ± SEM.

Rotational analysis protocol.

Live tissue preparation

For acutely dissociated retinal cells, isolated retinas were incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS (Invitrogen, Carlsbad, CA) containing papain (40–45 U/ml, pH 7.4, Worthington, Lakewood, NJ) for 45 minutes at 37°C. Retinal pieces were transferred to a Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal bovine serum (Invitrogen), 1X penicillin-streptomycin-glutamine (Invitrogen), and DNase I (100 U/ml, pH 7.4; Worthington), and gently triturated to obtain suspensions of isolated cells. Cells were pipetted onto coverslips
coated with concanavalin A (1 mg/ml; Sigma-Aldrich, St. Louis, MO), and then incubated for 30-60 minutes at 37°C to allow the cells to adhere to the coverslips. For slices, retinas were isolated and placed GCL down on nitrocellulose paper (Millipore), and cut into 150-200 μm slices using a razor blade tissue chopper (Stoelting Tissue Slicer; Stoelting, Wood Dale, IL). Slices were rotated 90° and held in place by two lines of vacuum grease. For whole retina preparations, retinas were isolated from eyecups and transferred to a glass slide. The retina was flat-mounted GCL up and held down at the edges by a nitrocellulose paper (47 mm, type TCMF, 0.22-μm pores, Millipore) that had been hole-punched.

**Electrophysiological recordings**

A gravity-fed perfusion system delivered mammalian extracellular solutions to the chamber at 1.3 ml/min. Whole-cell voltage- and current-clamp recordings were made in retinal slices and retinal flat mounts from TH-RFP and OPN4-EGFP mice. Some whole-cell voltage-clamp recordings were made on isolated cells to confirm drug actions under conditions of complete space clamp. Drug responses differed in amplitude in some recordings made from cells in slices compared to isolated cells. The TH-RFP transgenic mouse line was used to identify DA amacrine cells (Zhang et al., 2004). The type 1 DA amacrine cells were identified by their large soma size and wide-field processes in stratum 1 of the IPL (Gustincich et al., 1997; Zhang et al., 2004; Newkirk et al., 2013). To identify M1 ipRGCs in the OPN4-EGFP transgenic mouse line we used several defining features: 1) dendrites that mono-stratify in stratum 1 of the IPL, 2) bright EGFP fluorescence 3) resting membrane potential ranging from -55 to -65 mV, and 4) and sharp, robust light response, all of which correspond to previous descriptions of M1 ipRGCs (Schmidt et al., 2008; Schmidt and Kofuji, 2009, 2011).

Labeled cells were identified by epifluorescence using a Zeiss Examiner upright microscope equipped with a 40X water-immersion objective 1.2 NA. Drugs were superfused until their actions reached steady state before recording their responses.
To record changes in K⁺ channel currents in DA amacrine cells and M1 ipRGCs, the extracellular bath solution contained (in mM): 120 NaCl, 3 KCl, 1 MgCl₂, 1.2 NaH₂PO₄, 10 glucose, 2 mM CaCl₂, and 25 NaHCO₃. No Ca²⁺ channel blockers were used in order to maintain a physiologically normal environment. In addition, the amplitude of Ca²⁺ channel currents reduced by SRIF and its agonists in 2 mM external CaCl₂ was estimated to be negligible compared to the increase seen in mean K⁺ currents. The intracellular pipette solution contained (in mM): 20 KCl, 120 K-gluconate, 2 MgCl₂, 0.2 EGTA, 10 HEPES, and 2 Na₂-ATP. The extracellular bathing solution was bubbled in 95% O₂-5% CO₂ at room temperature (21–25°C). To isolate changes in Ca²⁺ channel currents the extracellular solution contained (in mM): 110 NaCl, 5 KCl, 5 CsCl, 0.1 4-aminopyridine, 7.5 BaCl₂, 15 TEA Cl, 10 glucose, and 10 HEPES, whereas the intracellular pipette solution contained (in mM): 120 CsMeSO₃, 10 TEA Cl, 0.1 CaCl₂, 1 EGTA, 10 HEPES, 3 ATP-Mg, 0.3 GTP-Li, and 8 phosphocreatine. Tetrodotoxin (TTX, 0.5 - 1 µM) was added to block Na channels. A synapse-blocking cocktail used to isolate melanopsin-based light responses contained: 1 mM L(+)-2-amino-4-phosphonobutyrate (L-AP4), 50 µM (2R)-amino-5-phosphonovaleric acid (AP5), 50 µM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), 100 µM picrotoxin, 10 µM strychnine, and 50 µM 1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA). L-AP4 is an mGluR6 agonist, AP5 is a NMDA receptor antagonist, NBQX is an AMPA receptor antagonist, picrotoxin and TPMPA are GABAₐ and GABAₐ receptor antagonists, respectively, and strychnine is a glycine receptor antagonist. In the unlikely event that GABAₐ receptor activation might affect ipRGCs, the addition of TPMPA would preclude such an action.

Extracellular solutions were superfused via a fast perfusion system (VC8-S; ALA Scientific, Albany, NY). Patch electrodes with 5 to 10 MΩ tip resistance were pulled from fire-polished borosilicate glass capillary tubes using a micropipette puller (Sutter Instrument, Novato, CA). Cell voltage was clamped with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) using whole-cell capacitance and series resistance compensation. Holding
potential was set at −60 mV. In voltage-clamp experiments, voltage steps (40 ms) were made in 10 mV increments from −80 to +40 mV.

Current-clamp recordings of DA amacrine and M1 ipRGCs were obtained to measure resting potentials and action potential frequency using the same solutions for recording K+ currents described above. Hyperpolarizing current was injected in some cells to maintain the membrane potential near −50 mV for DA amacrine cells, and near −60 mV for M1 ipRGCs. Narrow-pass filtered blue light stimuli were delivered via the objective from a shutter-controlled mercury lamp and produced irradiance measured in the recording chamber of 3.4 X 10^{12} photons/s/μm^2 at 480nm.

All drugs and reagents were prepared in double-distilled water as stock solutions (frozen at -20°C) or prepared fresh before performing experiments. Tetrodotoxin (TTX) was purchased from Abcam Inc (ab120055, Cambridge, MA). Somatostatin-14 was obtained from Bachem Inc (Torrance, CA). L-054,264, L-803,087, and SKF38393 were from Tocris Bioscience (Minneapolis, MN).

Spontaneous spike activity was quantified for each cell as the average spike rate during 1 minute of recording before and during drug perfusion. The average waveform of the spikes was generated from 20-30 different cells in control or drug preparations. Photocurrent amplitudes were measured from the baseline (100 ms) before light onset to the peak of the current response. Data analysis for electrophysiology was performed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA). Graphing and statistical analysis were performed using Graphpad Prism 6.0F software (Graphpad Software, La Jolla, CA). Data are reported as the means ± SEM. Values of p < 0.05 were considered statistically significant. All data sets were compared using paired Student’s t-test.

Results
The intrinsic light response of M1 ipRGCs is independently modulated by SRIF and dopamine

In the presence of a pharmacological cocktail that blocks synaptic inputs throughout the inner retina, we examined the changes in the intrinsic light response of M1 ipRGCs. The recorded melanopsin ipRGCs shared a morphology similar to that reported for the M1 subtype (Schmidt et al., 2011). The melanopsin-based response to 480 nm stimulation showed a characteristic inward photocurrent that averaged 85.3 ± 13.2 pA (N = 5 retinas; 25 cells; Fig. 1, black). The photocurrent was suppressed in the presence of the D1 agonist SKF38393 (5 µM), by about 36% (control vs SKF: 86.7 ± 12.3 pA vs 55.4 ± 8.1 pA; N = 4 retinas; 16 cells; p = 0.0002; Fig. 1A), consistent with a previous report (Van Hook et al., 2012).
Figure 1. The inhibitory and independent modulatory actions of SRIF and the dopamine D1 receptor agonist SKF38393 on intrinsic melanopsin-based light response in M1 ipRGCs revealed by the selective sst4 agonist L-803,087 and the D1 receptor antagonist SCH23390.

Representative traces of a paired M1 ipRGCs light response in control (synaptic blocking cocktail; black) or (A) SKF38393 (5 µM), (B) SRIF (100 nM), (C) L-803,087 (1 µM), (D) SRIF (100 nM) and SKF38393 (5 µM), (E) L-803,087 (1 µM) and SKF38393 (5 µM), (F) SCH23390 (5 µM) or (G) both SCH23390 (5 µM) and L-803,087 (1 µM). Lower panels in A-G show the average photocurrent amplitudes and significance of photocurrent reduction in the presence drug(s) represented with * (* indicates p < 0.05). Recordings from intact OPN-EGFP retinas.
We found that SRIF (100 nM) also caused a 23% reduction in the photocurrent (control vs SRIF: 85.2 ± 13.2 pA vs 65.8 ± 12.9 pA; N = 4 retinas; 14 cells; p = 0.0068; Fig. 1B). SRIF is reported to inhibit the excitability of RGCs through ion channel modulation mediated by sst_4 receptors (Farrell et al., 2010; Farrell et al., 2014). Therefore, to test if SRIF directly affects the M1 ipRGC light response, we applied L-803,087 (1 µM) an sst_4 agonist. Application of L-803,087 decreased the photocurrent amplitude by 27% (control vs L-803,087: 99.3 ± 16.4 pA vs 72.9 ± 13.9 pA; N = 3 retinas; 6 cells; p = 0.0143; Fig. 1C). This suggests that SRIF actions occur directly on M1 ipRGCs and that these cells receive inhibitory inputs from both SRIF and DA amacrine cells.

To examine the combined contributions of SRIF and DA on the melanopsin-based photocurrent, we applied SRIF (100 nM) and SKF38393 (5 µM) together, and L-803,087 (1 µM) and SKF38393 (5 µM) together. Applying both SRIF and SKF38393 together, as well as L-803,087 and SKF38393 together, caused 21% and 47% decrease in the photocurrent, respectively (control vs SRIF and SKF38393: 78.5 ± 17.3 pA vs 58.9 ± 15.9 pA; N = 4 retinas; 10 cells; p = 0.0206; control vs L-803,087 and SKF38393: 107.7 ± 21.2 pA vs 56.8 ± 13.0 pA; N = 4 retinas; 10 cells; p = 0.0371; Fig. 1D,E). Since there was no additive reduction in the photocurrent when we tested SRIF and SKF38393 together, we next sought evidence for a parallel signaling mechanism by applying L-803,087 with the D1 antagonist, SCH23390 (5 µM). Application of SCH23390 (5 µM) alone did not change the photocurrent amplitude (control vs SCH23390: 80.7 ± 8.5 pA vs 73.8 ± 7.2 pA; N = 4 retinas; 11 cells; p = 0.1057; Fig. 1F), suggesting that in these retinas, there was a low level of basal DA release. However, application of L-803,087 (1 µM) and SCH23390 (5 µM), together, which isolates SRIF input onto M1 ipRGC, resulted in a 47% decrease in the photocurrent (control vs L-803,087 and SCH23390: 100.0 ± 9.2 pA vs 52.3 ± 6.8 pA; N = 4 retinas; 11 cells; p = 0.0003; Fig 1G). This suggests that dopamine and SRIF independently modulate the intrinsic light response of M1
ipRGCs. Photocurrent attenuation was significant in the presence of all drugs individually and in combination, with the exception of SCH23390 (p = 0.1057; Fig. 1A-G, graphs).

**Contribution of SRIF and L-054,264 (a selective sst2A agonist) to ion channel modulation in DA amacrine cells**

In consideration of the similarly scaled reductions in M1 ipRGC photocurrent amplitude in the presence of SRIF, SKF38393, or both together, we tested whether SRIF modulates DA amacrine cells, which in turn, would feed inhibition onto M1 ipRGCs. To identify the specific changes in DA amacrine cells following SRIF receptor activation, we first applied the endogenous agonist, SRIF, to isolated DA amacrine cells from TH-RFP retinas or TH-RFP retinal slices. Whole-cell voltage-clamp recordings of DA amacrine cells using depolarizing steps produced outward delayed rectifier-like K\(^+\) currents in control (Fig. 2A, left). In the presence of SRIF (100 nM) there was an increase in outward K\(^+\) currents (Fig. 2A, right). We also examined the effects of SRIF on inward Ca\(^{2+}\) channel currents, which were isolated using K\(^+\) channel blockers and with Ba\(^{2+}\) as a charge carrier. DA amacrine cell Ca\(^{2+}\) channel currents were diminished in the presence of SRIF (Fig. 2C). The effects of SRIF on K\(^+\) and Ca\(^{2+}\) channel currents can be seen in the mean I-V relations (Fig. 2B,D). SRIF significantly increased K\(^+\) currents by 39% (control vs SRIF in slices: 1337.0 ± 137.2 pA vs 1854.0 ± 106.8 pA; N = 7 cells; p = 0.0103; Fig. 2B). SRIF significantly decreased Ca\(^{2+}\) channel currents by 35% (control vs SRIF in slices: -183.4 ± 24.9 pA vs -132.9 ± 16.1 pA; N = 6 cells; in isolated cells: -80.1 vs -48.9 pA; N = 1 cell; p = 0.0141; Fig 2D).
Figure 2. Modulation by SRIF of $K^+$ and $Ca^{2+}$ channel currents on DA amacrine cells in TH-RFP retinal slices and isolated RFP cells.

