The mechanism underlying bipolar cell subtype specification

by

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Abstract

The mammalian central nervous system (CNS) has a high degree of complexity and cell type diversity that enables sophisticated processing of sensory information, circuit formation, and behaviour. While much is known about the patterning and specification of the major neuronal classes in the CNS, through processes such as morphogen gradient signaling and transcription factor combinatorial coding, much less is known about how subtypes within each cell class are specified. Bipolar cells are one of the main classes of interneurons in the vertebrate retina and consist of fifteen different subtypes based on their physiological function, morphology, and unique gene expression. The cellular mechanisms behind the specification of these subtypes are not fully known. In this thesis, I examine these mechanisms by investigating the role of extrinsic and intrinsic factors on the specification and differentiation of bipolar cell subtypes. We hypothesize that the specification of bipolar cell subtypes occurs in a multi-step manner and is dependent on non-cell autonomous (extrinsic) signals. To test this hypothesis, I conducted a series of experiments on the early postnatal mouse retina, which is the period when bipolar cells are generated. First, I examined whether bipolar cell marker onset was temporally ordered as would be predicted in a multi-step model. Postnatal day 3 (P3) mice were injected with EdU (5-ethynyl-2'-deoxyuridine), a thymidine analog that labels proliferating cells and then dissociated and fixed the retinal cells 24-120 hours after injection. My results show that *Vsx2-5.3-PRE-Cre*, a marker of pan-bipolar cells specification, is first detected 36 hrs after cell cycle exit, whereas specialized bipolar subtype-specific markers are expressed 48-60 hrs post-EdU injection. This observation is consistent with the idea that bipolar cells develop in a stepwise manner, first as an unspecified, pan-bipolar cell intermediate and then into one of the 15 subtypes. To further investigate this possibility, I developed a novel dissociated retinal culture assay that enabled me to accurately track retinal progenitor cells and postmitotic precursor cells and determine the requirement of cell autonomous and non-cell autonomous mechanisms during bipolar cell subtype specification. This assay involves culturing dissociated retinal cells from P3 EdU-injected mice at high density (abundant cell contact) or low density (scarce cell contact) at various timepoints, thereby allowing me to probe the role of these mechanisms in RPCs, early postmitotic cells, and late postmitotic cells. My findings revealed the first 24-48 hrs post cell cycle exit to be a critical, cell contact-dependent period for the specification of bipolar cell subtypes. This assay also allowed us to test the effect of blocking or activating the Notch and the

Sonic Hedgehog (Shh) signal transduction pathways by using pharmacological compounds and recombinant ligands. Co-activation of Notch and Shh pathways increased the specification of *Vsx1*+ subtypes suggesting they play a role in their specification. Altogether, our results suggest that bipolar cell subtype specification follows a multi-step model, through an undifferentiated bipolar cell intermediate, and that cell contact plays a role in the specification mechanisms of bipolar cell subtype development. This is a novel finding that provides insight into the mechanisms underlying retinal neuronal subtype development and possibly in other neuronal cell types throughout the CNS.

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List of Abbreviations

EdU	5-ethynyl-2’ -deoxyuridine
BC	Bipolar cell
bHLH	Basic helix-loop-helix
CBC	Cone bipolar cell
CNS	Central nervous system
CKO	Conditional knock out
DAPT	*N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester
DLL1	Delta-like 1
DLL4	Delta-like 4
DMSO	Dimethyl sulfoxide
E	Embryonic
EthD-1	Ethidium homodimer-1
GCL	Ganglion cell layer
GDF11	Growth differentiation factor 11
HBSS	Hank’s balanced salt solution
HC	Horizontal cell
HD	Homeodomain
HEK293T	Human embryonic kidney 293T
Hh	Hedgehog
Hes1	Hairy and enhancer of split-1
INL	Inner nuclear layer
IS	Inner segment
IPL	Inner plexiform layer
KO	Knock out
Mash1	Atonal homolog 1
Math5	Atonal homolog 5
mGluR6	Metabotropic glutamate receptor 6
miRNA	microRNA
ONL	Outer nuclear layer
OPL	Outer plexiform layer

OS	Outer segment
Otx2	Orthodenticle homebox 2
p27kip1	Cyclin dependent kinase inhibitor 1B
P	Postnatal
Pax6	Paired box protein
PBS	Phosphate-buffer saline
PFA	Paraformaldehyde
PKCα	Protein kinase C alpha
RBC	Rod bipolar cell
RGC	Retinal ganglion cell
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
Shh	Sonic hedgehog
Smo	Smoothened
TF	Transcription factor
Vsx1	Visual system homeobox 1
Vsx2	Visual system homebox 2
WT	Wild-type

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Chapter 1: Introduction

1.0 Overview

Cell fate specification in the vertebrate retina is a highly complex process that involves many different types of cellular events. Cell-autonomous (intrinsic) and non-cell-autonomous (extrinsic) mechanisms, or often a combination of both, are responsible for the specification of the retinal cell classes (Cepko 1999). The cellular mechanism underlying the specification of fully differentiated retinal cell subtypes, however, is still poorly understood. In this thesis, I examined the mechanism underlying the specification of bipolar cell subtypes, a major class of retinal interneuron with 15 different subtypes.

1.1 Cell type diversity in the central nervous system

The mammalian central nervous system (CNS) is composed of millions and sometimes billions of cells divided into thousands of specialized cell types based on their unique function, structure, and morphology (Poulin et al. 2016). From an evolutionary perspective, the specialization of cells into cell types makes perfect sense. Having each cell focus on performing a specific task, rather than perform many tasks, is a robust advantage for any multicellular organism. This division of labor allows a subset of cells to have their own role and thereby improve the overall ability of the organism to integrate common neural processes such as sensory information, functional integration, circuit formation and behavior (Arendt et al. 2016).

One of the first pieces of evidence demonstrating the astounding cell type diversity of the nervous system was the work by pioneering neuroscientist Santiago Ramon y Cajal at the end of the 19th century (Gil et al. 2014). His microscopic drawings of stained cortical neurons perfectly exemplified the morphological diversity of the CNS. Today, classifying the general cell type of a neural cell is an easy task thanks to incredible technological advancements that allow researchers to histologically examine cells such as brightfield, confocal, and electron microscopy as well as cell imaging. Simply looking at the morphology of a neural cell stained with a membrane dye under the microscope gives us enough information to accurately estimate what general type of cell class they belong to. Common morphological features used to define neural cell types can be axonal and dendritic shape, branching patterns, soma size and spine density.

However, cell type classification goes beyond morphological features. Cells can be classified based on their physiological features such as resting potential, firing rate and biophysical properties. Structural connectivity is another defining property of cell types, albeit harder to examine in some scenarios. Most importantly, cells are classified based on their molecular properties such as protein and mRNA composition (Zeng & Sanes 2017). These last two features are incredibly important, since often there might be morphologically identical cells with different functions that can only be determined by looking at their molecular properties. Alternatively, there could be morphologically distinct cells with the same molecular composition. Within each cell type, there are often cases in which finer distinctions within one cell type can be made and these can be subclassified as cell subtypes, recent transcriptomic analyses have demonstrated the individual transcriptome of single cells is an excellent way to characterize specific subtypes within a population of cell classes (Rheaume et al. 2018; Yan et al. 2020; Shekhar et al. 2016).

Cell classification can be simplified with a hierarchical model. Generally, the broadest way a cell can be categorized is as a "class", examples of neural cell classes are cortical excitatory neurons, cortical inhibitory interneurons, retinal photoreceptors, and retinal bipolar cells (BCs). Cell classes share general main functional features but can be further broken down based on specific distinctions. The second, finer way to categorize cells are cell "types", examples of neural cell types are L4 excitatory neurons, VIP+ inhibitory interneurons, retinal GABAergic amacrine cells, and retinal direction-selective ganglion cells. Cell types share more features with each other such as synaptic partners, morphology, and molecular structure. Lastly, neural cells can be further classified into cell "subtypes" (e.g., retinal cone BC1A, BC1B and star pyramid excitatory cortical neuron) which can often only be correctly distinguished by specific immunohistochemical markers and transcriptomic analysis and are therefore almost identical in function, structure, morphology, and gene expression.

1.2 Development of the central nervous system

In vertebrates, the development of the central nervous system (CNS) during embryogenesis occurs during a long period and involves many intricate processes. For decades, the spinal cord has been the experimental focus in studying many of the now well-known developmental processes behind the development of the CNS such as neural patterning, cell fate

specification along the dorsal-ventral (D-V), anterior-posterior (A-P) and left-right (L-R) axes via morphogens and transcriptional coding (Jessel 2000; Lee & Pfaff 2001; Ulloa & Briscoe 2007).

Neural patterning, the process by which spatially distinct cells acquire different identities during development, occurs across the A-P, D-V, and L-R axes (Altman & Brivanlou 2001). Extrinsic signals play a very important role in stablishing cell fate in the spinal cord. The spinal cord has distinct cell types at defined positions along the D-V axis of the neural tube during development, and they are specified by the signaling activities of the notchord and mediated by a secreted protein called Sonic Hedgehog (Shh) (Patten 2000). Experiments in which Shh was ectopically expressed *in vivo* and *in vitro* induces the differentiation of floor plate cells, motor neurons and ventral interneurons (Marti et al. 1995; Roelink et al. 1995; Ericson et al. 1996). In contrast, removal of Shh signaling by gene targeting prevents the differentiation of these cell types (Chiang et al. 1996). Additionally, these cell fates are affected by the concentration of Shh; neurons generated in progressively more ventral regions require a higher concentration of Shh for their induction, exemplifying the role of Shh as a morphogen during early CNS development (Ericson et al. 1997).

The combined expression of specific transcription factors plays a role in the specification of certain cell fates, this process is referred to as combinatorial coding. This mechanism enables a relatively small number of transcription factors (TFs) to combine in different ways to promote different neural cell fates. For example, the combinatorial expression of various transcription factors specifies the identity of five progenitor cell domains in the ventricular zone of the neural tube (Stifani 2014). Various cell lineage studies using genetic markers such as *Cre* and *LacZ* demonstrate different types of interneurons and motor neurons arise from each of the distinct progenitor domains; V0-V3 interneurons are derived from p0-p3 progenitor domains in the neural tube, respectively, whereas MNs (motor neurons) are derived from the pMN progenitor domain (Cecile et al. 1999; Stifani 2014). The strategy used to correctly establish this progenitor domains involves concentration gradients of TFs. Cross-repressive interactions between different TF pairs results in spatially exclusive distinct domains giving rise to different cell fates (Stifani 2014).

Even though four different classes of embryonic interneurons (V0-V3) originate from the same region during early development, their final adult phenotype is unexpectedly

heterogeneous. For example, V1-derived interneurons in the adult mouse spinal cord are in different cortical layers and include distinct types of inhibitory interneurons (Alvarez et al. 2005). Similarly, V2-derived interneurons give rise to both excitatory and inhibitory interneuron populations based on immunohistochemical analysis and lineage tracking experiments (Al-Mosawie et al. 2007). Altogether, these results suggest these embryonically related interneurons have the capacity to diversity into distinct population of cell types, suggesting the presence of many undiscovered developmental mechanisms downstream of main cell class specification.