(A) Outward $I_K$ recording from a DA amacrine cell in the absence (left) and presence (right) of SRIF peptide (100 nM). (B) Mean I-V relationship for $I_K$ in the absence (black) and presence (red) of SRIF. (C) Inward $I_{Ca}$ recordings in the absence (left) and presence (right) of SRIF. (D) Mean I-V relationship for $I_{Ca}$ in the absence (black) and presence (red) of SRIF. Recordings were made in the presence of TTX (500 nM). Voltage command protocol is shown below the current trace. $N = 4$-$7$ cells/group. * $p < 0.05$. 
Using the selective sst$_{2A}$ agonist L-054,264, we investigated the effects of activating sst$_{2A}$ receptors on isolated DA amacrine cells and in retinal slices from TH-RFP mice. Previous immunohistochemical studies have shown that the sst$_{2A}$ receptor is localized to DA amacrine cells (Johnson et al., 1998; Johnson et al., 1999; Johnson et al., 2000). Peak K$^+$ currents at +40 mV increased 34% when L-054,264 (1 µM) was applied (Fig. 3A), as also seen in the mean I-V relation of K$^+$ currents (control vs L-054,264 in slices: 1331 ± 100.6 pA vs 1782 ± 30.4 pA; N = 5 cells; p = 0.0265 Fig. 3B). We also examined inward Ca$^{2+}$ channel currents in the absence and presence of L-054,264 (Fig. 3C). Average peak inward Ca$^{2+}$ channel currents showed a significant reduction (25%) with L-054,264 compared to the control recordings (control vs L-054,264 in slices: -187.6 ± 18.0 pA vs -140.4 ± 21.77 pA; N = 6 cells; p = 0.0092; Fig. 3D). These results suggest SRIF acts on sst$_{2A}$ receptors in DA amacrine cells to modulate ion channel activity that promotes inhibition of DA amacrine cells, making it unlikely that SRIF amacrine cells inhibit ipRGCs via intermediary DA amacrine cells.
Figure 3. Effects of L-054,264, selective sst$_{2A}$ agonist, on K$^+$ and Ca$^{2+}$ channel currents on DA amacrine cells in TH-RFP retinal slices and isolated RFP cells.

(A) Outward $I_K$ recording from a DA amacrine cell in the absence (left) and presence (right) of L-054,264 (1 µM). (B) Mean I-V relationship for $I_K$ in the absence (black) and presence (red) of L-054,264. (C) Inward $I_{Ca}$ recordings in the absence (left) and presence (right) of L-054,264. (D) Mean I-V relationship for $I_{Ca}$ in the absence (black) and presence (red) of L-054,264. Recordings were made in the presence of TTX (500nM). Voltage command protocol is shown below the current trace. $N = 4$-$7$ cells/group. * $p < 0.05$. 
Effects of SRIF and sst_{2A} agonist on action potential generation in DA amacrine cells

DA amacrine cells generate action potentials spontaneously, and these spikes are thought to trigger dopamine release (White, 1996). Therefore, in consideration of our previous finding that SRIF modulates ion channels in DA amacrine cells, we examined whether SRIF changes the firing properties of DA amacrine cells. In current-clamp recordings, DA amacrine cells in retinal slices produced tonic spiking activity and had an average membrane potential of -55 ± 1.15 mV (without current injection; n = 28 cells), which was similar to previous findings (Newkirk et al., 2013). Application of SRIF reduced the number of spikes, slightly hyperpolarized the cells and revealed the presence of small transient depolarizations (Fig. 4A, red). The average spike waveform did not change (Fig. 4C, red), and the average peak amplitude and half-width showed no significant alterations (Fig. 4D,E). However, quantitative analysis of DA amacrine spike rates revealed a significant increase in the average interevent interval (from 190.2 ms to 685.8 ms; N = 5 retinas; 10 cells; p < 0.01; Fig. 4F), and the spike frequency was greatly reduced (6.1 to 2.6 Hz; N = 5 retinas; 11 cells; p < 0.05; Fig. 4G).
Figure 4. SRIF and L-054,264 decrease spontaneous spiking in DA amacrine cells from TH-RFP slices.
(A) An example trace of spontaneous spiking from a DA amacrine cell in the control (left) and SRIF (100 nM; right).  (B) Spontaneous spike activity from a DA amacrine cell in absence (left) and presence (right) of L054,264 (1 µM).  (C) Average waveform of spikes in control (gray), SRIF (red), and L054,264 (blue). Insets show an overlay of an average spike waveform in control with SRIF or L-054,264. There was no significant difference in the peak amplitude (D) or half-width (E) in the spikes from DA amacrine cells when treated with SRIF or L-054,264. Quantification of interevent intervals (F) and spike frequency (G) in control compared to SRIF or L-054,264 revealed a significant difference and opposite trends. * p < 0.05.
To determine whether SRIF’s effects on resting membrane properties of DA amacrine cells were mediated by sst2A receptors, we applied L-054,264 onto current-clamped DA amacrine cells in retinal slices. L-054,264 reduced the number of spikes compared to control (Fig. 4B) and did not significantly change the average waveform, peak amplitude or half-width (Fig. 4C, D, and E). As was the case with SRIF, L-054,264 significantly increased the interval between spikes (253.9 to 1203 ms; N = 3 retinas, 7 cells, p < 0.05) and decreased spike frequency (5.2 to 2.3 Hz; N = 3 retinas; 7 cells, p < 0.01; Fig. 4F and G). These results suggest that SRIF, acting through sst2A receptors, can strongly inhibit the spontaneous activity of DA amacrine cells, an action that could down-regulate dopamine release and, feedback inhibition onto M1 ipRGCs.

Fasciculation of SRIF and TH processes in stratum 1 of the inner plexiform layer

To establish an anatomical basis for this synaptic interaction, we sought next to determine the relationship between SRIF amacrine cells and the TH immunoreactive DA amacrine cells and their processes in stratum 1 of the IPL. Rarely occurring SRIF immunoreactive amacrine cell somata were located in the inner nuclear layer (INL) and displaced to the GCL. Their processes, characterized by multiple varicosities, formed a continuous network in stratum 1 of the IPL (Fig. 5A, red) and a few processes were in strata 3 and 5 of the IPL. TH immunoreactive amacrine cell somata and processes were located in the INL, and their processes primarily ramified in stratum 1 of the IPL (N = 9 retinas; Fig. 5A, green). A few processes were also found in stratum 3 of the IPL and some processes crossed the INL and ramified in the OPL (not shown). Retinal sections double labeled with SRIF and TH antibodies showed that the SRIF and TH immunoreactive processes were in close proximity in stratum 1 of the IPL with numerous contacts between these processes (Fig. 5A, A’, arrows). In addition, SRIF immunoreactive processes contacted TH immunoreactive somata, and few SRIF and TH immunoreactive processes were in very close proximity and formed contacts in stratum 3 of the
IPL (Fig. 5A). The SRIF and TH immunoreactive processes in stratum 1 of the IPL were closely aligned in whole mounted retinas (Fig. 5B"") and were characterized by the juxtaposition of SRIF immunoreactive processes (N = 9 retinas; Fig. 5B) to both fine and large diameter TH processes (Fig. 5B') in all retinal regions. Notably, the fascicles of SRIF and TH immunoreactive processes formed ring-like patterns around the AII cell bodies (Fig. 5B, insets) (Voigt and Wässle, 1987; Contini and Raviola, 2003; Debertin et al., 2015).

To test whether the contacts between the SRIF and TH immunoreactive processes were non-random, we quantified the fluorescent density of contacts between SRIF and TH immunoreactive processes in a 3D reconstruction of an image stack taken through the IPL (Fig. 5C, C'; see Materials and Methods: Quantification of contacts). SRIF and TH immunoreactive processes made significantly more contacts in the original (0°) image reconstruction compared to each of the rotated (90°, 180° and 270°) image reconstructions (N = 6 retinas; p < 0.01, 0.02, and 0.01, respectively). The decrease in % fluorescent density of contacts upon rotation was present when normalized to TH (0.79 ± 0.11 % at 0°, 0.21 ± 0.04 % at 90°, 0.21 ± 0.04 % at 180°, and 0.25 ± 0.04% at 270°; N = 6 retinas) or SRIF fluorescent density (3.80 ± 0.82 % at 0°, 1.11 ± 0.29 % at 90°, 0.99 ± 0.18 % at 180°, and 1.21 ± 0.26% at 270°; N = 6 retinas; Fig. 5D, E). Collectively, SRIF and DA amacrine cell processes extensively fasciculate in stratum 1 throughout the entire retina, and their processes have numerous contacts that are likely to be sites for neurotransmission and cell modulation.
Figure 5. SRIF and TH immunostaining show closely apposed SRIF and TH immunoreactive amacrine cell processes in stratum 1 of the IPL.
(A) SRIF amacrine cell processes (red) and TH immunoreactive DA amacrine cell processes (green) are in close proximity in stratum 1 of the IPL. (A’) A 3X magnification of box in (A) reveals multiple contacts between SRIF amacrine cell and DA amacrine cell processes in stratum 1 of the IPL. Whole mount retina shows varicose and fine SRIF amacrine cell processes (B) and large and fine caliber DA amacrine cell processes (B’). (B”) Merged images highlight the juxtaposition of the SRIF amacrine and DA amacrine cell processes. Contacts are more frequent near the varicosities of the DA amacrine cell processes. Arrows show the points of contacts between SRIF and DA amacrine cell processes. Insets show perisomatic rings formed by SRIF and TH immunoreactivity. Asterisks mark the soma that are surrounded by SRIF and DA amacrine cell processes. (C) A 3D model of SRIF and DA amacrine cells from an image stack taken through the IPL of a retinal whole mount was generated to determine contacts between the processes. (C’) Omitting the red channel reveals SRIF immunoreactive processes and puncta that make contact with DA amacrine cell processes. Pink labeling shows contacts between SRIF and DA amacrine cell processes. (D) Quantification of fluorescent density created from the contacting processes was normalized to the fluorescent density of the TH immunoreactivity. Rotated reconstructions (90°, 180°, 270°) had significantly less fluorescent density generated from the contacting processes. (E) Fluorescent density of the contacts was also normalized to the fluorescent density of SRIF immunoreactivity. Fluorescent density of contacts following rotation was significantly less than un-rotated image. Scale bar: (A) 20 µm, (A’) 10 µm, (B) 10 µm, and (C) 10 µm. * p < 0.05.
sst\textsubscript{2A} localization at DA amacrine cell somata and their processes

To establish sst\textsubscript{2A} receptor expression by DA amacrine cells, retinal sections were double immunolabeled with sst\textsubscript{2A} and TH antibodies. Consistent with previous studies, sst\textsubscript{2A} immunoreactivity was found in rod bipolar cell cells including their dendrites, axon and axonal terminals (Johnson et al., 2000) (N = 5 retinas; Fig. 6A). Although the intense rod bipolar cell labeling obscures many of the DA amacrine cell processes, double immunolabeling showed sst\textsubscript{2A} immunoreactivity was localized to TH immunoreactive amacrine cell somata located in the INL adjacent to the IPL (Fig. 6B-B''), and their processes in stratum 1 of the IPL (Johnson et al., 1998; Johnson et al., 1999; Johnson et al., 2000)(Fig. 6C-C''). These results confirm that DA amacrine cells express the SRIF receptor subtype sst\textsubscript{2A} on their soma in the INL and processes in stratum 1 of IPL.
Figure 6. sst$_{2A}$ immunoreactivity is co-localized with TH immunoreactivity in a retinal section.