While much is known about the development of general cell classes during embryogenesis, there is a considerable gap in our knowledge about the mechanisms behind the specification of specialized subpopulations of cell types/subtypes in the central nervous system. Important questions remain unanswered: How is final neural cell subtype identity achieved? Is this a direct, intermediate, or multi-step model? Recent single-cell RNA-sequencing analyses studies suggest the existence of a postmitotic precursor cell in the specification of mature neural subtypes in the cortex and the retina (De Anda et al. 2016; Roaux & Arlotta 2013; Shekhar et al. 2022). However, experimental evidence is lacking. The vertebrate murine retina is an excellent model to start addressing these unanswered questions due to its well characterized hierarchical cell classification (Figure 1) that will be described in detail in the next section.

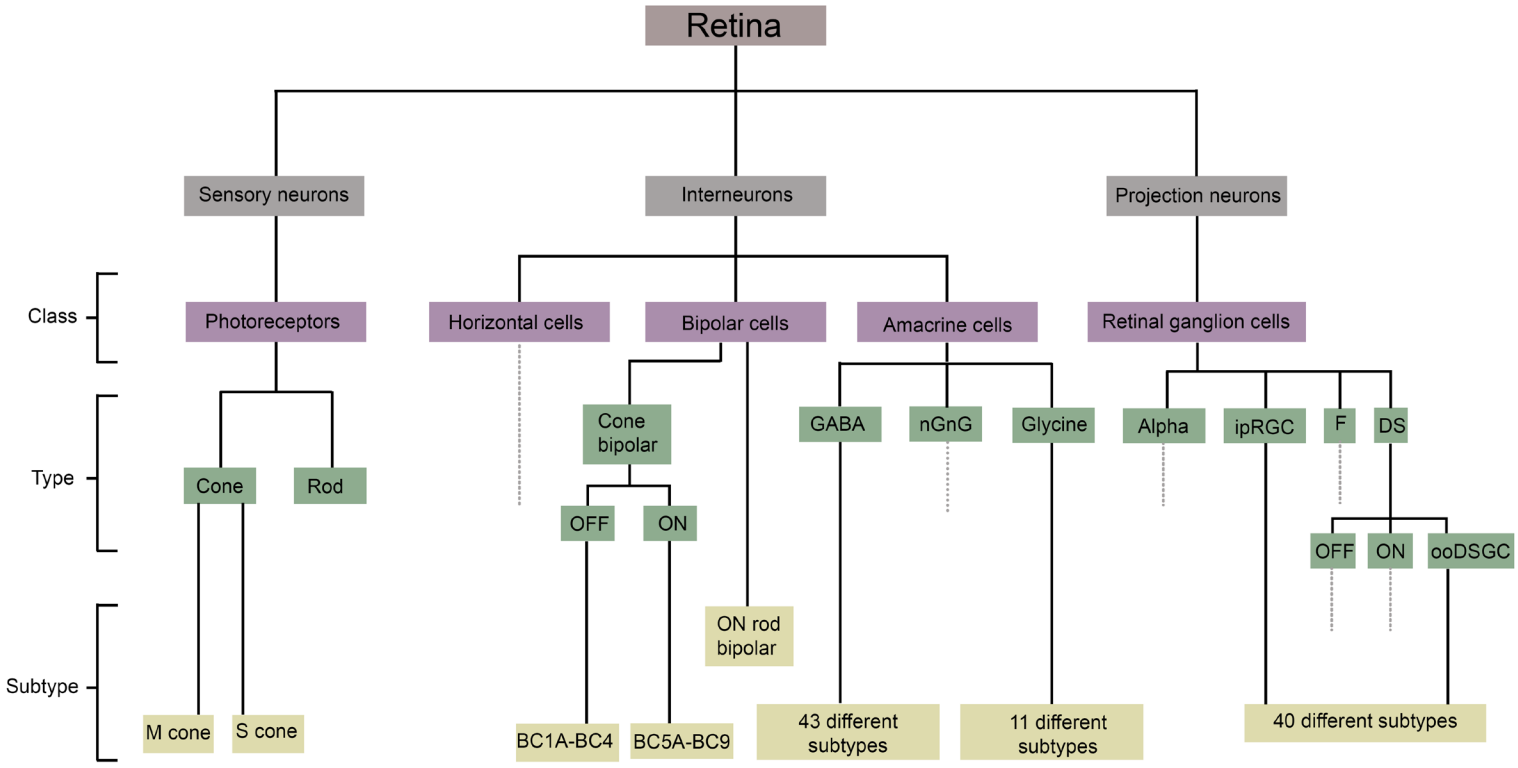

Figure 1 Hierarchical classification of mouse retinal neurons

Proposed hierarchical classification of retinal neurons. Retinal neural cells can first be grouped into classes (purple boxes), and then further classified into types and subtypes (green and yellow boxes, respectively) based on distinct morphological, structural, and molecular features. Dashed lines indicate additional cell types that were not labeled. DS, direction-selective retinal ganglion cells; F, forkhead box P2 (*Foxp2)*-expressing RGCs; nGnG, non-GABAergic-non-glycinergic amacrine cells; ooDSGC, ON-OFF direction-selective RGCs; ipRGC, intrinsically photosensitive retinal ganglion cell; BC, bipolar cell. Modified from Zeng & Sanes 2017.

1.3 Anatomy and physiology of the Vertebrate Retina

1.3.1. Organization and functional structure of the retina

The retina is a light-sensing tissue located at the back of the eye that is divided into five distinct layers. The posterior-most layer is called the outer nuclear layer (ONL), and this is where the nuclei of rod and cone photoreceptors reside and send their axons towards the outer plexiform layer (OPL) where they synapse with bipolar cells and horizontal cells. The nuclei of

these two cell types (BCs and HCs) reside in the inner nuclear layer (INL) and send their projections towards the inner plexiform layer (IPL) to synapse with retinal ganglion cells (RGCs) and amacrine cells. Lastly, RGC nuclei reside in the ganglion cell layer (GCL). Amacrine cell bodies reside in both the INL and the GCL. The nuclei of Muller glial cells, which play an important role in regulating homeostasis and metabolic processes, reside in the INL but send their projections vertically across all layers of the retina (Figure 2).

There are five main classes of retinal neurons, and they all play a unique role in detecting visual signals. Light detection, the most basic function of the retina, starts with cone and rod photoreceptors responding to light. This information is then processed and sent to the main interneurons of the retina responsible for the vertical transmission of visual signals, the bipolar cells (BCs). These signals may be processed by amacrine cells and then sent to retinal ganglion cells (RGCs) whose axons converge to form the optic nerve. All these cells work together to orchestrate the mechanism behind light detection and visual signaling. Lastly, visual signals transmitted by RGCs axons via the optic nerve are project to several regions in the central nervous system and allow us to experience vision and to extract other forms of visual information.

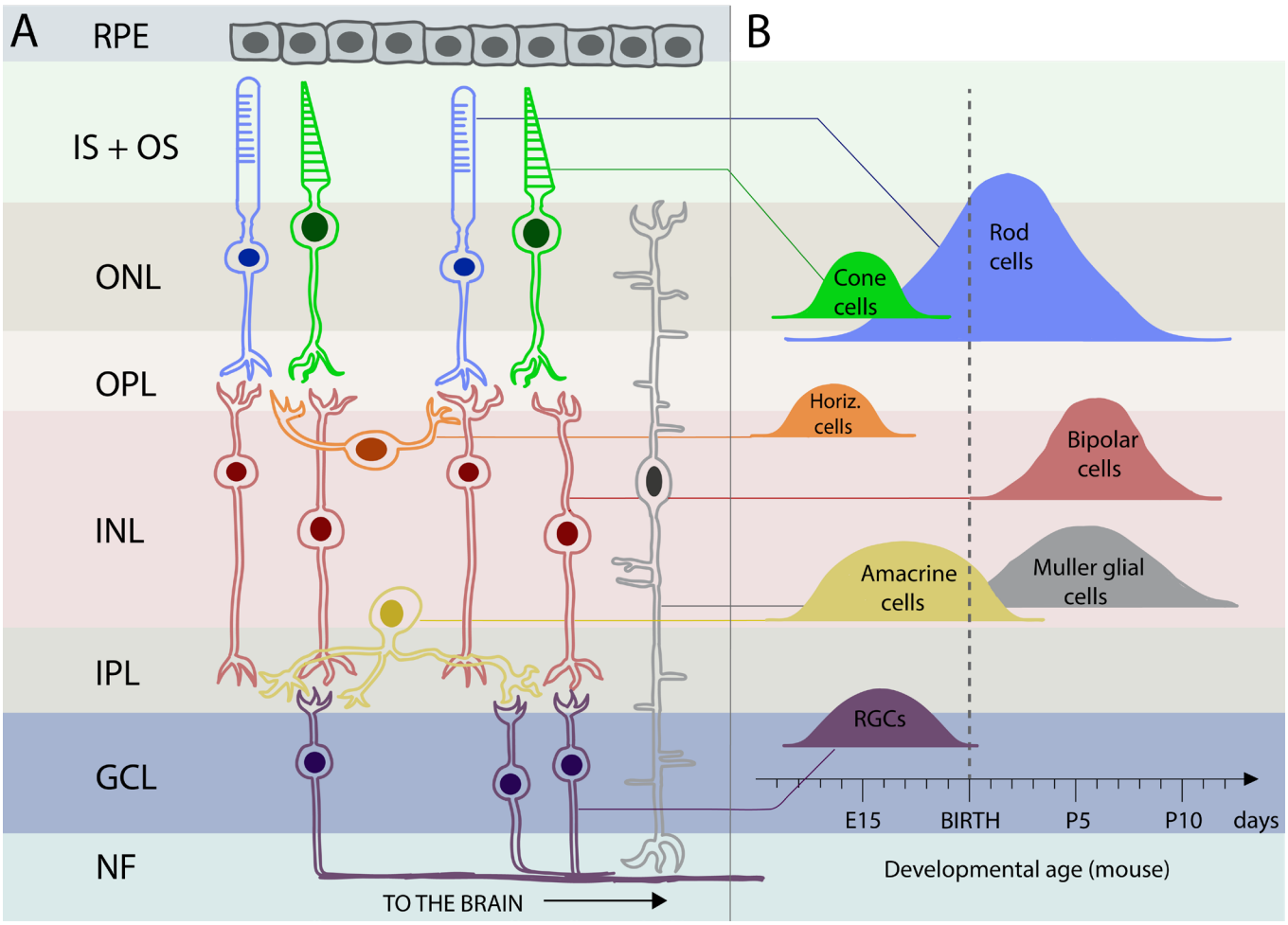

Figure 2. Structure of the mature retina and temporal order of cell birth in the mouse.