(A) Merged image of sst$_{2A}$ immunoreactivity (red) and TH immunoreactivity (green) shows sst$_{2A}$ strongly immunostained rod bipolar cell axons and is co-localized with a TH immunoreactive amacrine cell somata (B) that is located in the proximal INL and processes (C) that primarily ramify in stratum 1 of the IPL. (B') TH immunoreactivity robustly labels DA amacrine cell somata in the INL. (B'') sst$_{2A}$ immunoreactivity labels the same DA amacrine cell soma. (C') TH immunoreactive processes ramify in stratum 1 of the IPL. (C'') sst$_{2A}$ immunoreactivity is found in DA amacrine cell processes in stratum 1 of the IPL. Arrows highlight the co-localized soma and process of sst$_{2A}$ and TH immunoreactivity. Scale bar: 10 µm.
Modulation of K\textsuperscript{+} and Ca\textsuperscript{2+} channels in M1 ipRGCs by SRIF and the sst\textsubscript{4} agonist, L-803,087

In view of SRIF’s effect on M1 ipRGC light responses, we examined the SRIF receptor activation on K\textsuperscript{+} and Ca\textsuperscript{2+} channel currents in M1 ipRGCs in OPN4-EGFP retinas. We first performed whole-cell voltage-clamp on EGFP fluorescent cells in the presence and absence of SRIF. Voltage-clamped EGFP fluorescent cells showed a 37% increase in peak outward K\textsuperscript{+} currents at +40 mV when SRIF (100 nM) was applied (Fig. 7A, right) compared to control (Fig. 7A, left). This effect of SRIF is shown in mean I-V relations (control vs SRIF in slices: 648.2 ± 114.9 pA vs 1031.0 ± 161.9 pA; N = 9 cells; p = 0.0095; Fig. 7B). In addition, EGFP fluorescent cells showed smaller inward Ca\textsuperscript{2+} channel currents during application of SRIF (Fig. 7C, right) compared to control (Fig. 7C, left). There was a 39% attenuation of peak inward Ca\textsuperscript{2+} channel current (control vs SRIF in slices: -111.0 ± 26.3 pA vs -49.0 ± 36.7 pA; N = 2 cells; in isolated cells: -72.8 ± 13.9 pA vs -44.0 ± 7.7 pA; N = 6 cells; p = 0.0163; Fig. 7D).
Figure 7. SRIF modulation of K\(^+\) and Ca\(^{2+}\) channel currents in M1 ipRGCs in OPN-EGFP retinas and isolated OPN-EGFP cells.

(A) Outward I\(_K\) recording from a M1 ipRGC in the absence (left) and presence (right) of SRIF (100 nM). (B) Mean I-V relationship for I\(_K\) in the absence (black) and presence (red) of SRIF. (C) I\(_{Ca}\) recordings in the absence (left) and presence (right) of SRIF. (D) Mean I-V relationship for I\(_{Ca}\) in the absence (black) and presence (red) of SRIF. Recordings were made in the presence of TTX (500 nM). Voltage command protocol is shown below the current trace. N = 4-7 cells/group. * p < 0.05.
Similarly, when we tested for the effects of the selective SST4 agonist L-803,087, on M1 ipRGCs, they produced (Fig. 8A, left) outward K⁺ currents that were potentiated in the presence of L-803,087 (1 µM; Fig. 8A, right). The changes in K⁺ currents with the application of L-803,087 can be seen in the mean I-V relations (control vs L-803,087 in slices: 1141 ± 212.8 pA vs 1336 ± 199.6 pA; N = 4 cells; p = 0.0064; Fig. 8B). In addition, M1 ipRGCs displayed a reduction in Ca²⁺ channel currents when we compared control recordings (Fig. 8C, left) to those made with the application of L-803,087 (Fig. 8C, right). This is shown in the mean I-V relations in Figure 8D (control vs L-803,087 in slices: -74.9 ± 23.9 pA vs -33.11 ± 20.3 pA; N = 2 cells; in isolated cells: -75.3 ± 12.1 pA vs -62.5 ± 9.0 pA; N = 3 cells; p = 0.0343). Thus, SRIF acting via SST4 receptors modulates K⁺ and Ca²⁺ ion channels in M1 ipRGCs.
Figure 8. The selective sst₄ agonist L-803,087 modulated K⁺ and Ca²⁺ channel currents on M1 ipRGCs in intact OPN-EGFP retinas and isolated OPN-EGFP cells.

(A) Outward Iₖ recording from an M1 ipRGC in the absence (left) and presence (right) of L-803,087 (1 µM).  (B) Mean I-V relationship for Iₖ in the absence (black) and presence (red) of L-803,087.  (C) IₖCa recordings in the absence (left) and presence (right) of L-803,087.  (D) Mean I-V relationship for IₖCa in the absence (black) and presence (red) of L-803,087. Recordings were made in the presence of TTX (500 nM). Voltage command protocol is seen below the current trace. N = 4-7 cells/group. * p < 0.05.
Effects of SRIF and L-803,087 on M1 ipRGC excitability

At rest, the EGFP labeled ipRGCs from which we recorded in retinal slices had a resting membrane potential of -63 ± 1.7 mV (n = 30 cells), similar to that reported for M1 ipRGCs (Zhao et al., 2014). The recorded M1 ipRGCs exhibited an intrinsic spontaneous firing rate of 1.5 ± 0.16 Hz (n = 30 cells). Current-clamped M1 ipRGCs fired spontaneously at rates that decreased during application of SRIF (Fig. 9A, black vs red). The averaged waveform was altered in the presence of SRIF, with a slight reduction in spike amplitude and a small increase in after hyperpolarization (Fig. 9C, red). Quantification of the average peak amplitude of spikes in the presence of SRIF revealed a non-significant trend toward reduction (Fig. 9D). Analysis of spike half-widths in control or in the presence of SRIF also showed no significant differences (Fig. 9E). However, SRIF significantly increased the interevent interval between spikes (501 to 1300 ms) dramatically reducing the spike frequency in M1 ipRGCs (1.9 to 0.6 Hz; Fig. 9F, G).
Figure 9. Spontaneous spike activity in M1 ipRGCs from OPN-EGFP retinas was modulated by SRIF and L-803,087.

(A) Representative trace of spontaneous spiking activity of a M1 ipRGC without (left) and with (right) the application of SRIF (100 nM).  (B) An example of a spontaneously spiking M1 ipRGC in absence (left) and presence (right) of L-803,087 (1 µM).  (C) The average waveform of spikes from M1 ipRGCs in control (gray), SRIF (red) and L-803,087 (blue). Insets show an overlay of an average waveform in control with SRIF or L-803,087. Quantitative analysis of peak amplitude (D) and half-width (E) of spikes from M1 ipRGCs show no significant differences in the presence of SRIF or L-803,087. The interevent intervals (F) and spike frequency (G) significantly increased and decreased, respectively, in the presence of SRIF, L-803,087, and in combination with the D1 agonist SKF38393 (5 µM).  * p < 0.05.
We next examined if the actions of SRIF in regulating the resting properties of M1 ipRGCs were mediated through sst4 receptors. In the presence of L-803,087, M1 ipRGCs in retinal slices displayed a decrease in the number of spikes and a larger, more transient spike (Fig. 9B). However, there was no significant change in the average waveform, peak amplitude and half-width (Fig. 9C blue, D, and E). Nevertheless, M1 ipRGCs showed the characteristic effects of SRIF on spontaneous activity, which include an increased interevent interval (675 to 1693 ms) and decreased spike frequency (1.6 to 0.6 Hz) during application of L-803,087 (Fig. 9F,G).

Next we addressed changes in the resting properties in M1 ipRGCs in the presence of SRIF and DA amacrine cell input. We tested changes in spike properties in the presence of SRIF and the D1 agonist SKF38393, as well as L-803,087 and SKF38393. There were no significant changes in the peak amplitude and half-width (Fig. 9D,E). The interval between spikes increased and the spike frequency decreased compared to control (Fig. 9F,G). There was no significant additive effect of SKF38393 upon the actions of SRIF or L-803,087 (Fig. 9F,G). Together, these results suggest that SRIF acts through the sst4 receptor to directly regulate M1 ipRGC resting properties, and this action is unaffected by DA amacrine cell input.

**Contacts of SRIF processes and M1 ipRGC dendrites in stratum 1 of IPL**

To test if SRIF amacrine cell processes formed contacts with M1 ipRGC dendrites, we examined the distribution of SRIF amacrine cells and melanopsin immunoreactive M1 ipRGCs in both retinal sections and whole mounts. Melanopsin immunoreactivity was localized to a small number of somata in the GCL (N = 4 retinas; Fig. 10A, green, asterisk) and in the proximal INL (not shown), and to dendrites in strata 1 and 5 of the IPL (Fig. 10A, A', green). The appearance of the melanopsin immunoreactive cells with dendrites in stratum 1 of the IPL is consistent with their identity as M1 ipRGCs (Berson et al., 2010). SRIF immunoreactive processes (red) and melanopsin immunoreactive dendrites (green) were often in close proximity.
in stratum 1 of the IPL (Fig. 10A, A’). Rarely occurring SRIF processes in stratum 5 of the IPL were also adjacent to melanopsin immunoreactive dendrites (Fig. 10A). Some SRIF immunoreactive processes (N = 4 retinas; Fig. 10B) and melanopsin immunoreactive dendrites (Fig. 10B’) in whole mounted retinas were in juxtaposition, and they made multiple contacts in stratum 1 of the IPL (Fig. 10B” , arrows). In contrast to the numerous contacts between the SRIF and TH immunoreactive amacrine cell processes in stratum 1 of the IPL, there were fewer SRIF immunoreactive processes adjacent to melanopsin immunoreactive dendrites.

To test if the contacts between SRIF amacrine cell processes and melanopsin ipRGC dendrites were non-random, we quantified the fluorescent density of contacts between SRIF processes and melanopsin dendrites in a 3D reconstruction of an image stack taken through the IPL (Fig. 10C, C’). The number of contacts between the SRIF processes and melanopsin dendrites was significantly more in the original reconstruction (0°) compared to the rotated (90°, 180° and 270°) reconstructions (Fig. 10D,E; N = 11 retinas; p < 0.03, 0.04, and 0.04). The decrease in % fluorescent density of contacts upon rotation was consistent when normalized to melanopsin (0.90 ± 0.39 % at 0°, 0.12 ± 0.05 % at 90°, 0.09 ± 0.05 % at 180°, and 0.10 ± 0.05% at 270°; N = 11 retinas) or SRIF fluorescent density (1.34 ± 0.36 % at 0°, 0.66 ± 0.33 % at 90°, 0.69 ± 0.37 % at 180°, and 0.68 ± 0.31% at 270°; N = 11 retinas; Fig. 10D,E). On this basis, we conclude contacts between the apposing SRIF and melanopsin immunoreactive processes in stratum 1 of the IPL are putative sites of neurotransmission between SRIF amacrine cells and M1 ipRGCs.
Figure 10. SRIF and melanopsin immunostaining show the juxtaposition of SRIF amacrine cell processes and M1 ipRGC dendrites in stratum 1 of the IPL.

(A) SRIF amacrine cell processes (red) are mainly in stratum 1 of the IPL and a few processes are in strata 3 and 5 of the IPL. A melanopsin immunoreactive somata (asterisk) and its dendrites in strata 1 and 5 of the IPL. (A’) is a magnification of the inset in (A) showing melanopsin ipRGC dendrites and SRIF amacrine cell processes forming contacts in stratum 1 of the IPL. (B) SRIF immunoreactive varicose processes in stratum 1 of the IPL in a whole mount. (B’) Melanopsin immunoreactivity shows robust labeling of melanopsin ipRGC varicose dendrites. (B’’) Merged images show close contacts between SRIF amacrine cell processes often near the varicosities of the M1 ipRGC dendrites. (C) A 3D reconstruction of SRIF processes and melanopsin dendrites from an image stack taken through the IPL of a retinal whole mount shows many punctate SRIF amacrine cell processes near a displaced melanopsin immunoreactive ipRGC somata and dendrites. (C’) Omitting the red channel that labeled SRIF amacrine cells reveals the contacts (pink) formed with melanopsin ipRGC somata and dendrites. Arrows show the contacts between SRIF amacrine cell processes and melanopsin ipRGC dendrites. (D) Quantification of fluorescent density created from the contacting processes was normalized to the fluorescent density of the melanopsin immunoreactivity. Rotated images had significantly less fluorescent density generated from the contacting processes. (E) Fluorescent density of the contacts was also normalized to the fluorescent density of SRIF immunoreactivity. Fluorescent density of contacts following rotation was significantly less than un-rotated image. Scale bar: 10 µm. * p < 0.05.
Localization of sst₄ receptors on ipRGCs

To determine whether receptors for direct modulation by SRIF of M1 ipRGCs existed, we tested for the expression of sst₄ immunoreactivity in transverse retinal sections. We used the OPN4-EGFP mouse retinas (Schmidt et al., 2008) and processed them for sst₄ immunolabeling. sst₄ immunoreactivity was weakly expressed by medium and large diameter somata in the GCL, and more strongly expressed on numerous primary as well as secondary dendrites in the IPL. sst₄ immunoreactivity was robustly present in axons of the nerve fiber layer (N = 4 retinas; Fig. 11A, red). EGFP labeled somata were located in the GCL and their dendrites extended from the GCL across the IPL (Fig. 11B') to stratum 1 of the IPL (Fig. 11C'). The EGFP labeled cell somata and dendrites were sst₄ immunoreactive (Fig. 11B'', C''). In whole-mount OPN4-EGFP retinas, all EGFP fluorescent cells were sst₄ immunoreactive (not shown). Collectively, our data suggest that sst₄ is expressed by multiple ganglion cell types, including the EGFP-labeled ipRGCs.
Figure 11. Transverse section through an OPN4-EGFP retina showing co-localization of EGFP fluorescence with sst₄ immunoreactivity.