A) The structure of the mature retina consists of various layers. The most posterior region consists of the retinal pigment epithelium (RPE), followed by the inner and outer segments of the photoreceptors (IS+OS). The outer nuclear layer (ONL) is where the nuclei of rod and cone photoreceptors reside, and the outer plexiform layer (OPL) is where they send their axon terminals towards to interact with the dendritic arbors of bipolar cells and horizontal cells. The nuclei of bipolar, horizontal and amacrine cells all reside in the inner nuclear layer (INL) and the inner plexiform layer (IPL) consists of the axon terminals of bipolar cells and amacrine cells interacting with the dendritic arbors of retinal ganglion cells (RGCs). The processed signals are RGCs in the ganglion cell layer (GCL), whose axons converge into a neural fibre to send this processed information to the brain. **B)** The orderly yet overlapping birth order of each corresponding cell type throughout retinal development (Embryonic day (E) 9.5 – Postnatal day(P) 10). The height of each curve represents the relative number of each cell type in the

retina. Cone photoreceptors, amacrine cells, horizontal cells and RGCs are born embryonically, and Muller glial and bipolar cells are born postnatally. Figure modified from Cepko 2014

1.3.2 Retinal cell type diversity

The function of the retina goes beyond simply sending information to the visual cortex, and this is demonstrated by its vast neural cell type diversity, one of the main landmarks of the vertebrate retina (Gollisch & Meister 2009). Even though there are only six major cell classes in the mouse retina, five neuronal and one glial, each of these cell classes has been further subdivided into a total of more than a hundred specialized retinal cell subtypes based on the most recent single-cell RNA sequencing analyses (Rheaume et al. 2018; Yan et al. 2020; Shekhar et al. 2016). In the inner plexiform layer alone, where bipolar cells reside, there are over 70 different subtypes of neurons (Visser et al. 2015). This diversity makes sense from a functional perspective since a very intricate neuronal circuit is required to compute and extract the large amounts of visual information that living organisms are constantly exposed to. Light detection, motion detection, object motion and approaching motion, among others, are all examples of incredibly important visual processes required for survival. Each of these processes requires its own specialized circuit for proper functioning (Gollisch & Meister 2009).

An example of a complex retinal mechanism is the ON and OFF pathways which start with bipolar cells. ON bipolar cells depolarize in response to an increase in illumination whereas OFF BCs depolarize in response to a decrease in illumination. The molecular basis for these pathways originates from their unique response to the neurotransmitter glutamate released by photoreceptors. ON BCs express the metabotropic glutamate receptor mGluR6 whereas OFF BCs express ionotropic glutamate receptors such as AMPA and kainite receptors. Photoreceptors are constantly releasing glutamate unless exposed to light and ionotropic receptors respond to glutamate by exciting OFF BCs. In contrast, the metabotropic receptors in ON BCs respond to glutamate by preventing cell excitation during a dark stimulus. These pathways are important for differentiating the contrast between an image and its background, which is a fundamental aspect of vertebrate vision. This functional difference is associated with its structure: ON BCs send their axons towards the deepest sublayers of the INL, whereas OFF BCs send theirs to the

outermost sublayers. ON BCs interact with ON ganglion cells and OFF BCs interact with OFF ganglion cells (Kolb 2003).

1.3.3 Bipolar cell subtype classification

Bipolar cells have been classified into 15 different subtypes based on their unique morphology, function, and gene expression (Shekhar et al. 2016). Cell subtypes are sometimes referred as "types" in the literature but in this thesis, I will refer to the general retinal cell classes (i.e., bipolar cells, photoreceptors, etc.) as "types" and specialized retinal types (i.e. rod bipolar cell) as "subtypes". The most basic way of classifying bipolar cells is based on their preferred synaptic partners: cone vs. rod. Next, their morphology and function come into play: bipolar cells projecting their axons to sublaminae 1 and 2 of the inner plexiform layer are called OFF bipolar cells, while those whose axons project to layers 3,4 and 5 are called ON bipolar cells (Figure 3). As previously mentioned, ON BCs depolarize in response to an increase in illumination whereas OFF BCs depolarize in response to a decrease in illumination. Subtypes are also classified based on their morphology; however, this method of identification is often unreliable as morphologically similar subtypes could be molecularly and physiologically distinct. For this reason, the most important way to classify BCs is by their unique gene expression. As of today, single cell sequencing analysis has revealed a total of 15 molecularly unique types. 14 cone, one rod, 9 ON and 6 OFF (Shekhar et al. 2016).

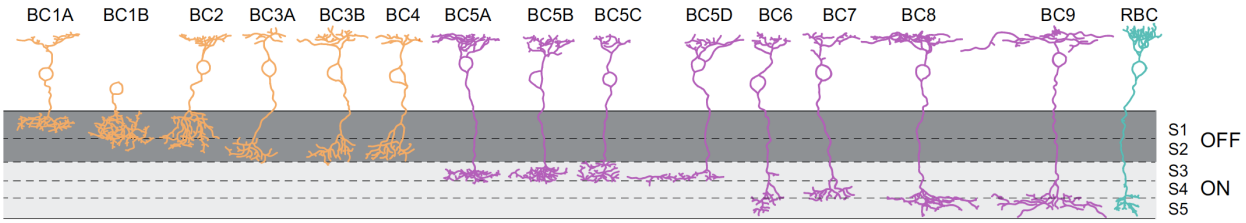

Figure 3. Bipolar cell subtypes.

Bipolar cells (BCs) can be classified based on their synaptic connections with photoreceptors. Cone BCs (BC1A – BC9) are shown in yellow and purple. Rod bipolar cell is shown in blue. Additionally, they are classified based on their morphology, gene expression and physiological function. OFF BCs are shown in yellow and send their axons to sublaminae S1 and S2 of the IPL. ON BCs are shown in purple and blue and send their axons to sublaminae S3-S5 of the IPL. Figure from Shekhar et al. 2016

1.4 Retinal development

1.4.1 Birth order of retinal cell types in vertebrates

Landmark experiments using radiolabeled thymidine to track cell fate demonstrated that retinal cell birth overlaps but still follows a chronological order embryonically and postnatally (Sidman R.L. 1961, Young et al. 1985a,b). In mice, retinal ganglion cells (RGCs) are born first, followed by cone photoreceptors, horizontal cells, amacrine cells and some rod photoreceptors. Bipolar cells, the rest of rod photoreceptors and Muller glia are all born postnatally (Figure 2). Experiments using retroviral vector-mediated gene transfer to track cell lineage in mice suggested that all retinal cells derived from a multipotent progenitor cell (Turner & Cepko 1987, Turner et al. 1990). Further research on other species have confirmed this to be the case as well (Wetts et al. 1988). Following this discovery, a large amount of research has been performed to try to understand the mechanism behind retinal development, and while many molecular regulators have been discovered, the cellular mechanism is still not fully known.

Throughout this thesis, I will be mentioning terms such as cell specification, differentiation, and determination. It is important to clearly define them to avoid confusion. Cell determination refers to a progenitor cell being fully committed to giving rise to a certain type of

daughter cells, even when placed in different environments. Specification refers to an RPC, or a newly post-mitotic cell becoming biased towards a specific cell class (e.g., bipolar cell precursor). I will refer to differentiation as a process that occurs downstream of specification and leads to the process of a cell becoming a fully specialized subtype (e.g., rod bipolar cell). Some transcription factors are required exclusively for the specification of a single retinal cell type, whereas others are expressed by a subset of different cell types and play multiple roles (Cepko 2014).

1.4.2 Retinal progenitor cell determination

Even though it is known retinal progenitors are multipotent, meaning they give rise to all retinal cell types, the exact mechanism behind this process has not been determined. Two general types of mechanisms can be considered: intrinsic or extrinsic. Intrinsic mechanisms (referred to as cell autonomous mechanisms) are molecular pathways or cues present within a cell such as transcription factors or micro RNAs that influence cell fate. On the other hand, extrinsic mechanisms (referred to as non-cell autonomous mechanisms) are signals coming from outside of the cell in the extracellular environment with the potential to influence cell fate determination, specification and/or differentiation. Examples of extrinsic mechanisms can be secreted molecules, membrane-bound ligands or growth factors activating signaling pathways. Generally, both intrinsic and extrinsic mechanisms are believed to play role in neural development (Belliveau & Cepko 1999; Cepko 2014). The major question is the relative contribution of extrinsic and intrinsic mechanisms to the specification of cell types.

A few possible models have been proposed to explain how RPCs undergo cell fate determination into one of the six types of retinal interneurons and one glial type. One model proposes RPCs undergo different “competence” states throughout retinal development (Cepko et al. 1996). Competence state refers to the capacity of an RPC to give rise to a particular cell type at a particular time. In this model, RPCs intrinsically undergo a temporal order of different competence states to give rise to a different subset of retinal cell types at each state. According to this model, genetically distinct RPCs are present at different stages of development that enable the different competency states. Another intrinsic model proposes RPCs are established early in development and that distinct RPCs undergo an intrinsic mechanism to give rise to a particular fate. Alternatively, there could be a fully extrinsic RPC determination model in which all RPCs

always have the same level of competence and are dependent on extracellular cues for determination (Cepko 2014).

Many studies have helped examine these models for retinal cell fate determination (Basset & Wallace 2012; Cepko 2014). When embryonic retinal progenitors are cultured in a postnatal environment, they are still able to give rise to embryonic cell fates (Belliveau & Cepko 1999) and postnatal progenitors still give rise to postnatally born cells even when cultured in an embryonic environment (Belliveau et al. 2000). Additionally, RPCs can give rise to their temporally appropriate progeny even when isolated in culture based on immuno-staining analysis (Cayouette et al. 2003; Gomes et al. 2011). However, in some of these experiments the ratio of some of the cell types produced was affected, suggesting environmental signals might play a role in stablishing cell fate across the retina. Nevertheless, these results suggest there is an important intrinsic component affecting RPC competence states.

1.4.2.1 Intrinsic regulators of RPC competence

Several studies have demonstrated that transcription factors from the basic helix-loop-helix (bHLH) family involved in RPC intrinsic competence by acting as transcriptional activators and repressors (Cepko 1999; Hatakeyama & Kageyama 2004). A helix-loop-helix is the structural protein motif characteristic of these transcription factors often involved in regulating growth by affecting cell proliferation and differentiation in eukaryotes. Mice with a targeted deletion of the bHLH gene *Math5* show an almost complete loss (>80%) of RGCs and an increase in the number of amacrine cells differentiated, suggesting it is required for RGC differentiation (Wang et al. 2000). Another transcription factor from the same family with an opposite effect is the mammalian hairy and enhancer of split homologue 1 *Hes1.* RPCs with ectopic expression of Hes1 do not differentiate into mature retinal cell types. In contrast, Hes1-null retinas demonstrate premature differentiation of progenitors into immature retinal cell types (Tomita et al. 1996a). These results suggest Hes1 is required for maintaining progenitor identity by acting as a transcriptional repressor. Additionally, another bHLH transcription factor, *Olig2* helps bias progenitor cells towards cell cycle exit embryonically during retinal development (Hafler et al. 2012).

Another important group of proteins involved in RPC competence is the homeodomain (HD) family of transcription factors (Zagozewski et al. 2014). A homeodomain is a DNA-

binding structural motif characteristic of these transcription factors with important roles in embryogenesis and cell type differentiation. For instance, the highly conserved HD transcription factor *Pax6* plays a crucial role during eye development (Chow et al. 1999; Marquardt et al. 2001). Misexpression of Pax6 in *Xenopus laevis* is sufficient to generate ectopic eyes with mature cell types and a similar morphology to endogenous eyes, suggesting its presence alone is enough to activate lens and eye development (Chow et al. 1999). Pax6 is also crucial for retinal cell type development; Cre-mediated loss of *Pax6* in RPCs results in an exclusive generation of amacrine cells, suggesting *Pax6* expression is required for maintaining RPC multipotency (Marquardt et al. 2001). Conditional knock-out of the HD transcription factor *Otx2* in progenitors results in a fate switch from photoreceptors to amacrine-like cells (Nishida et al. 2003). Another homeobox gene essential for retinal development is visual system homeobox 2, *Vsx2* (also known as *Vsx2*). Mice lacking *Vsx2* show reduced RPC proliferation and a complete loss of bipolar cells, suggesting it plays a role in both RPC proliferation and bipolar cell fate (Burmeister et al. 1996).

1.4.2.2 Extrinsic regulators of RPC competence

The contribution of intrinsic factors in establishing RPC competence is undoubtedly significant. However, many studies demonstrate extrinsic regulators still play an important role in retinal development. Extrinsic cues have been found to be important for feedback inhibition by limiting the generation of certain cell types (Poggi et al. 2005; Waid & McLoon 1998: Belliveau & Cepko 1999; Cepko 2014). In the chick, the production of RGCs is decreased when progenitors are cultured with adjacent older RGCs, suggesting there is an important factor secreted by these cells that inhibit its own production (Waid & McLoon 1998). A similar result is found in zebrafish; when progenitor cells are transplanted into a mutant retina lacking RGCs, their division rate is accelerated, and their spatial distribution widened (Poggi et al. 2005). This phenomenon is also seen in rats; P0 amacrine cells decrease the number of amacrine cells being born when co-cultured with embryonic progenitors (Belliveau & Cepko 1999). Altogether, these results suggest there is a cell non-autonomous mechanism inhibiting the production of specific cell types in the developing retina, specifically by adjacent older retinal cells secreting factors. The next challenge becomes discovering the identity of these secreted molecules.

Sonic hedgehog (Shh) is a secreted protein that activates the Hedgehog signaling pathway, it functions as a mitogen during retinal development and as an important morphogen in many other regions (Johnson & Tabin 1995; Jensen & Wallace 1997). In chick, retroviral overexpression of Shh results in a decrease in RGC cell numbers both in vivo and in vitro (Zhang & Yank 2001). A similar phenotype is observed in mice when Shh is overexpressed; treatment of retinal explants with a recombinant N-terminal fragment of Shh results in a decrease in RGCs numbers (Wang et al. 2005). Additionally, deletion of Shh results in a depleted pool of RPCs, due to precocious cell cycle exit (Wang et al. 2005). Interestingly, an opposite effect is seen in zebrafish: Shh signaling leads to cell cycle exit (Shkumatava & Neumann 2005). This difference could be due to functional differences among these two species that have led to evolutionary changes in the role of Shh. In summary, Shh is an important molecule that plays a role in two important retinal development mechanisms: 1) retinal proliferation and 2) RGC specification.

Another important signaling pathway involved in retinal development is the Notch cell-cell signaling pathway. This is a highly evolutionarily conserved pathway named after the Notch receptor which is the main transmembrane receptor of the pathway. The pathway was first discovered over a hundred years ago, and it has been found to play a crucial developmental role in both invertebrates and vertebrates (Artavanis-Tsakonas et al. 1999). In the retina, the main Notch receptor of interest is Notch1, and it is expressed by RPCs and Muller glial cells (Furukawa et al. 2000). Conditional deletion of *Notch1* during embryonic retinal development results in a decrease in the number of RPCs and an increase in the number of cone photoreceptors generated, suggesting *Notch1* plays a role in maintaining cells in a progenitor state and inhibiting photoreceptor cell fate (Jadhav et al. 2006; Yaron et al. 2006). Additionally, the increase in cone photoreceptors seen in *Notch1*-CKO mice appears to be at the expense of other retinal cell types, since there was a decrease in the number of amacrine cells, bipolar cells, Muller glial cells and RGCs (Yaron et al. 2006). Altogether, these results suggest *Notch1* is required for maintaining RPCs in a progenitor-like state and for inhibiting the cone photoreceptor cell fate and allow the generation of other retinal cell types.

Growth and differentiation factor 11 (GDF11) is a member of the transforming growth factor-β superfamily of secreted signaling molecules, and it's been found to be a negative regulator of RGC number (Kim et al. 2005). GDF11-null retinas have an abnormally highly

dense ganglion cell layer early in development and by P0, a 50% increase in the number of RGCs and a decrease in amacrine cells and photoreceptors without affecting RPC proliferation (Kim et al. 2005). Additionally, the expression of *Math5*, a transcription factor required for RGC differentiation, is seen for longer than usual. (Kim et al. 2005). Altogether, these results suggest GDF11 acts as a negative regulator of RGCs by controlling the expression of cell-intrinsic components of RPCs.

1.5 Bipolar cell development

1.5.1 Gene regulatory network of bipolar cell development

The HD transcription factor *Vsx2* (or *Chx10*) is an essential component for bipolar cell specification. *Vsx2* is expressed by RPCs during development and remains expressed in adult bipolar cells (Burmeister et al. 1996). The role of *Vsx2* on retinal proliferation seems to be different from its role in bipolar cell specification. Mice lacking both *Vsx2* and *p27kip1* (a negative regulator of proliferation in the retina) show a rescue in the abnormal retinal proliferation normally seen in *Vsx2*-null mice (Green et al. 2002). However, these mice still have no bipolar cells present, suggesting *Vsx2* plays a different, independent role in bipolar cell specification.

Ectopic expression of *Vsx2* in neonatal rats promotes bipolar cell differentiation at the expense of photoreceptors without affecting cell proliferation, suggesting the presence of a binary fate choice between these two cell types (Livne-bar et al. 2006). The zinc-finger transcription factor *Blimp1* is required to establish the correct ratio of photoreceptors and bipolar cells during retinal development in *Otx2*+ cells. Experiments in which *Blimp1* was conditionally knocked out in retinal progenitor cells showed a decrease in the number of photoreceptors and an increase in bipolar cells based on immunostaining analysis (Brzezinski et al. 2010; Katoh et al. 2010). Further analysis of *Blimp1* CKO retinas carrying the rod-specific transgene *Nrl-GFP* showed an increase in the number of cells expressing *Vsx2*+GFP when compared to control retinas (Brzezinski et al. 2010). These results suggest photoreceptors re-specified to bipolar cells in the absence of *Blimp1*. Altogether, these observations demonstrate that *Blimp1* regulates photoreceptor and bipolar cell numbers potentially by repressing genes involved in bipolar cell specification in *Otx2*+ cells and thus stabilizing the photoreceptor cell fate.

Deletion of TF binding sites in *Blimp1* demonstrates the presence of a gene regulatory network in the binary fate choice between BCs and photoreceptors in newly postmitotic

progenitor cells (Wang et al. 2014). *Otx2* upregulates *Blimp1* expression in progenitors, but in newly postmitotic cells *Blimp1* inhibits itself, *Vsx2*, and *Otx2*. These low levels of *Otx2* and *Vsx2* inhibition led to rod photoreceptor cell fate. In contrast, when BCs are specified, *Notch1* seems to inhibit *Blimp1*, allowing high *Otx2* and *Vsx2* expression, resulting in bipolar cell fate specification (Wang et al. 2014). This data suggests there are two potential cell fates in these newly postmitotic RPCs, however, the mechanisms behind how this gene network is kept in balance to allow the right number of each cell type to be generated remain unknown.

The bHLH transcription factors *Mash1* and *Math3* play a crucial role in the Muller glia vs. bipolar cell fate. *Mash1*$^{-/-}$ KO retinas significantly reduce the number of PKCα+ (Rod BCs) cells. Double KO retinas (*Mash1*$^{-/-}$/*Math3*$^{-/-}$) had no bipolar cells s and an increase in Muller glial cell numbers (Vimentin+) (Tomita et al. 1996; Tomita et al. 2001). These results suggest that *Mash1* and *Math3* are necessary for neurogenesis and their downregulation might play a role in gliogenesis. Misexpression of *Vsx2* in *Mash1-Math3* double mutant retinas increased the number of Muller glia in the inner nuclear layer (INL) but not bipolar cells. Misexpression of *Mash1* or *Math3* did not rescue BPC numbers either. In contrast, misexpression of both *Vsx2* together with *Mash1* and *Math3* rescued the number of mature bipolar cells and decreased the number of Muller glia (Hatakeyama et al. 2001). Altogether, these results suggest *Mash1* and *Math3* might play a role in neuronal cell type specification in the INL cells.

1.5.2 Bipolar cell subtype development

1.4.2.1 Intrinsic regulators of bipolar cell subtype development

Many studies have demonstrated the involvement of many transcription factors in the differentiation and/or survival of some bipolar cell subtypes. This section addresses the known role of each transcription factor involved in bipolar subtype development.

Isl1. The LIM-homeodomain transcription factor Isl1 plays a crucial role in the development of amacrine, retinal ganglion cells and ON and OFF bipolar subtypes. In bipolar cells (BCs), Isl1 expression starts at around P5 and is later restricted to ON bipolar cells in the adult retina (Elshatory et al. 2007). *Isl1*-null mice retinas have normal numbers of bipolar cells at P6, but after three weeks there is a significant decrease in the number of bipolar cells. Further immunostaining analysis revealed a significant decrease in many ON and OFF bipolar subtypes (Elshatory et al. 2007). These results suggest Isl1 is required for the survival and/or maturation of some BPC subtypes but is not required for general bipolar cell specification.

Bhlhb4. The transcription factor *Bhlhb4* is required for the survival of rod bipolar cells (RBCs) in mice. Experiments in which *Bhlhb4* was knocked out by replacing its coding region with the *LacZ* reporter gene demonstrated a complete loss of RBCs, as shown by the lack of the RBC-specific marker PKCα. Additionally, these mutant retinas displayed physiological abnormalities. However, rod bipolar cells appeared to be born at normal numbers, and only after P8 did the numbers decrease dramatically (Bramblett et al. 2004). These results suggest *Bhlhb4* is not involved in the specification of rod bipolar cells but is rather required for their survival.