(A) OPN4-EGFP retinas contain EGFP fluorescent (green) M1 ipRGCs. sst₄ immunoreactivity (red) is localized to RGC somata and to numerous processes throughout the IPL, and RGC axons in the NFL. Inset (B) is magnified in (B' and B'') showing a M1 ipRGC dendrite ascending to stratum 1 of the IPL that is co-labeled with sst₄ immunoreactivity. Inset (C) is magnified in (C' and C'') which shows co-localization between M1 ipRGC and sst₄ immunoreactivity in stratum 1 of the IPL. Arrows indicate the co-localized OPN-EGFP fluorescent process with sst₄ immunoreactivity. Scale bars: (A) 20 µm, (B) 5 µm, and (C) 10 µm.
Discussion

The DA amacrine cell-M1 ipRGC microcircuit provides reciprocal intraretinal interactions that participate in light adaptation and non-image forming vision. Our results indicate that both cell types can be inhibited directly by the wide-field SRIF amacrine cell output acting postsynaptically via two distinct SRIF receptor subtypes, sst\textsubscript{2A} and sst\textsubscript{4}, expressed by DA amacrine cells and ipRGCs, respectively. In addition to attenuating the intrinsic light response of M1 ipRGCs, we found that SRIF differentially increased K\textsuperscript{+} currents and decreased Ca\textsuperscript{2+} currents in both DA amacrine cells and M1 ipRGCs. These modulatory actions are inhibitory, the former acting to hyperpolarize the cell and reduce action potential generation, and the latter acting to decrease calcium-mediated transmitter release. SRIF produces similar modulatory actions on voltage gated K\textsuperscript{+} and Ca\textsuperscript{2+} channel currents in photoreceptors, bipolar cells, and other ganglion cells (Johnson et al., 1998; Akopian et al., 2000; Petrucci et al., 2001; Farrell et al., 2010), suggesting that SRIF signaling in the retina is inhibitory.

The parallel inhibitory actions of SRIF would compensate for the disinhibition of M1 ipRGCs, which arises from SRIF-mediated inhibition of DA amacrine cells (Fig. 13). In other words, when SRIF amacrine cells inhibit DA amacrine cells, the DA amacrine cells release less inhibitory signal onto the M1 ipRGCs, in effect maintaining the M1 ipRGCs in a more excitatory state. To counteract this action, SRIF provides parallel inhibition onto M1 ipRGCs, which would add back the lost inhibitory influence. As a result, SRIF actions on DA amacrine cells could proceed with tuning retinal dopamine levels without leading to a destabilization of the M1 ipRGC light response. The identity of the long sought interneuronal subtype responsible for inhibition of DA amacrine cells (Pérez-León et al., 2006; Wong et al., 2007; Atkinson et al., 2013; Newkirk et al., 2013) is revealed to be the rarely occurring SRIF-containing wide-field amacrine cell described in this report.
Figure 12. Regulation of the DA amacrine cell - M1 ipRGC microcircuit by SRIF amacrine cells. The principle contacts at which SRIF amacrine cells mediate parallel inhibition (red arrows) of DA amacrine cells and M1 ipRGCs (whose reciprocal interactions are denoted with black arrows) occur within stratum 1 (S1) of the inner plexiform layer. Few contacts occur in strata 3 (S3) and 5 (S5). SRIF amacrine cells target M1 ipRGCs for inhibition in a manner that can compensate for the loss of DA mediated inhibition of M1 ipRGCs, which occurs when SRIF inhibits the DA amacrine cell.
SRIF inhibits excitability and intrinsic photosensitivity

Both DA amacrine cells and M1 ipRGCs spike spontaneously, leading to sustained neurotransmitter release (Gustincich et al., 1997; Feigenspan et al., 1998; Zhao et al., 2014). The spontaneous spiking of DA amacrine cells was reduced in the presence of SRIF and L-054,264, and M1 ipRGCs decreased their spiking in the presence of SRIF and L-803,087. These reductions in excitability caused by SRIF in both cell types underscore the inhibitory nature of SRIF modulation and are likely mediated principally by the K⁺ channel enhancement induced by SRIF. Adding to this action, a SRIF-induced Ca²⁺ current attenuation was also established here, which would reduce the release of neurotransmitter by both cell types. In the case of M1 ipRGCs, this would reduce release of the excitatory neurotransmitter glutamate onto DA amacrine cells (Zhang et al., 2008; Zhang et al., 2012; Atkinson et al., 2013), as well as their central retinal recipient nuclei (Gooley et al., 2001; Engelund et al., 2010; Gompf et al., 2015). In the case of DA amacrine cells, Ca²⁺ channel inhibition would reduce the release of dopamine as well as their co-transmitter GABA (Contini and Raviola, 2003; Hirasawa et al., 2009; Hirasawa et al., 2012).

In comparing the modulatory effects of SRIF, L-803,087, SKF38393, and SCH23390 on intrinsic melanopsin-based light response in M1 ipRGCs, we found that the actions of SRIF were carried out directly on the M1 ipRGCs, and were not mediated in any manner by the DA amacrine cell. We showed that the intrinsic light response of M1 ipRGCs is inhibited by dopamine acting directly on D1 receptors in mouse retina, consistent with findings in the rat retina (Van Hook et al., 2012), and we showed that SRIF also inhibited the melanopsin-based light response in M1 ipRGCs. Inhibition of the M1 ipRGC photocurrent by the selective sst₄ agonist L-803,087 and the D1 agonist SKF38393 individually means that the inhibitory inputs from DA amacrine cells and SRIF amacrine cells act independently on M1 ipRGCs.

Co-application of SRIF and SKF38393, or L-803,087 and SKF38393, did not produce an additive reduction of the M1 ipRGC photocurrent, possibly reflecting a common signaling
pathway that increases cAMP (Feigenspan and Bormann, 1994). In addition, blocking dopamine responses in M1 ipRGCs with the D1 antagonist SCH23390, which might be expected to increase the light response, had no effect, possibly due to a low basal release of dopamine from DA amacrine cells and the possibility of other inhibitory pathways converging on the M1 ipRGC. We also found that L-803,087, applied in the presence of SCH23390, continued to significantly attenuate the M1 ipRGC photocurrent to the same degree as L-803,087 alone. These data highlight the non-additive and independent inhibitory actions of dopamine and SRIF on M1 ipRGC photocurrents.

It is also interesting to note that SRIF’s parallel inhibitory actions occur at different mechanistic levels. SRIF and dopamine both inhibit M1 ipRGCs, but via different intracellular mechanisms: SRIF modulates voltage-gated K⁺ and Ca²⁺ channel currents (Farrell et al., 2010), while dopamine decreases Na⁺ currents and increases hyperpolarization-activated cation currents (Hayashida and Ishida, 2004; Chen and Yang, 2007; Hayashida et al., 2009; Van Hook et al., 2012).

**Infrastructure for distinct sst receptor-mediated inhibition**

In addition to the presumed locations for reciprocal interactions of DA amacrine cells and M1 ipRGCs in stratum 1 of the IPL (Østergaard et al., 2007; Vugler et al., 2007; Wong et al., 2007; Zhang et al., 2008; Van Hook et al., 2012), there are other GABAergic, glycinergic, and glutamatergic cells ramifying in stratum 1 of the IPL that could provide regulatory signaling of the SRIF-DA-ipRGC microcircuit (Dumitrescu et al., 2009; Contini et al., 2010; Newkirk et al., 2013). Whether other cells modulate this microcircuit and alter the light response of the M1 ipRGC or adjust their glutamatergic signaling (Gompf et al., 2015) to DA amacrine cells is an important question for future study.

Here we showed the processes of the SRIF amacrine cells and DA amacrine cells are closely aligned and form fascicles in stratum 1 of the IPL, and that they are also in close
proximity to the dendrites of M1 ipRGCs. These findings are congruent with our evidence supporting synaptic communication between SRIF amacrine cells and DA amacrine cells, as well as SRIF amacrine cells and M1 ipRGCs.

We confirmed DA amacrine cells express the sst$_{2A}$ receptor (Johnson et al., 1998; Helboe and Møller, 1999; Cristiani et al., 2002; Thermos, 2003). Although others have reported localization of sst$_5$ receptors on DA amacrine cells (Ke and Zhong, 2007; Wu et al., 2012; Chen et al., 2014), we have not found sst$_5$ expression on DA amacrine cells (data not shown), thus we focused on the physiological effects of sst$_{2A}$, and further studies will be needed to confirm localization of sst$_5$ and its functional effects on DA amacrine cells. We also showed that M1 ipRGCs express the sst$_4$ receptor, corresponding to previous reports that sst$_4$ is the only SRIF receptor subtype localized to ganglion cell somata and processes (Cristiani et al., 2002; Farrell et al., 2010). Anatomically, DA amacrine cells and M1 ipRGCs bear the appropriate receptors for modulation by the neuropeptide SRIF, which is solely released from the SRIF amacrine cells.

**Alternative regulatory signaling by SRIF and DA amacrine cells**

Although we show that SRIF could mediate parallel inhibition of DA amacrine cells and M1 ipRGCs, SRIF amacrine cells also contain GABA (Cristiani et al., 2002; Thermos, 2003). Further investigation is needed to explore the implications of the SRIF and GABA co-immunoreactive processes that are apposed to DA amacrine cell or M1 ipRGC processes. Others have shown GABA-mediated amacrine cell signaling was enhanced in the presence of other co-transmitters including vasoactive intestinal peptide, dopamine, and somatostatin (Veruki and Yeh, 1992; Feigenspan and Bormann, 1994; Contini and Raviola, 2003; Hirasawa et al., 2009; Hirasawa et al., 2012). It is possible that the inhibitory effects of SRIF are a means for refining fast GABAergic signaling from the SRIF amacrine cells onto DA amacrine cells and M1 ipRGCs.
Impacts of SRIF-mediated inhibition

Inhibition of the microcircuit (DA amacrine cells - M1 ipRGCs) by SRIF is of special importance since the two cell types mediate critical functions in the visual system. The dopamine system modulates actions including circadian photoentrainment, gap junction coupling, and light- and dark adaptation (Ruan et al., 2008; Jackson et al., 2012; Jin et al., 2015). M1 ipRGCs, which produce robust light-evoked depolarizations, project to the suprachiasmatic nucleus of the hypothalamus and olivary pretectal nuclei, implicating central regulation of circadian rhythms and pupillary light reflex, respectively (Schmidt et al., 2008; Schmidt and Kofuji, 2009). In addition, ipRGCs project to other brain regions that influence sleep, mood, cognition, pain and addiction (Hattar et al., 2006). Compensatory inhibition of the M1 ipRGCs could therefore be expected to stabilize its broad actions on targets in the brain as well as its local targets in the retina.
References