Bhlhb5. The basic-loop-helix transcription factor *Bhlhb5* is expressed by both GABAergic amacrine cells and Type 2 OFF cone bipolar cells (CBCs) in the adult mice and plays an important role in their specification (Huang et al. 2014). *Bhlhb5*-null mice show a significant decrease in the number of Type 2 BPCs early during development at P6 (Feng et al. 2006). Although these results suggests that *Bhlhb5* is required for the formation of Type 2 BCs, it is not clear whether this cell type is absent in the *Bhlhb5*-null retina as no knock-in reporter gene as used in this study. Additionally, lineage-tracing studies demonstrated not all Vsx1+ cells originated from Bhlhb5 lineages (Type 2) are lost, suggesting the possibility of a non-cell-autonomous role in the specification of Vsx1+ Type 2 BCs (Huang et al. 2014).

Prdm8 is required for the survival of RBCs and Type 2 OFF CBCs in mice. The initial expression of *Prdm8* in the retina starts at around E11.5 and is seen abundantly in various cell types. From P6 onwards though, its expression is restricted to Type 2 BCs and a subset of amacrine cells and retinal ganglion cells. *Prdm8*-null mice have normal RBCs and *Vsx1*+ Type 2 cell numbers at P6, but by adulthood, a significant number of these cells are lost (Jung et al. 2015). This suggests the specification of these BCs is normal, but their survival is impaired by the loss of *Prdm8*. This is a very similar phenotype to the one seen in *Bhlhb4*-null mice, but since Prdm8 is expressed earlier (P3 vs P5) it is suggested Prdm8 works upstream of Bhlhb4 in their role in the survival of these BCs.

Vsx1, a homologue of *Vsx2*, is a paired-type homeodomain and CVC domain transcription factor expressed exclusively by Types 2,6 and 7 and plays an important role in their terminal differentiation (Chow et al. 2001, Chow et al. 2004, Shi et al. 2011). *Vsx1*-null retinas showed normal BPC specification and cell numbers. However, the terminal differentiation of OFF BCs was incomplete as shown by the lack of expression of some subtype markers. These mice also showed physiological abnormalities shown by electroretinography (Chow et al. 2004).

Additionally, Vsx1 seems to be required for the activation of OFF bipolar genes and repression of ON BPC genes (Shi et al. 2011).

Irx5, Irx6. Like *Vsx1,* the Iroquois homeobox TFs *Irx5* and *Irx6* are required for the terminal differentiation of some OFF bipolar subtypes. *Irx5*-deficient mice show a defect in the expression of some immunohistological markers for Type 2 and Type 3 OFF BPCs, suggesting it is required for the terminal differentiation of these types. However, the loss of *Irx5* or *Vsx1* does not seem to affect the expression of each other, suggesting these genes regulate two independent genetic pathways (Cheng et al. 2005). Like *Irx5, Irx6*$^{lacZ/lacZ}$ mutant mice show defects in the expression of some markers for Type 2 and 3a OFF BCs. Additionally, *Irx6*-deficient increased *Vsx1* expression and decreased *Bhlhb5* expression. Interestingly, the loss of *Irx6* resulted in a hybrid type2/3a bipolar cell, suggesting *Irx6* is important for defining specific differences between these two cell types (Star et al. 2012). Altogether, these results suggest these transcription factors are part of a complex gene regulatory network involved in regulating the development of Type 2 and Type 3 OFF BCs.

Table 1 Summary of transcription factors involved in the development of bipolar cell subtypes

	Cone														Rod	
	OFF						ON									
	1a	1b	2	3a	3b	4	5a	5b	5c	5d	6	7	8	9	RB	Role
Bhlhb4															+	Survival
Bhlhb5			+													Specification
Irx5			+	+	+											Terminal differentiation
Irx6			+	+	+											Terminal differentiation
Isl1							+	+	+	+	+	+	+	+	+	Survival and terminal differentiation
Prdm8			+												+	Survival
Vsx1			+								+	+				Specification and terminal differentiation
Vsx2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Specification

1.5.2.2 Extrinsic regulators of bipolar cell subtype development

The role of extrinsic factors on bipolar cell subtype specification and differentiation has not been fully explored. However, there is some evidence that the Notch and Sonic hedgehog signaling pathways could be involved. Being one of the last retinal cell types born, other retinal cell types could potentially be involved in subtype development. Additionally, there are many cell-surface recognition molecules required for generating the correct synaptic connections in the retina, and even though there is no evidence of their involvement in bipolar cell specification, it is still a possibility (Sanes & Zipursky 2020).

The Notch signaling transduction pathway plays an important role in retinal proliferation, inhibits photoreceptor cell fate, and could play a role in bipolar cell subtype specification. Analysis of *Notch1* CKO retinas demonstrated that the loss of *Notch1* caused abnormal retinal morphology, reduced eye size and increased numbers of photoreceptor precursors (Yaron et al. 2006). Loss of *Notch1* in newly post-mitotic retinal cells in the postnatal retina results in a decrease in the number of BCs and Muller glia and an increase in rod photoreceptors (Mizeracka et al. 2013). These results suggest *Notch1* might play a role in the post-mitotic specification of BCs, but more thorough research is required to reach a conclusion. The Notch signaling pathway is further covered in section 4.1.

The role of other retinal cell types on the specification and differentiation of bipolar cell subtypes is controversial. Deletion of both RGCs and amacrine cells in rats at P0 does not seem to disturb the formation of the segregated ON and OFF bipolar cell projections based on immunostaining, these results suggest these cells might not be required for subtype development (Gunhan-Agar et al. 2000; Gunhan et al. 2002). However, neither of these studies performed immunohistochemistry against specific subtype markers, and the total number of BCs was decreased. Additionally, since RGCs were deleted in P0, any previously secreted molecules might remain in the retinal environment. In *Atoh7* KO, RGC-deficient mice, there is a significant reduction in the number of cone bipolar cells and no difference in the number of rod bipolar cells, suggesting RGCs might play a role in the specification of cone bipolar subtypes (Moshiri et al. 2008; Bai et al. 2014). The role of other retinal cell types such as rod and cone photoreceptors has not been thoroughly studied.

Sonic hedgehog signaling has an important mitogenic effect in RPC proliferation and is involved in RGC specification (also described in section 1.2.2.2). Its effect on the specification

of other retinal cell types has not been fully investigated. Mice with *SmoM2*, a constitutively active form of the protein Smoothened (*Smo),* an important component of the Shh signaling pathway, have increased numbers of BCs, amacrine cells and Muller glial cells (Yu et al. 2006). This result suggests Shh signaling could influence the specification of these cells, but it is difficult to know for certain since this phenotype could be the effect of reduced RPC proliferation. The source of Shh in the retina are RGCs and their deletion results in a decrease in the number of cone bipolar cells (Bai et al. 2014). However, activation of *Smo* in postmitotic BCs 24 hours after cycle exit does not seem to influence cone and rod bipolar cell subtype specification (Wu 2017). Altogether, these results suggest Shh signaling is required in RPCs and perhaps in early postmitotic bipolar cell precursors for cone bipolar cell subtype specification. The Shh signaling pathway is further covered in section 4.2.

Various adhesion molecules are required for the correct formation of bipolar cell dendrites and axonal arborization (Dunn & Wong 2012; Lee et al. 2011; Sanes & Zipursky 2020). Soon after cell cycle exit, early post-mitotic bipolar cell precursor cells have long apical and basal processes spanning the entire retina, and a live imaging study demonstrated their axons and dendrites are formed from these processes (Morgan et al. 2006). Knock-out of two members of the type II cadherin family, *Cdh8* and *Cdh9,* results in abnormal axonal arborization of BC2 and BC5 (Duan et al. 2014). Removal of most rod BCs results in an abnormal expansion of the dendritic field of the remaining rod BCs, suggesting homotypic signals between these cells are responsible for constraining their dendrites (Johnson et al. 2017). A similar phenomenon is observed in type 7 BCs, suggesting homotypic interactions between ON BCs play a role in the regulation of the connectivity of their dendritic field as well (Lee et al. 2011). The Down Syndrome cell adhesion molecule (*Dscam*) establishes the correct dendrite and axon tiling in OFF cone bipolar cells by inhibiting outgrowth (Simmons et al. 2017). All these results demonstrate the importance of cell adhesion molecules in synaptogenesis of bipolar cells. Based on the timing of these studies, it is suggested these mechanisms occur after the specification of bipolar cells. However, the effect of these cell adhesion molecules on bipolar cell specification has not been thoroughly studied, and since they are an important component of the retinal environment, it is possible they might be involved.

1.5.2.3 A multi-step model involved in bipolar cell subtype specification

One of the main features of mature neurons is their inability to re-enter the cell cycle once they have fully differentiated into a specialized cell type. But the precise timepoint at which irreversible neural differentiation occurs and the degree of cell fate plasticity of postmitotic cells remains unknown. Recent research suggests that post-mitotic neural cells have the capacity to re-specify into various cell types for some time before fully committing to one neural cell fate (De Anda et al. 2016; Roaux & Arlotta 2013; Shekhar et al. 2022). Newly born neurons in the cortex continue to drive genes from a neural progenitor promoter days after cell cycle exit, suggesting that achieving a final cellular fate is a slow process (De Anda et al. 2016). Interestingly, these postmitotic cells have the capacity to re-specify into different subtypes for a short time via ectopic expression of the zinc finger transcription factor *Fezf2* (De la Rossa et al. 2013; Rouaux & Arlotta 2013). Altogether, these results demonstrate cortical postmitotic precursors have a high degree of cell fate plasticity after cell cycle exit for a few days after cell cycle exit, which strengthens the possibility of a multi-step model behind the specification of cell subtypes in the retina.

A single-cell RNA-sequencing analysis revealed that mouse retinal ganglion cells (RGC) do not differentiate into mature types after cell cycle exit, but rather their final cell fate is gradually acquired (Shekhar et al. 2022). The expression of the post-mitotic bipolar-specific transgene *Vsx2-5.3-PRE-Cre* occurs before the expression of various other subtype-specific markers in BCs and is also expressed in some mature photoreceptors, suggesting there was a post-mitotic cell fate switch and the presence of a bipolar cell post-mitotic precursor prior to subtype differentiation (Nickerson et al. 2011). In the cortex, progenitor cells originating from the medial, caudal, and lateral ganglion eminences give rise to distinct non-overlapping populations of cells, and a single-cell RNA-sequencing analysis across different developmental timepoints discovered these progenitors undergo different precursor states across all eminences prior to becoming unique, non-overlapping interneurons in the adult mouse (Mayer et al. 2018). As of today, no single-cell RNA-sequencing of postmitotic bipolar cell precursors has been done but it is possible, based on the previously mentioned research, that these precursor cells have a high degree of plasticity and undergo distinct cell states prior to becoming fully differentiated subtypes. Altogether, these results suggest there could be distinct postmitotic bipolar cell

precursor states prior to fully committing to a specialized subtype, suggesting the presence of a multi-step model behind the specification of bipolar cell subtypes.

1.6 Hypothesis, objectives, and significance

The molecular regulators behind the development of major neural cell classes have been studied extensively, but the mechanisms behind the specification of specialized neural cell subtypes is very understudied. The central theme of this study is to examine the role of extrinsic and intrinsic mechanisms in the specification of the fifteen bipolar cell subtypes. Postnatal cell birth, accessibility, simple structure, and being a non-vital tissue, all make the murine retina an ideal experimental model.

I hypothesize that the transition from an RPC to a fully differentiated bipolar cell subtype is a multi-step process involving non-cell autonomous mechanisms. This hypothesis is based on the findings discussed above which: (1) demonstrate the importance of various cell adhesion molecules in retinal circuit synaptogenesis (Dunn & Wong 2012; Lee et al. 2011; Sanes & Zipursky 2020), (2) suggest Notch and Shh signaling pathways are important for bipolar cell specification (Mizeracka et al. 2013; Wu 2017; Bai et al. 2014), and (3) indicate the presence of neural postmitotic precursors prior to becoming fully specialized types, suggesting a multi-step model (De Anda et al. 2016; Roaux & Arlotta 2013; Mayer et al. 2018; Shekhar et al. 2022).

My objectives are as follows:

1) To determine the onset of expression of bipolar cell subtype markers, with the goal to determine if there is a specific pattern.
2) To determine if cell-cell contact or extracellular signals are required for bipolar cell subtype specification.
3) To determine if the Notch and/or the Shh signaling pathways are required for bipolar cell subtype specification.

1.6.1 Significance

The mechanism of neuronal cell subtype specification is very understudied but very important for our understanding of basic neural development and potentially for various therapeutic and medical purposes in neural stem cell research. Our study in the retina is the first to functionally investigate the mechanisms behind a well-defined population of neural cell subtypes. The mechanisms studied here could have similar roles in other areas of the central nervous system.

Chapter 2: Materials and Methods

2.1 Mouse strains

All animal experiments were conducted with the approval of the University of Victoria's Animal Care Committee, by the Canadian Council for Animal Care. All mice used were from a mixed genetic background from two different strains: 129S1/Svlmj (strain # 002448, The Jackson Laboratory) and C57BL/6J (strain #00064, The Jackson Laboratory). All experiments involved mice possessing two distinct transgenes:

1) *Vsx2-5.3-PRE-Cre:* mouse *Vsx2* control regions direct expression of Cre recombinase to post-mitotic, undifferentiated bipolar cells (Nickerson et al. 2011). Founder mice were generated by backcrossing into 129S1 for 8 generations (strain: 026200, The Jackson Laboratory)

and:

2) *mGluR6:NLS-LacZ:* Express nuclear-localized β-galactosidase under the control of mouse *Grm6* in ON bipolar cells (Ueda et al. 1997). Founder mice were generated by backcrossing into 129S1 for more than 9 generations (strain # 026707, The Jackson Laboratory)

2.2 PCR genotyping

DNA was harvested from either ear or tail biopsies from mice. Samples were denatured in 75 µl of 50 mM NaOH at 95°C for 10 minutes; 25 µl of 0.5 M Tris-HCl (pH 8.0) was added to the samples. DNA samples were vigorously shaken and 1µl was added to a PCR reaction, run on a T3 Thermocycler (Biometra, USA). The PCR program for both transgenes genotyped was 120 s at 94°C for initial denaturation, followed by 32 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s with final extension at 72°C for 2 min. DNA samples were separated via gel electrophoresis in a sodium borate buffer gel with 1.1% agarose.

The genotyping primers for *Vsx2-5.3-PRE-Cre* were: Forward: 5' TGATGAGGTTCGCAAGAACC 3' Reverse: 5' CCATGAGTGAACGAACCTGG 3'. For *mGluR6:NLS-LacZ*: Forward: 5' TCTTTTGGCAATGTGAGGGC 3' Reverse: 5' TGAATACGCTTGAGGAGAGC 3'.

2.3 EdU (5-ethynyl-2'-deoxyuridine) injection

P3 mice were injected with 10 µg/g body weight of 1 µg/µl EdU (AbCam, Cat. # ab146186). Injections were performed subcutaneously in the back between the spine and a visible vein using a 1mL insulin syringe.

2.4 Dissection of the retina

The following dissection, dissociation and culture protocols were modified from a previous published retinal dissociation protocol (Jolicoeur & Cayouette 2014). Mice pups were anesthetized via inhalation of isoflurane followed by decapitation. Heads were briefly immersed in 70% ethanol and then placed in Ca+, Mg+ - free 1xHBSS (Cat. # 14175-145) in a Biosafety cabinet Class II. Eyes were enucleated; the cornea was carefully pierced with the sharp end of a pair of thin forceps (Dumont #55) and slowly teared apart with another pair of forceps until the retina was isolated; the lens was then removed.

2.5 Retinal dissociation and culture

Isolated retinas were incubated for 5 minutes at 37°C in a 15 mL tube with a solution containing 10 mL of 1xPBS, 100 U/mL of papain (Cat. # LS003126, Worthington), 100 µl of 0.4% DNase (Cat. # LS002007, Worthington) and 2 mg of L-cysteine (Cat. # C7352, Sigma). After the incubation, the supernatant was carefully aspirated without disrupting the retinas settled at the bottom, and 4 mL of LO-OVO solution: 9 mL of 1xPBS, 100 µl of 0.4% DNase and 1 mL of 10x Lo Ovomucoid [4 mL 1xPBS, 600 mg of BSA (Cat. # A-4161, Sigma), 600 mg of Trypsin inhibitor (Cat. # T2011, Sigma)] was added slowly to the tube with the retinas. The retinas were allowed to settle down, and the supernatant aspirated again. Another 1 mL of LO-OVO solution was added and the retinas were gently triturated with a P1000 pipette until the retinas broke down apart until the solution appeared uniformly cloudy without any visible clumps, about 15-20 times. 5 mL of LO-OVO solution was added to the cell suspension and the tube was centrifuged 8 minutes at 500 x *g* at room temperature.

The supernatant was aspirated, and the cell pellet was resuspended in 1 mL of pre-warmed cell culture medium [10 mL of Neurobasal Medium (Cat. # 21103, ThermoFisher), 10 mL of DMEM/F-12, GlutaMAX Medium (Cat. # 10565018, ThermoFisher), 200 µl of B27 supplement (Cat. # 17504044, ThermoFisher) and 200 µl of Penicillin-Streptomycin (Cat. # 15140122). The cell suspension was passed through a 30µm cell strainer (Cat. # 130-098-458, Miltenyi Biotech) to remove cellular clumps. Total number of cells was counted using a

hemacytometer and cells were plated at their corresponding density by diluting down suspension in additional media if necessary (high density: ~300K cells/mL, low density: ~10K cells/mL) by adding 125 µl of the cell suspension onto Poly-D-lysine + mouse laminin coated 12 mm coverslips (Cat. # NEU-GG-12-1.5-LAMININ) in 24-well plates (Cat. # 734-22324, VWR) for 2 hours in an incubator at 37°C and 8% CO_2 to allow the cells to settle down. 1 mL of culture medium was added to each well with a coverslip for long-term culture. Half of the media was replaced with fresh media every 48 hours for as long as needed.

2.5.1 Co-culture experiments

HEK293T cells were grown at 37°C and 8% CO_2 before co-culture experiments; HEK293T cells were diluted in cell media and counted using a hemacytometer until a high 100K cells/mL density was achieved. EdU-injected dissociated retinal cells were diluted at low-density (10K cells/mL) in 1) cell media only, 2) EdU negative high-density dissociated retinal cells from sibling mouse or 3) HEK293T cells at high-density. Cells were then plated as usual and cultured for 48 hours. Half of the media was replaced with fresh media every 48 hours for as long as needed.

2.5.2 Retinal cell culture treatments

Various pharmacological cell culture treatments were used to upregulate or downregulate the Notch and Sonic Hedgehog pathway *in vitro*, these are summarized in the table below (Table 1).

Table 2 Summary of the pharmacological treatments used in dissociated retinal cells cultures to upregulate or downregulate the Notch and/or the Sonic Hedgehog pathway.

Treatment	Final concentration in media	Mode of action
Addition of DAPT (Cat. # 72082, StemCell) in cell media.	10 µM diluted from a 10mM stock in DMSO	Inhibits ɣ-secretase, resulting in the indirect inhibition of the Notch pathway.
Addition of Cyclopamine (Cat. # 239803, Sigma) in cell media.	10 µM diluted from a 10 mM stock in DMSO	Binds to and blocks the Smoothened protein, a crucial component of the Sonic Hedgehog pathway.

Coated coverslips with recombinant mouse DLL1 (Cat. # 5026-DL-050) and recombinant mouse DLL4 (Cat. # 1389-D4-050).	5 µg/mL diluted from a 500 mg/mL stock in 1XPBS	Upregulation of the Notch signaling pathway by addition of DLL1 and DLL4, two of the main ligands of Notch 1.
Addition of Purmorphamine (Cat. # SML0868) in cell media.	10 µM diluted from a 10 mM stock in DMSO	Binds to and activates the Smoothened protein, a crucial component of the Sonic Hedgehog pathway.

2.5.3 Cell viability assay

The LIVE/DEAD™ Viability/Cytotoxicity Kit (ThermoFisher, USA, Cat. # L3224) was used to test the viability of cultured dissociated retinal cells. Two dyes were used in this kit: Calcein AM (ex/em ~495 nm/~515 nm) to distinguish live cells and ethidium homodimer-1 (EthD-1, ex/em ~ 495 nm/635 nm) to distinguish dead cells. This method relies on the presence of intracellular esterase activity in living cells, which enzymatically converts calcein AM to the intensely green fluorescent protein calcein. EthD-1 enters damaged cell membrane and binds to nucleic acids, producing a bright red fluorescence. 2 µM of calcein AM and 4 µM of EthD-1 were combined in a solution with 1xPBS. 100 µL of this solution was added to a 12 mm coverslip with living cultured dissociated cells. Coverslips were incubated for 45 minutes at room temperature. 10 µM of 1xPBS were added after incubation and the coverslips were carefully mounted on a microscope slide. Cells were immediately analyzed via confocal microscopy. The proportion of live/dead cells was quantified.

2.6 Cell fixation, immunocytochemistry and EdU detection

2.6.1 Cell fixation

Culture medium was aspirated, and 4% PFA (Electron Microscopy Science, USA, Cat. # 157-08) was added to the retinal cells for fixation for 10 minutes on ice. The retinal cells were washed three times with 1XPBS and stored at 4°C.