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Chapter 4

Vasoactive intestinal polypeptide-expressing amacrine cells in mouse retina*

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*In preparation
Abstract:
Visual processing in the retina is mediated by multiple microcircuits that are composed of different retinal cell classes that collectively perform computations on visual stimuli prior to further processing by the brain. In order to understand how retinal microcircuits, or the interaction between cell types, can mediate the processing of visual information, we must understand how individual retinal cell types work. Amacrine cells form the most diverse retinal cell type with ~30 different populations; however, they are the least understood type in terms of their connectivity, cellular properties, and role in bipolar to ganglion cell interactions. In this investigation, we identify amacrine cells that express vasoactive intestinal polypeptide (VIP) using a VIP-tdTomato transgenic mouse line, and determine their intrinsic properties and synaptic partners. There are three morphologically distinct types of VIP amacrine cells. Mainly, there are two monostratified types and one bistratified type. Electrophysiological reveals the presence of specific Na⁺, K⁺, and Ca²⁺ channels on the major subtypes. In addition, VIP amacrine cells receive glutamatergic excitatory inputs, GABAergic and glycinergic inhibitory inputs. Anatomical studies confirm glutamatergic input by means of synaptic interactions with rod bipolar cells, type 2-OFF cone bipolar cells, and type 6-ON cone bipolar cells. Together, these results provide detailed anatomical and electrophysiological descriptions of the properties and microcircuit partners of VIP amacrine cells. These findings form the basis of a connectivity map involving VIP amacrine cells with other retinal neurons, as well as understanding the role of VIP amacrine cells in visual processing.
Introduction:

Processing of images presented to the brain is dependent on the complex signaling of retinal cells. Broadly speaking there are five retinal cell types: photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. The retina is organized into three neuronal layers and two synaptic/plexiform layers. Inhibition in the inner plexiform layer (IPL) consists of amacrine cell signaling onto bipolar and ganglion cells, as well as other amacrine cells. Amacrine cells are estimated to consist of 30-40 different morphological types, which are categorized as narrow-, medium-, or wide-field types based on the lateral extent of their processes, as well as their neurochemical composition (GABA or glycine) (MacNeil and Masland 1998; Masland 1988; Masland 2012b; Wässle 2004). In addition, recent studies revealed types of amacrine cells that express glutamate, and non-GABA and non-glycine amacrine cells (Haverkamp and Wässle 2004; Johnson et al., 2004; Kay et al., 2011). Despite the revelations of different morphological amacrine cell types, we still do not have a clear understanding of the physiological function and synaptic partners of each amacrine cell type (Masland 2012a; Masland 2012b).

A group of amacrine cells are neuropeptide expressing amacrine cells that co-express a classical neurotransmitter, such as GABA, in addition to a neuropeptide, such as somatostatin, vasoactive intestinal peptide (VIP), neuropeptide Y, substance P, and pituitary adenylate cyclase-activating polypeptide (Bagnoli et al., 2003). Earlier extracellular recordings from rabbit retina showed that VIP increased activity in ganglion cells under most light conditions (Jensen 1993), yet it enhanced inhibitory GABA-induced currents and modulated GABA_A receptor function on rod bipolar and ganglion cells (Veruki and Yeh 1992; Veruki and Yeh 1994). In addition, VIP has been shown to have developmental and neuroprotective roles following retinal damage (Szabadfi et al., 2012). These studies suggest VIP has an extensive and long-term influence on multiple retinal circuits.

Cre/lox recombination approaches has aided in the genetic labeling of specific retinal cell populations, and the investigation of their functional role in the retina (Gompf et al., 2015;
Ivanova et al., 2010; Lee et al., 2014; Lu et al., 2013). Using a transgenic mouse line expressing Cre under the control of the VIP promoter (Taniguchi et al., 2011) we found two main VIP amacrine cell types in the INL, and a third cell type in the GCL. These cells have multiple K+ current types including TEA-sensitive delayed rectifier Kv, 4-AP/Cd2+-sensitive A-type. VIP amacrine cells also have TTX-sensitive Na+ currents, though many had no or very small Na+ currents. Most cells lacked action potentials under current clamp, but had single spikes in response to depolarizing current injections. There was also the presence of verapamil-sensitive sustained inward L-type Ca2+ channel currents. Inhibitory inputs were dominated by GABA<sub>A</sub> and glycine receptors. Finally, immunohistochemical studies revealed pre- and post-synaptic interactions between OFF-cone type 2 and ON-cone type 6 bipolar cells, and rod bipolar cells with VIP amacrine cell process in strata 1, 3, and 4 of the IPL, consequently demonstrating presence of putative excitatory synaptic inputs. These findings will aid in identifying the influence of VIP modulation on retinal microcircuit activity.

**Materials and Methods**

*Animals:* Animal housing and all experiments were in accordance with the guidelines and policies for the welfare of experimental animals prescribed by the UCLA Animal Research Committee, UCLA Division of Laboratory Animal Medicine and the U.S. Public Health Service Policy on Humane Care and use of Laboratory Animals.

Adult mice of both sexes (20-30g; < 3 month old) were used for these studies. VIP-tdTomato and VIP-ChR2 mouse lines were generated by crossing a VIP-Cre transgenic mouse line (VIP<sup>tm1(cre)Zjh</sup>/J; VIP-internal ribosome entry site (IRES)-Cre; JAX #10908) with a Cre-dependent tdTomato (B6.Cg-Gt(Rosa)26Sor<sup>tm9(CAG-tdTomato)Hze</sup>/J; Ai9; JAX #7909) or ChR2-EYFP (B6;129S-Gt(Rosa)26Sor<sup>tm32(CAG-COP4*H134R/EYFP)Hze</sup>/J;JAX #012569) reporter mouse line (Madisen et al., 2012; Madisen et al., 2010; Taniguchi et al., 2011) (Jackson Laboratories, Bar Harbor, ME). The mice were maintained on a 12-hour light/dark cycle.
Mice were deeply anesthetized using 1-3% isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL) and killed by decapitation or cervical dislocation. Eyes were enucleated, and the cornea and lens were removed in Hank’s Balanced Salt Solution (HBSS) (ThermoScientific, Waltham, MA).

**Tissue Preparation for vertical retinal sections:**
Eyecups were immersion fixed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 for 15-60 minutes. Eyecups were subsequently washed in 0.1 M PB for 30 minutes and stored in 30% sucrose overnight at 4°C, and then embedded in optimal cutting temperature medium (Sakura Finetek Inc., Torrance, CA) and sectioned vertically at 12 µm. Retinal sections were placed onto gelatin-coated slides and stored at -20°C until used for immunohistochemistry.

**Immunohistochemistry**
Retinal sections were processed for immunohistochemical labeling using an indirect immunofluorescence method (Vuong et al., 2015). Frozen retinal sections were thawed for 10 minutes at 37°C on a warming plate, then washed three times for 10 minutes with 0.1 M PB (pH 7.4). Sections were then incubated in a blocking solution of 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X-100 in 0.1 M PB for 1 hour at room temperature. Following removal of the blocking solution, the primary antibody solution was immediately added to the sections and incubated for 12-16 hours at 4°C. Primary antibody solution contained 3% NGS, 1% BSA, 0.05% sodium azide and 0.5% Triton X-100 in 0.1M PB. Retinal sections were then washed three times for 10 minutes in 0.1 M PB. The sections were then incubated with their corresponding secondary antibodies goat anti-rabbit, -rat or -mouse conjugated to Alexa 568 or 488 (1:1000; Invitrogen, Grand Island, NY) for two hours at room temperature. The secondary antibody was removed and sections were washed three times in 0.1 M PB for 10 minutes per wash. Sections were air-dried and mounted using Aqua Poly/Mount.
To control for nonspecific binding of the secondary antibody, the primary antibodies were omitted in the single-labeling studies.

For immunohistochemistry controls, all antibodies were tested using mouse retina in single immunostaining experiments to confirm specificity and optimize concentration prior to performing any double labeling experiments. Experiments omitting primary antibodies eliminated specific immunostaining. For double labeling controls, one of the two primary antibodies used for double labeling was omitted during the primary incubation step. In this case, only the immunostaining by the remaining primary antibody was detected.

**Antibodies:** The following antibodies were used in this study: 1) rabbit polyclonal against vasoactive intestinal polypeptide (VIP; #7913 and #7916, CURE UCLA), 2) goat polyclonal choline acetyltransferase (ChAT; #AB144P, Millipore, Temecula, CA) 3) mouse monoclonal against tyrosine hydroxylase (TH; MAB5280, Millipore), 4) rabbit polyclonal against GABA (#A2052; Sigma-Aldrich, St. Louis, MO), 5) rat polyclonal against glycine (#IG1002; ImmunoSolution, Queensland, Australia), 6) guinea pig polyclonal against RNA binding protein with multiple splicing (RBPMS; #1832-RBPMS, PhosphoSolutions, Aurora, CO), 7) rabbit polyclonal against PKC α (ab4124, Cambridge, MA), 8) mouse monoclonal against synaptotagmin II (ZNP-1; Zebrafish International Resource Center, University of Oregon, Eugene, OR), 9) mouse monoclonal against Go- α (MAB3073; Millipore), and 10) mouse monoclonal against C-terminal binding protein-2 (CtBP2/RIBEYE; BD Transduction Laboratories, San Jose, CA).

**Confocal image acquisition**

The immunostaining images were acquired with a Zeiss 510 Meta or 710 confocal laser scanning microscope (LSM Carl Zeiss, Thornwood, NY) equipped with 488, 543, and 633 nm laser lines. Confocal scans were captured using a C-Apochromat 40X 1.2 NA corrected water
objective and a Plan-Apochromat 63X 1.4 NA corrected oil objective. Projections of three images (1024 X 1024 pixels or 2048 X 2048 pixels) with a total of 0.9-1.0 µm thickness (z-axis step between 0.3-0.5 µm) were collected and adjusted for brightness and contrast in Adobe Photoshop CS2 v.9.02 (Adobe Systems, San Jose, CA).

Live tissue preparation
To make retinal slices, retinas were isolated and placed ganglion cell layer down on a piece of nitrocellulose paper (Millipore, Bedford, MA), and cut into 150-200 µm slices using a razor blade tissue chopper (Stoelting Tissue Slicer; Stoelting, Wood Dale, IL). Slices were rotated 90° and held in place by two lines of vacuum grease.

Electrophysiological recordings
A gravity-fed perfusion system delivered mammalian extracellular Ringer’s and test solutions to the chamber at 1.3 ml/min. Whole-cell voltage- and current-clamp recordings were made in retinal slices from VIP-tdTomato and VIP-ChR2 mice.

Labeled cells were identified by epifluorescence using a Zeiss Examiner upright microscope equipped with a 40X water-immersion objective 1.2 NA. Drugs were superfused until their actions reached steady state before recording their responses.

To record changes in K+ channel currents the extracellular bath solution contained (in mM): 120 NaCl, 3 KCl, 1 MgCl₂, 1.2 Na₂HPO₄, 10 glucose, 2 mM CaCl₂, and 25 NaHCO₃. The intracellular pipette solution contained (in mM): 20 KCl, 120 K-gluconate, 2 MgCl₂, 0.2 EGTA, 10 HEPES, and 2 Na2-ATP. The extracellular bathing solution was bubbled in 95% O₂-5% CO₂ at room temperature (21–25°C). To isolate changes in Ca²⁺ channel currents the extracellular solution contained (in mM): 110 NaCl, 5 KCl, 5 CsCl, 0.1 4-aminopyridine, 7.5 BaCl₂, 15 TEA Cl, 10 glucose, and 10 HEPES, whereas the intracellular pipette solution contained (in mM): 120
CsMeSO₃, 10 TEA Cl, 0.1 CaCl₂, 1 EGTA, 10 HEPES, 3 ATP-Mg, 0.3 GTP-Li, and 8 phosphocreatine. Tetrodotoxin (TTX, 0.5 - 1 µM) was added to block Na channels.

Extracellular solutions were superfused via a fast perfusion system (VC8-S; ALA Scientific, Albany, NY). Patch electrodes with 5 to 10 MΩ tip resistance were pulled from fire-polished borosilicate glass capillary tubes using a micropipette puller (Sutter Instrument, Novato, CA). Cell voltage was clamped with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) using whole-cell capacitance and series resistance compensation. Holding potential was set at −60 mV. In voltage-clamp experiments, voltage steps (40 ms) were made in 10 mV increments from −80 to +40 mV. Current-clamp recordings were obtained to measure resting potentials and action potential frequency using the same solutions for recording K⁺ currents described above. Depolarizing current injection was used to monitor generation of action potentials and spike properties.

AMPA (100 µM) was puffed onto neighboring cells in the slice preparation through a 2-5 MΩ pipette with a Picospritzer. AMPA puffs elicited inhibitory postsynaptic currents (IPSCs) from VIP amacrine cells that were voltage-clamped to 0 mV, the reversal potential for cation channel currents. To isolate inhibitory receptor inputs, 3 µM gabazine (SR95531), 3 µM strychnine, and 50 µM (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA) was bath applied. Gabazine and TPMPA are GABA_A and GABA_C antagonists, respectively, and strychnine is a glycine receptor antagonist. Contributions of different inhibitory inputs onto VIP amacrine cells were determined by analyzing average charge transfer (Q), in VIP-tdTomato cells in response to AMPA puffs.

Amplitudes and gating properties of established currents were measured and correlated with VIP amacrine cell morphology and revealed by Lucifer Yellow (LY) intracellular filling and confocal microscopy to reveal dendritic stratifications of recorded cells.

All drugs and reagents were prepared in double-distilled water as stock solutions (frozen at -20°C) or prepared fresh before performing experiments. Tetrodotoxin (TTX) was purchased.
from Abcam Inc (Cambridge, MA), gabazine, strychnine, and TPMPA were purchased from Sigma-Aldrich (St. Louis, MO).

Data analysis for electrophysiology was performed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA). Graphing and statistical analysis were performed using Graphpad Prism 6.0F software (Graphpad Software, La Jolla, CA). Data are reported as the means ± SEM. Values of p < 0.05 were considered statistically significant. All data sets were compared using paired Student’s t-test.

Results

Localization of VIP amacrine cells in transgenic VIP-tdTomato mouse line

VIP amacrine cells were visualized in a transgenic VIP-tdTomato mouse line that was generated by crossing the VIP-Cre (VIP-IRES-Cre) mouse line (Taniguchi et al., 2011) with the Ai9 Cre-dependent tdTomato reporter mouse line (Madisen et al., 2010) (Fig. 1). To determine the specificity of the tdTomato-expressing cells, VIP-tdTomato retinas were labeled with a VIP antibody (Fig. 1A). All VIP immunoreactivity co-localized with the tdTomato fluorescent cells (Fig. 1A,B). VIP immunoreactivity was faint in cell bodies and processes compared to tdTomato fluorescence. There was strong tdTomato fluorescence in the cell bodies in the INL (average soma diameter = ~ 9.8 µm) and GCL (average soma diameter = ~8.8 µm) (Fig. 1C). The tdTomato fluorescent cell processes that emanated from cell bodies in the INL had a primary branch that ramified in strata 3 and 4 of the IPL (Fig. 1B). Most tdTomato fluorescent processes were located in strata 1, 3, and 4 of the IPL (Fig. 1C). There were also a few fluorescent processes sparsely distributed in stratum 5 of the IPL (Fig. 1C). The fluorescent processes were characterized by numerous varicosities. TdTomato fluorescence was not detected in the outer retina, photoreceptors, bipolar, horizontal, and Müller cells. These findings indicate that Cre activity was restricted specifically to VIP-immunoreactive amacrine cells.
Figure 1: VIP antibody labeling compared to transgene expression in retinas of VIP-tdTomato transgenic mice.

(A) VIP antibody labeling of cells in INL and GCL, and processes of the IPL. (B) Transgene labeling of the same cells shown in A. (C) VIP amacrine cells in the VIP-tdTomato mouse retina show tdTomato fluorescent somata localized to the INL and GCL. VIP amacrine cells have processes in ON- and OFF-layers of the IPL. IPL: inner plexiform layer. INL: inner plexiform layer. GCL: ganglion cell layer. Scale bar: 20 µm
To test whether there were multiple amacrine cell types, general markers for amacrine cells were used including GABA, glycine, choline acetyltransferase (ChAT), and tyrosine hydroxylase (TH) (Kay et al., 2011; Pérez de Sevilla Müller et al., 2007; Strettoi et al., 1992; Vaney and Young 1988; Vuong et al., 2015). TdTome fluorescent cell bodies co-localized with GABA immunoreactivity in the INL and GCL (not shown). TH immunoreactive processes in stratum 1 of the IPL overlapped with tdTomato fluorescent processes (not shown). There was also overlap of tdTomato fluorescent processes with calretinin immunoreactive processes in stratum 3 of the IPL, and calretinin- and ChAT-immunoreactive processes in stratum 4 of the IPL and some in stratum 5 of the IPL. The tdTomato fluorescent cell bodies did not co-localize with glycine, ChAT or TH immunoreactive cell bodies (not shown). These findings suggest tdTomato fluorescent cells in the VIP-tdTomato mouse retinas correspond to GABA-containing amacrine cells that stratify in stratum 1, 3, 4, and a few processes in 5 of the IPL.

In addition, to test if the VIP-tdTomato cells were ganglion cells, VIP-tdTomato retinal sections were labeled with RBPMS, a ganglion cell marker (Rodriguez et al., 2014; Vuong et al., 2015). The tdTomato fluorescent cells did not co-localize with RBPMS immunoreactivity (not shown). This result indicates the fluorescent labeling in VIP-tdTomato retinas are specific for amacrine cells, and no ganglion cells are labeled in this line.

Intrinsic electrophysiological properties of VIP amacrine cells

I next examined the types of voltage-gated ion channels present on VIP amacrine cells. I whole-cell voltage-clamped VIP-tdTomato fluorescent somata in retinal slices using an electrode filled with Lucifer yellow to confirm that I was recording from a labeled cell (Fig. 2A,B). These recordings revealed multiple voltage-gated ion channels that were differentiated based on current kinetics. There were different forms of $K^+$ currents including outward $K^+$ currents that were TEA-sensitive $K_v$ (Fig. 2C). I also saw currents that resembled inwardly rectifying K channels ($K_{IR}$), though the amplitude of these currents were nominal (Fig. 2C). At high voltage
steps (+40 to +100 mV) there were Ca$^{2+}$-activated K$^+$ currents in some cells (Fig. 2D). Some VIP amacrine cells also exhibited K$^+$ currents that had the A-type waveform, which were characterized with a relatively fast inactivating outward current (Fig. 2E).
Figure 2: VIP amacrine cells have multiple types of K⁺ channels.

Whole-cell patch clamp recordings were made from VIP-tdTomato cells in retinal slices with Lucifer yellow-filled pipettes to visualize their dendrites. Relative expression of K⁺ current types differed between VIP cells. **A.** Image of targeted VIP-tdTomato cell. **B.** Same bistratified cell filled with Lucifer yellow. **C.** Large outward TEA-sensitive Kᵥ currents and negligible Kᵢᵢ currents. **D.** K (Ca²⁺) currents evident at +30 mV. **E.** 4AP-sensitive A-type K⁺ currents isolated by digital subtraction (before and after block by Cd²⁺). Voltage steps: 100 ms; Scale = 100 pA in C, 50 pA in D, 40 pA in E.
Since there were discernable inward currents we next tested for the presence of TTX-sensitive Na$^+$ currents and hyperpolarization-activated cation currents ($I_h$). Voltage clamped VIP-tdTomato cells had small inward Na$^+$ currents that were less than 200 pA in response to depolarizing steps. These inward Na$^+$ currents were eliminated with the application of TTX (0.5 - 1 µM) indicating these cells have TTX-sensitive Na$^+$ channels (Fig. 3A). In response to hyperpolarizing steps, voltage clamped VIP tdTomato cells showed slowly activating inward currents that resembled the $I_h$ channel currents (Van Hook and Berson 2010). These inward currents were subsequently blocked in the presence of Cesium (Cs$^+$) (1 mM), confirming that these were $I_h$ currents (Fig. 3B).

The TTX-sensitive Na$^+$ currents and the presence of $I_h$ channel currents suggest that VIP amacrine cells may be spiking cells. To test this VIP-tdTomato cells were current clamped, and at resting membrane potential (-60 mV) these cells displayed infrequently occurring miniature spikelets (Fig. 3D). These spikelets had an average amplitude of ~15 mV, and were not considered spikes or action potentials. On the other hand, with injection of depolarizing current steps the VIP-tdTomato cells showed damped ~100 Hz oscillations (Fig. 3C).

There was also a subset of current-clamped VIP-tdTomato cells that showed a single spike (Fig. 4). The single spikes were elicited by increasing steps of depolarizing current injections. The 75 mV spike was followed by a plateau phase around -5 mV (Fig. 4B). These single spikes were eliminated in the presence of TTX (1 µM) (not shown).
Figure 3. Electrophysiological properties of ion channels

(A) Voltage clamp of TTX-sensitive $I_{\text{Na}}$, amplitudes of which varied greatly between cells, was typically 100 pA or less. Step duration 20 ms. (B) Voltage clamp of tdTomato cells have Cs$^+$-sensitive $I_h$. (C) Under current clamp, a typical cell responded to increasing depolarizing current steps with damped $\sim$100 Hz oscillations. $V_{\text{rest}} = -54$ mV. (D) Spikelets were often observed in unstimulated cells, possibly due to gap junction coupling ($V_{\text{rest}} = -43$ mV). Scale = 25 ms, 20 mV in C; 5 min, 10 mV in D. Step duration: 50 ms; Scale = 100 pA in A.
Figure 4. Single spike activity in VIP amacrine cells

(A) Few tdTomato cells responded to increasing depolarizing current steps with a single spike. $V_{rest} = -54$ mV (B) An enhanced depiction of the single spike elicited by depolarizing current injection shown in A.
Specific Ca$^{2+}$ channel currents in VIP amacrine cells

Voltage-gated calcium channels are important regulators of synaptic transmission. We next investigated for the presence of different voltage-gated calcium channels on VIP amacrine cells. In voltage-clamped VIP-tdTomato cells there were inward Ba$^{2+}$ currents that had a peak amplitude at 0 mV of ~350 pA (Fig. 5A,B, control). These inward Ba$^{2+}$ currents were blocked by the L-type calcium channel blockers verapamil and nifedipine (Fig. 5A,B). Quantitative analysis of the peak amplitude of the Ca$^{2+}$ channel currents in nifedipine and verapamil showed ~50% decrease (Fig. 5C). Application of the P/Q-type calcium channel blocker ω-agatoxin caused a significant decrease (~40%) in the inward Ba$^{2+}$ currents (Fig. 5C). Ba$^{2+}$ currents in control compared to the N-type calcium channel blocker ω-conotoxin did not show a significant decrease (Fig. 5C). These findings suggest there are differential contributions of voltage gated Ca$^{2+}$ channels to the inward Ba$^{2+}$ currents in VIP amacrine cells, and the Ca$^{2+}$ channels detected are likely L- and P/Q-type voltage gated calcium channels (Fig. 5). The results of these recordings suggest VIP amacrine cells contain a heterogeneous set of ion channels that aid in its signaling in the inner retina.
Figure 5. Different Ca\(^{2+}\) channel currents in VIP amacrine cells

(A) Inward Ba\(^{2+}\) currents in the absence (control) and presence of L-type Ca channel blocker verapamil. (B) Ba\(^{2+}\) induced inward Ca\(^{2+}\) channel currents in the absence (control) and presence of L-type Ca channel blocker nifedipine. (C) Normalized peak Ca channel current at 0 mV. * P < 0.05.
Optogenetic responses of VIP amacrine cells

Using the VIP-ChR2-EYFP transgenic mice, which were generated by crossing the VIP-Cre line with the Cre dependent channelrhodopsin-EYFP reporter line, we could optogenetically activate VIP amacrine cells. The VIP amacrine cells in the VIP-ChR2-EYFP retinas were identified based on their EYFP fluorescence (Fig. 6A). These cells were activated by a bright ChR2-activating stimulus that consisted of a 450 nm blue laser. Current-clamped VIP-ChR2-EYFP cells in retinal slices that were exposed to increasing intensity of the blue laser stimuli for 200 ms showed a characteristic single spike followed by damped oscillations, which were similar to the single spikes that we recorded in VIP-tdTomato cells (Fig. 4 and 6B). Voltage-clamped VIP-ChR2-EYFP cells in retinal slices during a 200 ms blue light stimuli displayed inward currents with increasing light intensity (Fig. 6C). At maximal blue light stimulation these cells had inward currents with a peak amplitude of ~ 100 pA. These results demonstrate an effective tool to non-invasively and reliably target and activate VIP amacrine cells. The VIP-ChR2-EYFP mouse line can be utilized to understand VIP amacrine cells output to bipolar and ganglion cells.
Figure 6. Activity of VIP-ChR2-EYFP amacrine cells

(A) An example of a VIP-ChR2-EYFP cell patched clamped in slice. (B) Current clamp responses to increasing intensity blue laser stimuli for duration of 200 ms bar showed damped oscillations. (C) Voltage clamp of an EYFP cell during similar stimuli shows inward currents increasing with light intensity.
Inhibitory synaptic inputs of VIP amacrine cells are mediated by GABA$_A$ and glycine receptors

In order to understand the functional connectivity between VIP and other amacrine cells we used AMPA puffs to illicit inhibitory postsynaptic currents (IPSCs) in VIP tdTomato cells. AMPA activates a subtype of ionotropic glutamate receptors that is present on most amacrine cell types (Dumitrescu et al., 2006; Peng et al., 1995). To ensure that the AMPA puffs were functionally activating the cells, VIP-tdTomato cells were voltage-clamped to -60 mV, the reversal potential of Cl$^-$ ions. Puffing on AMPA (100 µM) while holding the cell at -60 mV produced an inward AMPA current (Fig. 7A, inset). This confirmed that the AMPA puffs were functionally activating retinal cells, and subsequently demonstrating VIP amacrine cells contain AMPA receptors.