2.6.2 Immunocytochemistry

Prior to immunostaining, the coverslips were incubated with a permeabilization/blocking solution (20X horse serum + 0.4% Triton in 1XPBS) for 30 minutes at room temperature (RT). Primary antibodies were applied to the coverslips and incubated for two hours at 37°C and then were washed off three times with 1XPBS before applying secondary antibodies. Secondary antibodies were applied to the coverslips and incubated for one hour at RT. The primary antibodies were prepared in 20X horse serum and 0.4% Triton in 1XPBS; the secondary antibodies were prepared in 1XPBS and DAPI (1mg/ml). Table 2 lists the primary and secondary antibodies used.

Table 3 List of primary antibodies

Antigen	Antiserum	Supplier, cat. #	Working dilution
Cre	Rabbit anti-Cre	Biolegend (908001)	1:500
Cre	Mouse anti-Cre	Millipore Sigma (MAB3120)	1:500
β-Galactosidase	Rabbit anti-β-gal	MP Biomedicals (5576)	1:15,000
PKC α	Rabbit anti-PKC α	Sigma Aldrich (P4334)	1:30,000
Vsx1	Mouse anti-Vsx1	Santa Cruz (sc-393699)	1:100

Table 4 List of secondary antibodies

Antibody	Supplier, cat. #	Working dilution
Donkey anti-Mouse IgG (H+L), Alexa Fluor 555	Invitrogen (A-31570)	1:500
Donkey anti-Rabbit IgG (H+L), Alexa Fluor 488	Invitrogen (A-21206)	1:2000
Goat anti-Mouse IgG subclass 3, Alexa Fluor 488	Jackson Immuno (115-545-209)	1:2000
Donkey anti-Rabbit IgG Alexa Fluor 555	Invitrogen (A-31572)	1:500

2.6.3 EdU detection

After secondary antibodies were applied, the coverslips were washed three times with 1XPBS. EdU detection solution (2M TBS, 0.4M $CuSO^4$, 10X Sodium ascorbate and 4M Alexa 647 azide in ddH_2O) was applied to the coverslips and incubated for 35 minutes at RT. The coverslips were washed three times with 1xPBS and mounted on a slide with Immunomount (Thermo Scientific, USA, Cat. # 9990402).

2.7 Confocal imaging and image analysis

Fluorescent microscopy was performed using a Nikon C2 confocal microscope. Images were taken using either a Plan Fluor 10X (NA 0.30) for low-density cultured cells or Plan APO 20X (NA 0.75) Nikon objective lens for high-density cultured cells and EZ-C2 imaging software. Image size was 2048, 1.2 pixel dwell and a laser power of 5. Images were taken in a Z series with each image having a 2µm step size and a total depth of ~15 µm for 20X images, and 8µm step size and a total depth of ~50µm for 10X images. These images were stacked and analyzed in FiJi image analysis software.

2.8 Quantification and statistical analysis

Cell counting in Z-series images was performed using FiJi image analysis software. Flat field correction of images was performed by dividing each channel image by a reference image from a fluorescent slide taken under the same conditions for each imaging session. Fluorescent colocalization was performed by setting a threshold (IJ_IsoData) from the maximum projection Z-stack of each channel. Total number of EdU+ cells were scored by overlaying a thresholded image of the EdU channel on top of a composite maximum projection, 32-bit version of the same image and manually selecting EdU+ cells via Cell Counter plugin (Cell Counter, *https://imagej.nih.gov/ij/plugins/cell-counter.html)*. Thresholded images were multiplied to each other to find colocalized marker expression. The result 8-bit image was overlaid on top of a composite maximum projection image with all channels to verify colocalization and manually score cells via the cell counter plugin. All EdU+ cells were considered progenitor cells at the time of injection. Cre (Vsx2-Cre) immunoreactive + cells were considered post-mitotic pan bipolar cells. Cre and PKCα+ cells were considered rod bipolar cells. Cre and β-gal+ cells were

considered ON bipolar cells. All Vsx1+ cells were considered bipolar cells since it is expressed exclusively on bipolar cells (Chow et al. 2001). The file names for all images were blinded prior to cell counting by using a program written by Dr. Bridget Ryan (University of Victoria). Each experiment was replicated at least three times. Data was documented in Microsoft Excel 2022. Graphs and t-tests were created in GraphPad Prism 9.

Chapter 3 Bipolar cell subtype specification is a multi-step mechanism involving cell contact

3.1 Does bipolar subtype specification occur in a stepwise manner?

3.1.1 Potential mechanisms involved in bipolar subtype specification

There are a few different ways in which an RPC could give rise to a fully differentiated bipolar cell subtype. The simplest mechanism is a "direct model" in which an RPC directly gives rise to a differentiated bipolar cell subtype. Alternatively, there could be an "intermediate model" in which an RPC, after exiting the cell cycle, gives rise to a transient, transcriptionally unique precursor cell prior to becoming a fully differentiated bipolar cell subtype. Lastly, the mechanism could involve a "multi-step model" in which an RPC, after exiting the cell cycle, gives rise to several precursor cells before becoming fully differentiated. In all these models, both extrinsic and intrinsic signals could be at play at different timepoints (Figure 4). Previous lab data suggests the presence of a post-mitotic precursor cell (Nickerson et al. 2011). Additionally, recent single-cell RNA sequencing analyses demonstrate post-mitotic precursor cells are an important component for the specification of fully specialized subtypes in the retina and have plastic properties (Shekhar et al. 2022; Sridhar et al. 2020; De Anda et al. 2016; Roaux & Arlotta 2013). We therefore hypothesize there is a multi-step model involved in the specification of bipolar cell subtypes. Our first aim was to look at the onset of expression of some of the major bipolar cell subtype markers in post-mitotic cells after cell cycle exit.

3.1.2 Experimental approach

We decided to inject EdU (5-ethynyl-2'-deoxyuridine, a thymidine analogue that gets incorporated into proliferating cells) into mice at P3, the peak of bipolar cell birth, and subsequently dissociated and fixed retinal cells at different timepoints afterwards and performed immunohistochemistry for a pan-bipolar marker and three bipolar subtype-specific markers described in detail in the next section (Figure 5,A). This approach allowed us to accurately track the cell fate of retinal progenitor cells at P3 (Figure 5,B). 24 hours after injection of EdU was our earliest analyzed timepoint because it is considered the earliest time after cell cycle exit post-mitotic bipolar cells start expressing markers (Nickerson et al. 2011). Retinal cells were dissociated at various time intervals after injection to pinpoint the earliest timepoint of marker

expression until P8, a timepoint in which bipolar cell birth is finished (120 hrs after injection) (Young, 1985a, b).

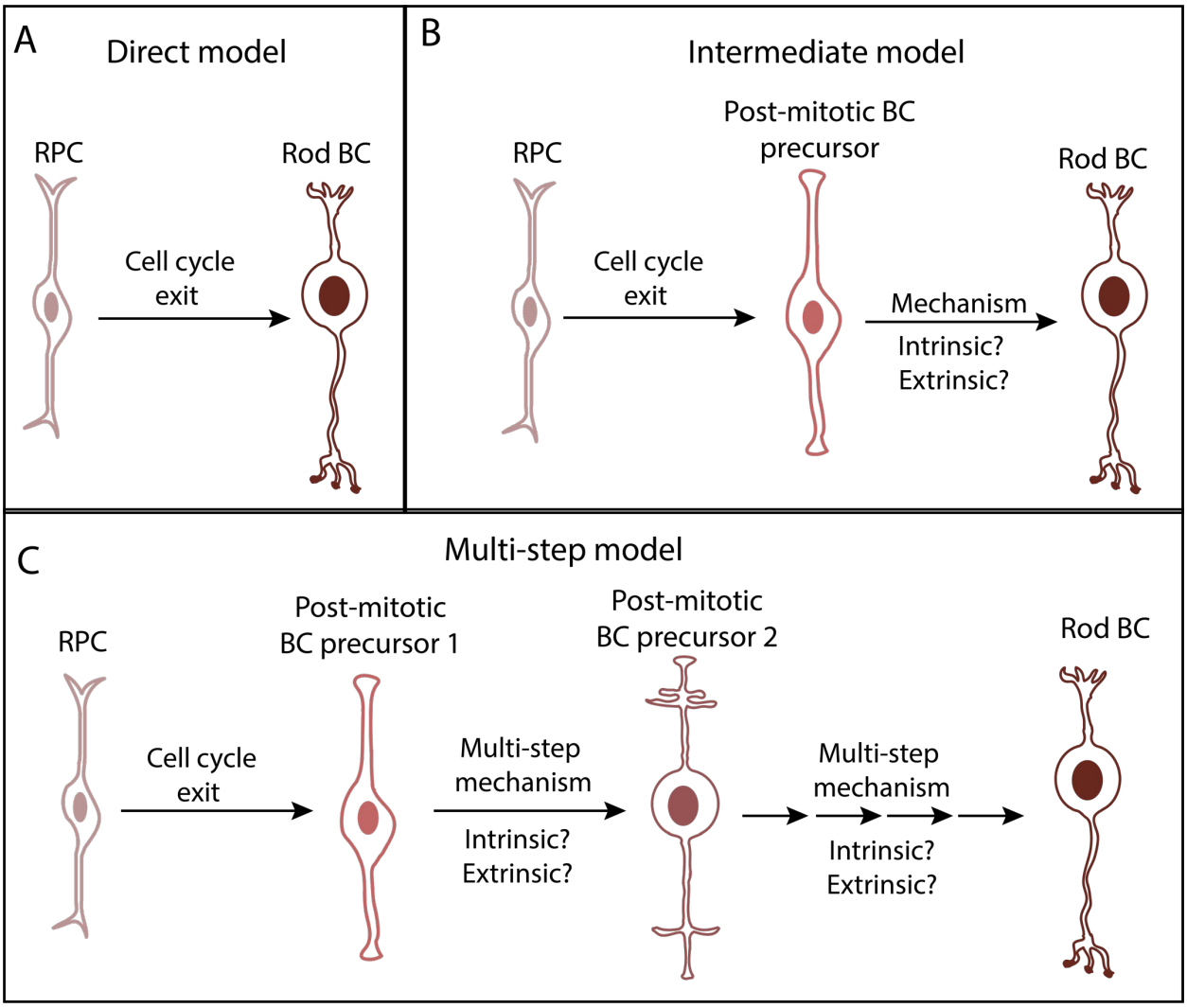

Figure 4 Potential mechanisms underlying bipolar cell subtype specification.

(A) Direct model: Retinal progenitor cell (RPC) directly giving rise to a fully differentiated rod BC after cell cycle exit. **(B)** Intermediate model: RPC giving rise to a post-mitotic BC precursor intermediate cell prior to becoming a rod BC via either intrinsic and/or extrinsic mechanisms. **(C)** Multi-step model: RPC giving rise to various post-mitotic BC precursor intermediate cells prior to giving rise to a fully differentiated rod BC via intrinsic and/or extrinsic mechanisms.