Contributions of different inhibitory inputs to VIP amacrine cells were determined by analyzing the average charge transfer (Q) or area under the elicited inhibitory postsynaptic currents (IPSCs) in VIP-tdTomato cells in response to AMPA puffs. AMPA puffs elicited outward IPSCs in VIP-tdTomato cells in retinal slices that were voltage clamped at 0mV (Fig. 7A). The Q of the VIP-tdTomato cells in control was 21 ± 1.24 pC. To determine the contribution of the different inhibitory receptors to the IPSCs we blocked GABA$_A$, GABA$_C$, and glycine receptors using gabazine (SR95531, 3 µM), TPMPA (50 µM), and strychnine (3 µM), respectively. There was a significant decrease in Q following application of gabazine and strychnine. Individually, gabazine and strychnine decreased Q by ~50% (Fig. 7B). The IPSCs were equally diminished by the application of gabazine and strychnine, and appeared to have an additive decrease in Q (~75% decrease), though the IPSCs were not completely removed (Fig. 7B). Application of TPMPA, on the other hand did not affect the presence of IPSCs in VIP-tdTomato cells (Fig. 7B). We also tested whether the IPSCs were a result of voltage-gated calcium channel mediated synaptic transmission by applying cadmium (200 µM), a non-specific voltage-gated calcium channel blocker. The presence of cadmium nearly completely eliminated the presence of IPSCs.
(Fig. 7B). We also confirmed that VIP-tdTomato cells contained GABA\textsubscript{A} receptors by examining the changes in the I-V relation in these cells in the absence and presence of muscimol (100 \textmu M), a GABA\textsubscript{A} agonist. Application of muscimol showed a slight shift in the I-V relation with a reversal potential at -60 mV, corresponding to Cl\textsuperscript{-} ions (Fig. 7C). These findings suggest VIP amacrine cells receive inhibition, likely from GABA and glycine containing amacrine cells that release GABA and glycine that act on GABA\textsubscript{A} and glycine receptors in VIP amacrine cells.
Figure 7. Inhibitory inputs to VIP-tdTomato cells

(A) AMPA (100µM) puff elicits mini inhibitory postsynaptic currents in VIP-tdTomato cells voltage clamped at 0mV. Inset: VIP-tdTomato cell voltage-clamped at -60mV show only inward AMPA current. Bar: 30 ms AMPA puff. (B) Average charge transfer (Q) of VIP-tdTomato cells in response to AMPA puffs, and in the presence of cadmium or SR (gabazine), TPMPA, strych (strychnine), which are GABA\textsubscript{A}, GABA\textsubscript{C}, and glycine receptor blockers, respectively. (C) Ramp I-V’s in control and muscimol, reversing at -60 mV, near $E_{Cl}$. 
**Excitatory synaptic inputs of VIP amacrine cells**

Amacrine cells receive excitatory glutamatergic input from bipolar cells, and the specific type of bipolar cell that provides input to amacrine cells is determined by the co-stratification of its processes in the different strata of the IPL. Based on the stratification pattern of VIP amacrine cell processes in strata 1, 3, and 4 of the IPL, we used bipolar cell markers that were specific for labeling bipolar cell axons that stratified in those same layers. More specifically, we aimed to find a pattern of labeling that consisted of VIP amacrine cell processes that were apposed to a bipolar cell axon that was co-localized to a synaptic ribbon marker.

**VIP amacrine cells and rod bipolar cells**

We first examined the relationship of VIP amacrine cell processes and rod bipolar cell axons in VIP-tdTomato retinal sections (Fig. 8A, pink vs yellow). Rod bipolar cells were labeled using an antibody against PKCα (Ghosh et al., 2004). The rod bipolar cells that were labeled had cell bodies in the distal INL and axons that extended through the IPL and terminated in strata 4 and 5 of the IPL. The axon terminals of the rod bipolar cells could be identified by their bulbous structures (Fig. 8A). Several tdTomato processes were apposed to the axons of the rod bipolar cells, and these apposition were not exclusively localized to the axon terminals, but were also found along the axon shaft of the rod bipolar cells (Fig. 8B,C, arrows). These contacts were further analyzed for synaptic interactions by using double immunohistochemistry. VIP-tdTomato retinal sections were stained with PKCα and the synaptic ribbon marker RIBEYE (Fig. 8, cyan). The triple labeled retinal sections revealed multiple putative synaptic contact sites, where tdTomato processes (pink) were apposed to PKCα-labeled rod bipolar cell terminals (yellow) that were co-localized to RIBEYE-labeled ribbon synapses (cyan) (Fig. 8D, boxes). There were also several sites where VIP-tdTomato processes were apposed to the rod bipolar cell axon shafts, but were no ribbon mediated synapses (Fig. 8B,C, arrows, and E,F boxes). These apposing processes between VIP amacrine cells and rod bipolar cells may be *en passant*
synapses that have been described previously (Dumitrescu et al., 2009). These results provide morphological evidence that suggests the apposing processes between VIP amacrine cells and rod bipolar cells are putative synaptic contact sites and possible en passant synaptic interactions.
Figure 8. VIP amacrine cells make en passant interactions with rod bipolar cells

Points of synaptic contact are found between VIP amacrine cell dendrites and rod bipolar cell axon shaft. (A) Mouse transverse section showing VIP-tdTomato amacrine cell bodies and processes (pink), RBC cell dendrites and axons, labeled by PKCα (yellow), and synaptic ribbons, labeled by RIBEYE (cyan). (B-C) Arrows: Potential points of synaptic contact as indicated by co-localization of RIBEYE (cyan) and PKCα (yellow) in close proximity to VIP amacrine cell process (pink). Primarily found in strata 4. Arrowhead: Closely aligned VIP amacrine cell process (pink) and RBC cell axon (yellow), but no synaptic ribbon marker. (D) Close up of points of potential synaptic contact. (E-F) Close up of closely aligned VIP amacrine cell process and RBC axon.
VIP amacrine cells and ON-cone bipolar cells

To determine the contacts between VIP amacrine cell processes and ON-cone bipolar cell axons we first used a non-specific marker that labels both ON-cone bipolar cells and rod bipolar cells, Go-α (Fig. 9A, green). Similar to the PKCα-labeled rod bipolar cells, the rod bipolar cells that were labeled with Go-α had axons that were closely apposed to the tdTomato processes (Fig. 9B, arrow, and D). The Go-α-labeled rod bipolar cell axons that were apposed to tdTomato processes did not co-localize with RIBEYE (Fig. 9B,D). The Go-α-labeled ON-cone bipolar cell terminals were localized to strata 3 and 4 (Fig. 9A,B,C, green) and many were found contacting tdTomato processes in those same layers (Fig. 9C, arrows). In order to determine whether these contacts could be ribbon-regulated synaptic interactions, we processed VIP-tdTomato retinal sections with antibodies against both Go-α and RIBEYE (Fig. 9, blue). Many of the Go-α processes in strata 3 and 4 were co-localized to RIBEYE, and these co-localizations were apposed to VIP-tdTomato processes (Fig. 9C,E,F). However, there were instances where VIP-td tomato processes that came in close contact with Go-α-labeled ON-cone bipolar cell axons, but neither cell type were co-localized with RIBEYE. Thus, those contacts may be non-specific associations between the two cells. Nevertheless, these findings indicate VIP amacrine cells are likely receiving synaptic input from ON-cone bipolar cells with axons that terminate in strata 3 and 4 of the IPL.
Figure 9. Many potential points of synaptic contact were found between VIP amacrine cell processes and ON-cone bipolar cells in strata 4 of the IPL.

(A) Mouse transverse section showing VIP-tdToma amacrine cell bodies and processes (red), ON-cone bipolar cell dendrites and axons (green), and synaptic ribbons, labeled with RIBEYE (blue). (B) Arrowhead: Closely aligned VIP-tdToma amacrine cell process and ON-bipolar cell axon. (C) Arrows: Many potential sites of synaptic contact as indicated by colocalization between Goα (green) and RIBEYE (blue) in close proximity to VIP amacrine cell process (red). Primarily found in strata 4 of the IPL. (D) Close up of indicated process in B showing lack of synaptic marker RIBEYE. (E-F) Points of potential synaptic contact.
VIP amacrine cells make contacts with type 2 OFF-cone bipolar cells and type 6 ON-cone bipolar cells

VIP-tdTomato retinal sections were labeled with ZNP-1, which is a non-specific marker for type 2 OFF- and type 6 ON-cone bipolar cells. ZNP-1 labeling was not visible in cell bodies, but was robust in bipolar cell axons and axon terminals in strata 1 and 4 of the IPL (Fig. 10A, yellow), which correspond to type 2 OFF- and type 6 ON-cone bipolar cells, respectively. VIP-tdTomato processes (Fig. 10A, pink) were in close contact with the axon terminals of type 2 OFF-cone bipolar cells in stratum 1 of the IPL (Fig. 10B,C). VIP-tdTomato processes were also in close contact with axons and axon terminals of type 6 ON-cone bipolar cells in stratum 4 of the IPL (Fig. 10B,D). We also labeled these sections with RIBEYE to demonstrate putative synaptic sites (Fig. 10, cyan). The triple labeled VIP-tdTomato retinal sections with ZNP-1 and RIBEYE revealed many contact sites that are ribbon-regulated synaptic interactions (Fig. 10, cyan). RIBEYE was found co-localized to the axons and axon terminals of both type 2 OFF- and type 6 ON cone bipolar cells (Fig. 10C,D), and many of these co-localized sites were apposed to VIP-tdTomato processes in strata 1 and 4 of the IPL (Fig. 10C,D, boxes). It is also interesting to note that there were many VIP-tdTomato processes that were co-localized to RIBEYE, and these co-localizations were apposed to the bipolar cell markers (Fig. 8D, 9D, and 10D), suggesting VIP amacrine cells may also provide reciprocal signaling to bipolar cells. In addition there were multiple contacts made between VIP-tdTomato processes and ZNP-1 labeled bipolar cell axons and axon terminals, however neither cell type was co-localized with RIBEYE. Together, these results support the idea of a synaptic interaction between VIP amacrine cells and cone bipolar cells, such that VIP amacrine cells receive input from specific type 2 OFF- and type 6 ON-cone bipolar cells, and possibly these bipolar cell types can receive VIP- or GABA-mediated signaling from VIP amacrine cells.
Figure 10. VIP amacrine cells make synaptic contact between type 2 OFF bipolar cells and type 6 ON bipolar cells.

(A) Mouse transverse section showing VIP-tdTomato amacrine cell bodies and processes (pink), type-2 OFF (strata 1) and type-6 ON (strata 4) cone bipolar cell dendrites and axons (ZNP-1, yellow), and synaptic ribbons (RIBEYE, cyan). (B) Arrow: Points of potential synaptic contact between VIP-tdTomato amacrine cell processes and Type-6 ON-cone bipolar cell axons found in strata 4. Arrowheads: Ribbon synapses at the axon terminal but not near any VIP amacrine processes. Outline Arrow: Potential point of synaptic contact between VIP amacrine cell process and Type-2 OFF-cone bipolar cell (C) Close up of potential point of synaptic contact in strata 1 (D) Close up of points of potential synaptic contact.
Discussion

In order to begin parsing the individual properties and the function of the ∼30-40 types of amacrine cells that are involved in retinal processing, it is critical to have well-characterized the transgenic mouse line that one can use to reliably target for experimental manipulations (Akrouh and Kerschensteiner 2015; Park et al., 2015; Zhu et al., 2014). In this study we paired electrophysiology and immunohistochemistry to characterize the intrinsic electrophysiological properties and synaptic partners of the VIP amacrine cells expressed in the transgenic VIP-tdTomato mouse line. We confirmed VIP amacrine cells are GABAergic, localized to both the INL and GCL, and have processes that mainly ramify in strata 1, 3, and 4 of the IPL (Park et al., 2015). We identified the presence of classical ion channels including K_{IR}, K_{v}, A-type K, I_{h}, TTX-sensitive Na^{+}, and L- and P/Q-type voltage gated Ca^{2+} channels. We showed that VIP amacrine cells are single spiking amacrine cells, and have oscillatory properties, likely due to the presence of TTX-sensitive Na^{+} channels or I_{h} channels, which have been shown to participate in oscillatory activity as well as stabilization of the resting membrane potential (Barnes and Werblin 1986; Koizumi et al., 2004; Robinson and Siegelbaum 2003; Trenholm et al., 2012).

VIP amacrine cells receive inputs from multiple retinal cell types. The presence of miniature excitatory postsynaptic potentiation in current-clamped tdTomato cells suggests VIP amacrine cells may be electrically coupled. Indeed, when Park et al. (2015) tested the gap junction blocker meclofenamic acid (MFA, 100 µM) (Manookin et al., 2008; Veruki and Hartveit 2009) in combination with inhibitory blockers, gabazine and strychnine, the voltage-clamped VIP amacrine cells lacked inhibitory response at +10 mV, the excitatory reversal potential. This confirms VIP amacrine cells receive inhibitory signaling through gap junctions.

VIP amacrine cells also receive inhibitory synaptic inputs from GABAergic and glycinergic amacrine cells, and excitatory synaptic inputs from, OFF- and ON-cone bipolar cell types. We showed VIP amacrine cells receive inhibitory inputs, which are mediated by GABA_{A} and glycine receptors, but not GABA_{C} receptors, and these results correspond with findings
from Park et al. (2015). With regards to excitatory inputs onto VIP amacrine cells, Park et al. (2015) voltage-clamped VIP amacrine cells and recorded responses to light stimulation. They found VIP amacrine cells had excitatory inward currents in response to light on and offset. The sluggish response to light offset suggested OFF bipolar cell input, whereas the excitatory inward current at light onset, which was blocked following the application of L-AP4 (blocks the ON pathway), confirmed ON bipolar cell input (Park et al., 2015). Our study contributes to the mapping of excitatory inputs to VIP amacrine cells. We showed VIP amacrine cells have putative excitatory synaptic interactions with rod bipolar cells and type 2 OFF- and type 6 ON-cone bipolar cells, as shown by co-localization of the bipolar cell markers with the synaptic ribbon marker RIBEYE. More specifically, VIP amacrine cells make contacts with putative synaptic release sites of type 2 OFF-cone bipolar cells and type 6 ON-cone bipolar cells in strata 1 and 4 of the IPL, respectively. The synaptic interactions of VIP amacrine cells with rod bipolar cells do not appear to be conventionally located at the synaptic terminal, rather putative synaptic interactions are localized along the axons of the rod bipolar cells, suggesting an *en passant* excitatory synaptic transmission. In addition, we found VIP amacrine cell processes also co-localized with RIBEYE, and were directly apposed to each of the bipolar cell markers. The curious placement of these apposed processes suggest VIP amacrine cells may also provide reciprocal actions onto bipolar cells (Dumitrescu et al., 2009). Further studies will be needed to deconstruct which bipolar cell types respond to VIP amacrine cell input, and how does their activity change in response to VIP amacrine cell input.

VIP acts through specific G-protein coupled receptors, however, their localization on specific retinal cell types remains controversial (D’Agata and Cavallaro 1998; Denis et al., 1991; Harmar et al., 2004; Lakk et al., 2012). There have been significant studies conducted to begin understanding the effects of VIP in inner retinal circuitry. Previous studies have shown application of VIP appears to potentiate GABA-mediated currents in horizontal cells, bipolar cells and amacrine cells, possibly by increasing cAMP production (Feigenspan and Bormann
Therefore, localizing specific VIP receptors on specific retinal cell types is an important future direction.

VIP amacrine cells in retinal circuit function: modulation of ganglion cell signaling

Stimulation of VIP amacrine cells would elicit the release of VIP or GABA. Though it may be difficult to determine the exact transmitter released upon stimulation, one could use a combination of pharmacology and optogenetic tools to isolate VIP amacrine cell output by recording changes in neighboring bipolar or ganglion cell activities (Chen et al., 2014; Zhu et al., 2014). A recent study by Park et al., (2015) used a cocktail of synaptic blockers that blocked glutamate receptors in the retina but left GABAergic synaptic transmission intact, and subsequently recorded morphologically identifiable ganglion cells in response to blue light stimulation of VIP-ChR2 amacrine cells. Multiple ganglion cell types had variable amplitudes of response to blue light stimulation (spontaneous inhibitory activity) including the OFF δ, ON-OFF direction selective, W3, OFF α, and ON α. Most of the spontaneous inhibitory responses were eliminated following application of gabazine, suggesting the inhibition was carried out via GABA release from VIP amacrine cells (Park et al., 2015). Moreover, the dendrites of these ganglion cells share stratification layers in the IPL (strata 1, 3, and 4) with VIP amacrine cells (Kim et al., 2015; Park et al., 2014; Sanes and Masland 2015; Zhang et al., 2012)

Collectively, VIP amacrine cells are classical inhibitory amacrine cells that display a heterogeneous set of ion channels including specific K⁺, Na⁺, and voltage gated Ca²⁺. The combination of these ion channels sets the resting membrane potential of these cells, and determine their unique spike properties. In addition, VIP amacrine cells are coupled to other amacrine cells, likely providing inhibitory signals. VIP amacrine cells also receive conventional GABAergic and glycinergic synaptic transmission from other amacrine cells. The excitatory inputs of VIP amacrine cells are mediated by type 2 OFF- and type 6 ON-cone bipolar cells.
Thus characterizing the intrinsic properties of VIP amacrine cells will further our understanding of amacrine cell function in visual processing.
References


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Chapter 5

Discussion
Discussion

The retina offers an accessible neural tissue that accompanies the modalities and network processing comparable to higher order structures in the brain. In the past decade development of genetically targeted labeling and manipulations of retinal cell types has greatly advanced our understanding of visual signaling.

It is much simplified, but for the most part an accurate description that the microcircuits in the retina responsible for processing visual stimuli is a fine balance of excitation and inhibition (Roska et al., 2006). Multiple channels of parallel processing mediate the signaling of light from the outer retina to the inner retina, but there is often crosstalk between and within the channels (Grimes et al., 2010; Grimes et al., 2015).

The goal of this dissertation was to contribute to understanding the role of amacrine cells. Each chapter of this report demonstrates tools currently used to investigate circuit interactions and will advance our understanding of amacrine cell-mediated retinal processing.

Identification of appropriate mouse models

The findings of this report highlight a technical challenge: the specificity of a transgenic mouse line. In order to determine the cellular and synaptic mechanisms by which amacrine cells modulate retinal circuit function it is critical that the mouse model used will serve to specifically target the cell of interest. By carefully characterizing four transgenic mouse lines driven by dopamine-related promoters using immunohistochemistry, soma/dendrite/axon measurements, and Neurobiotin cell fills, this study shows there is an abnormal amount of ectopic transgene expression. The results suggest there are more cells labeled with the transgene than are verified as dopamine amacrine cells. The possible explanations for this are reported in Chapter 2. These findings are not limited to the retina (Gelman et al., 2003; Lammel et al., 2015; Lindeberg et al., 2004; Savitt et al., 2005), and pose a dilemma for future investigators. The characterization of these lines provide an inventory of retinal cells that are labeled with the
transgene and will be of use to investigators looking to understand these labeled cells in retinal function.

**Investigation of non-image forming microcircuit and somatostatin-mediated inputs**

These studies used a combination of transgenic mouse lines, electrophysiology, pharmacology, and immunohistochemistry to parse out the specific interactions between three cell types in the dense plexus of stratum 1 of the IPL. The dopamine-releasing (DA) amacrine cell and intrinsically photosensitive retinal ganglion cells (M1 ipRGCs) are extremely integrated in their anatomical and functional relationships (Atkinson et al., 2013; Dkhissi-Benyahya et al., 2013; Sakamoto et al., 2005; Van Hook et al., 2012; Viney et al., 2007; Vugler et al., 2007; Zhang et al., 2012; Zhang et al., 2008). They share a stratification level in the IPL, they each express specific receptors for bi-directional modulation, and their intrinsic light response properties are mutually dependent.

The DA amacrine cell-M1 ipRGC microcircuit is modulated by somatostatin-releasing (SRIF) amacrine cells. Previous studies showed structural and physiological dependence between DA amacrine cells and M1 ipRGCs. This present study showed SRIF amacrine cells are also embedded in this microcircuit. First, SRIF amacrine cells make significant structural associations with both cell types, including apposition of its processes in stratum 1 of the IPL, and both DA amacrine cells and M1 ipRGCs express specific SRIF receptor subtypes. Secondly, SRIF modulates important ion channels on both cell types including attenuating Ca$^{2+}$ channel currents and potentiating K$^+$ channel currents by acting on its specific receptors. Finally, both SRIF and dopamine independently modulates melanopsin-based light response in M1 ipRGCs. Therefore, taking into consideration SRIF inhibits both cell types and both SRIF and dopamine inhibits M1 ipRGCs, the actions of SRIF are finely regulated to maintain different levels of inhibition of both cell types.
Targeted characterization of the intrinsic properties and connectivity of vasoactive intestinal polypeptide-containing amacrine cells

Though VIP amacrine cells were identified over three decades ago using antibody labeling, today the output of and input to VIP amacrine cells are studied using a Cre-expressing transgenic mouse line driven by the VIP promoter. Crossing of the VIP-Cre line with specific reporter lines have helped to target and activate VIP amacrine cells. Using VIP-tdTomato retinas, this report details the specific ion channels present on VIP amacrine cells. In addition, pharmacology and electrophysiology studies showed VIP amacrine cells receive GABAergic and glycinergic input from amacrine cells by acting on GABA_A and glycine receptors (Park et al., 2015). Moreover, immunohistochemical studies showed VIP amacrine cells form putative synaptic contacts with type 2 OFF- and type 6 ON- cone bipolar, and may be bi-directional; and there appears to be en passant contacts between VIP amacrine cells and rod bipolar cell axons. These findings are informational in understanding VIP amacrine cell connectivity and function in the retina.

Retinal circuit functions and future studies

The previous chapters of this report explore one method of microcircuit modulation: peptide mediated inhibition. Another area of study would be to investigate the actions of GABA, the co-transmitter expressed in SRIF amacrine cells, on the cells in the non-image forming circuit. These studies will provide physiological evidence suggesting neuropeptides work in conjunction with classical transmitters to set the gain of their signals (Feigenspan and Bormann 1994; Veruki and Yeh 1992).

As mentioned previously, neuropeptides are released from specific retinal cell types. SRIF and VIP are exclusively synthesized and released from amacrine cells, and morphologically their processes are confined to specific strata within the IPL. However, the peptides released from these amacrine cells can also be diffuse and work in a paracrine
fashion, thus acting on distant cells that express the appropriate receptors. This suggests there are many microcircuits that SRIF and VIP can modulate.

A possible candidate for SRIF amacrine cell modulation includes the ~20 different ganglion cell subtypes. As shown in this report and previous studies (Cristiani et al., 2002; Farrell et al., 2014; Farrell et al., 2010; Zalutsky and Miller 1990) ganglion cells express the sst$_4$ receptor subtype. Thus it would be interesting to see how somatostatin and sst$_4$ agonists shape ganglion cell receptive fields and light responses.

Future studies on the actions of VIP amacrine cells can include other amacrine cell types and bipolar cells. The findings of this study suggest there may be a bi-directional communication with bipolar cells (type 2 and 6). Moreover, VIP amacrine cell processes ramify in stratum 4 of the IPL. This stratum corresponds with blue or short wavelength ON cone bipolar cell axon terminal (Chen and Li 2012). Therefore it would be interesting to see whether VIP amacrine cells may provide VIP or GABA inputs onto the blue ON cone pathway, or if VIP amacrine cells receive glutamaterigc input from blue ON cone bipolar cells.

In addition, to isolating cell membrane changes in the presence of peptides, it would be beneficial to also determine the intracellular mechanistic changes, such as levels and activity of adenylyl cyclase and cAMP (Chen et al., 2014a; Chen et al., 2014b; Farrell et al., 2014; Mastrodimou et al., 2006; Schorderet et al., 1981; Vaquero et al., 2001; Zalutsky and Miller 1990)

In conclusion, the findings of this report provide the platform to further parse out how amacrine cells shape visual processing.
References