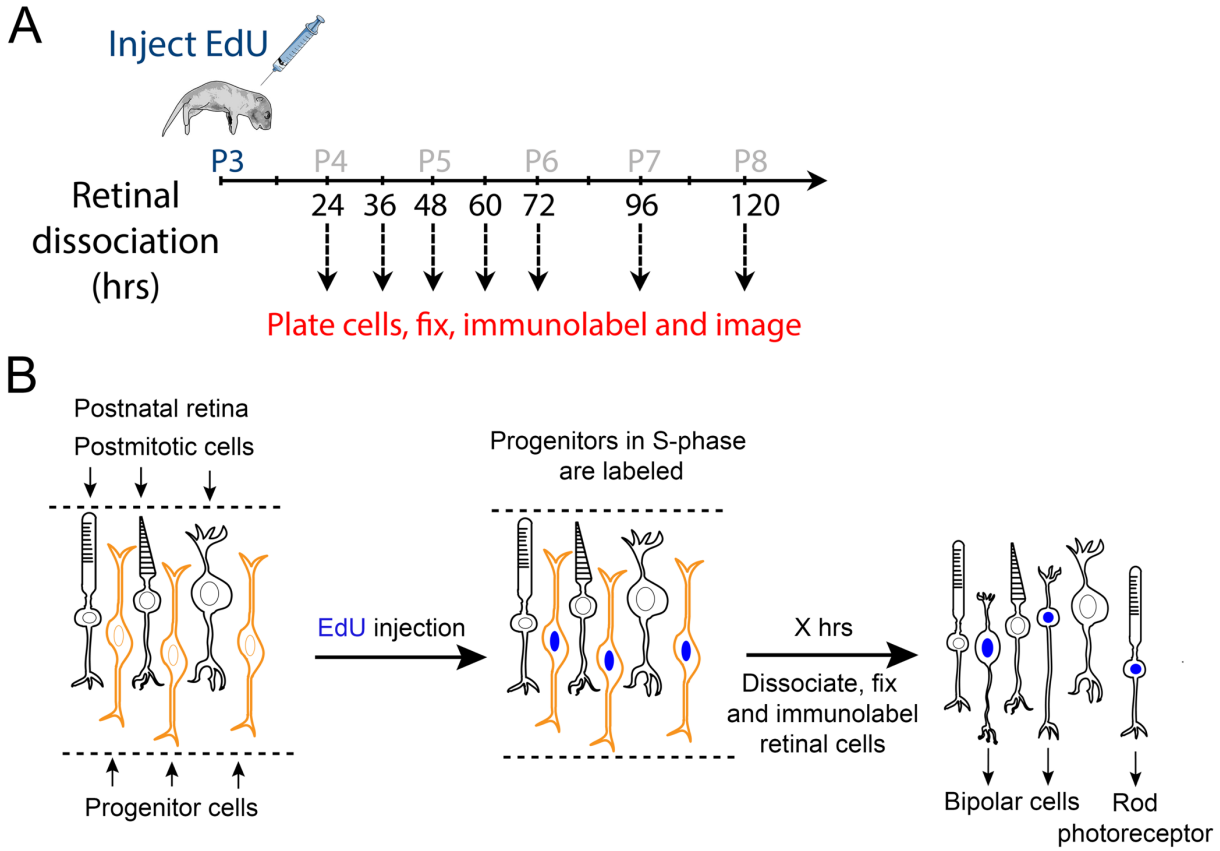

Figure 5 Experimental approach to examine the onset of expression of bipolar cell markers.

(A) Schematic of experimental approach; P3 pups were injected with EdU at P3 followed by retinal dissociation, fixing and immunolabeling of cells at different time points after injection. **(B)** EdU gets incorporated into the nuclei of cells undergoing S-phase during cell proliferation (blue). 24-120 hrs later, retinal cells were dissociated, fixed and immunolabeled against a pan-BC marker, and three BC-subtype specific markers.

3.1.2 Molecular markers of bipolar cell subtypes

Accurate identification of differentiated cell types is often a challenge in developmental biology due to the transient expression of molecular markers and mutual gene expression in different cell types. For example, *Grm6,* the gene that encodes the ON bipolar-specific metabotropic receptor mGluR6 is transiently expressed in RGCs. VSX2, the only pan bipolar cell marker, is also expressed by RPCs which makes accurate identification of bipolar cells during development a difficult task. Fortunately, our lab generated a transgenic mouse line

expressing Cre recombinase under the control of a postmitotic VSX2-BC specific enhancer, *Vsx2-5.3-PRE-Cre* (here on referred to as "Vsx2-Cre" or "Cre") circumventing this issue (Nickerson et al. 2011). Although cell-type specific Cre recombinase transgenic lines are often coupled with conditional reporter transgenic lines, we have found that conditional reporter expression (e.g. Ai9 tdTomato reporter expression) is sometimes erroneously expressed during embryonic development due to leaky Cre expression, making reporter alleles unreliable (Figure 6) Fortunately, the leaky Cre expression either occurs very early in development or at sub-threshold levels such that immunolabeling against Cre recombinase faithfully recapitulates VSX2 expression exclusively in BCs, making this marker an excellent tool for identification of post-mitotic BCs. Vsx2-Cre immunolabeling is a critical marker for my studies as it is a pan-BC marker (Shekhar et al. 2016; Nickerson et al. 2011) and is the earliest bipolar cell specific marker that has been identified to date, first appearing between 24-36 hrs after cell cycle exit (Nickerson et al. 2011).

In addition to Vsx2-Cre, three other bipolar cell molecular markers were used in my experiments to identify distinct subsets of bipolar cells: PKCα: a protein kinase expressed exclusively by rod BCs (and a subset of amacrine cells), *mGluR6:NLS-LacZ* (here on referred to as "mGluR6" or "βgal"): a transgenic line expressing nuclear β-galactosidase in ON bipolar cells, and VSX1: a transcription factor exclusively expressed by bipolar types 2, 6 and 7 (Chow et al. 2001, Chow et al. 2004, Shi et al. 2012). These markers were selected because they allow us to differentiate between the main major categories of subtypes: ON/OFF and Cone/Rod, as well as for a specific subset of bipolar cell types.

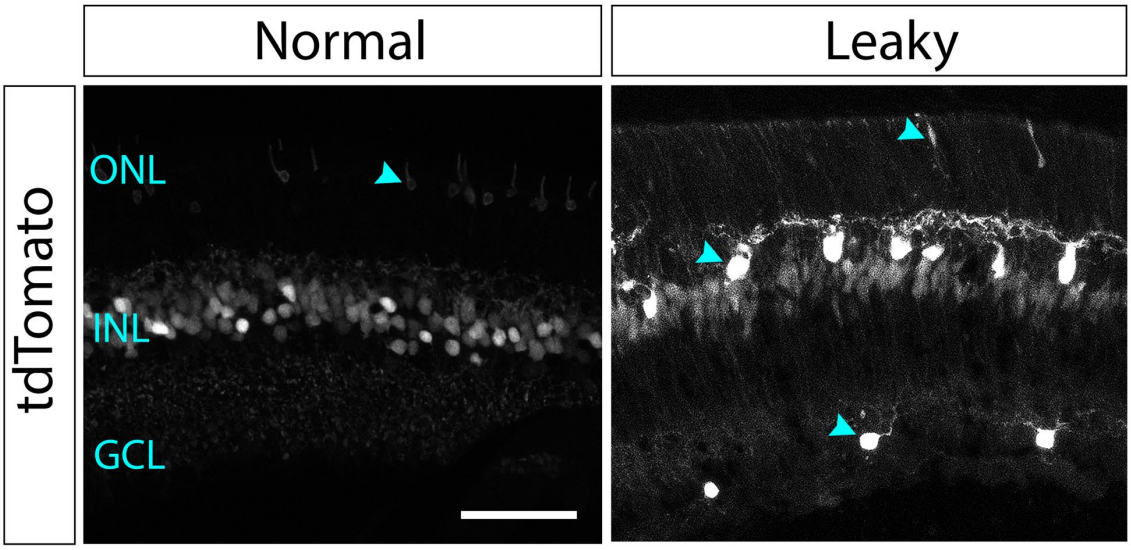

Figure 6 Adult retinal sections with leaky expression of VSX2-Cre in Ai9 tdTomato reporter mice

Retinal sections with normal (left panel) and leaky (right panel) VSX2-Cre tdTomato reporter expression in Ai9-tdTomato reporter adult mice. Strong reporter expression is normally exclusive to bipolar cells with only a few photoreceptors with weak expression, potentially due to post-mitotic cell fate switch (left panel, arrow). Leaky reporter expression is seen in some litter mates showing strong reporter expression not only in bipolar cells but also in retinal ganglion cells, horizontal cells, and photoreceptors (right panel, arrows). Scale bar = 50 µm.

3.1.3 Vsx2-Cre is expressed prior to other bipolar cell markers in post-mitotic cells

We analyzed the onset of expression of four major bipolar cell subtype markers by looking at the earliest time-point after cell cycle exit in which they are detectable via immunofluorescence. This was done by looking at the proportion of EdU+ cells co-expressing the marker of interest at each different timepoint. Our results show Vsx2-Cre, a pan post-mitotic bipolar cell marker, is first detectable 36 hours after cell cycle exit (Figure 7, B). Whereas mGluR6, PKCα, and Vsx1 are detected between 48-60 hours after cell cycle exit (Figure 7, E, H, K). The average proportion of EdU cells co-expressing Vsx2-Cre at 36 hours was 3.13% ± 0.05%. This number continuously increased at later timepoints, and then reached a plateau at 96

hrs post injection with an average proportion of 23.9% ± 1.96% of EdU cells expressing Cre. At 120 hrs after EdU injection, there was a small jump in the proportion of cells expressing Vsx2-Cre, for a final average proportion of 35.8% ± 3.95% of EdU cells expressing Cre at P8. The rest of the markers were seen at timepoints after the initial expression of Vsx2-Cre. The expression of VSX1, a transcription factor expressed solely by bipolar subtypes 2,6 and 7 was first seen 48 hours after cell cycle exit with an average proportion of EdU cells co-expressing VSX1 of 0.533% ± 0.393% This number continued to increase at later timepoints until reaching a proportion of 6.8% ± 3.02% at P8. The expression of PKCα, a marker for rod BCs, was first detected 60 hours after cell cycle exit (Figure 7, H). At this timepoint, the average proportion of EdU cells co-expressing PKCα was 0.667% ± 0.328%. This number continued to increase at later timepoints until reaching a total proportion 4.57% ± 0.546% at P8. mGluR6, a marker for ON BCs, was first detected 60 hours after cell cycle exit (Figure 7, K) with an average proportion of 2.37% ± 0.644% of EdU cells co-expressing mGluR6. This number continued to increase at later timepoints until reaching an average proportion of 19.2% ± 0.061% at P8.

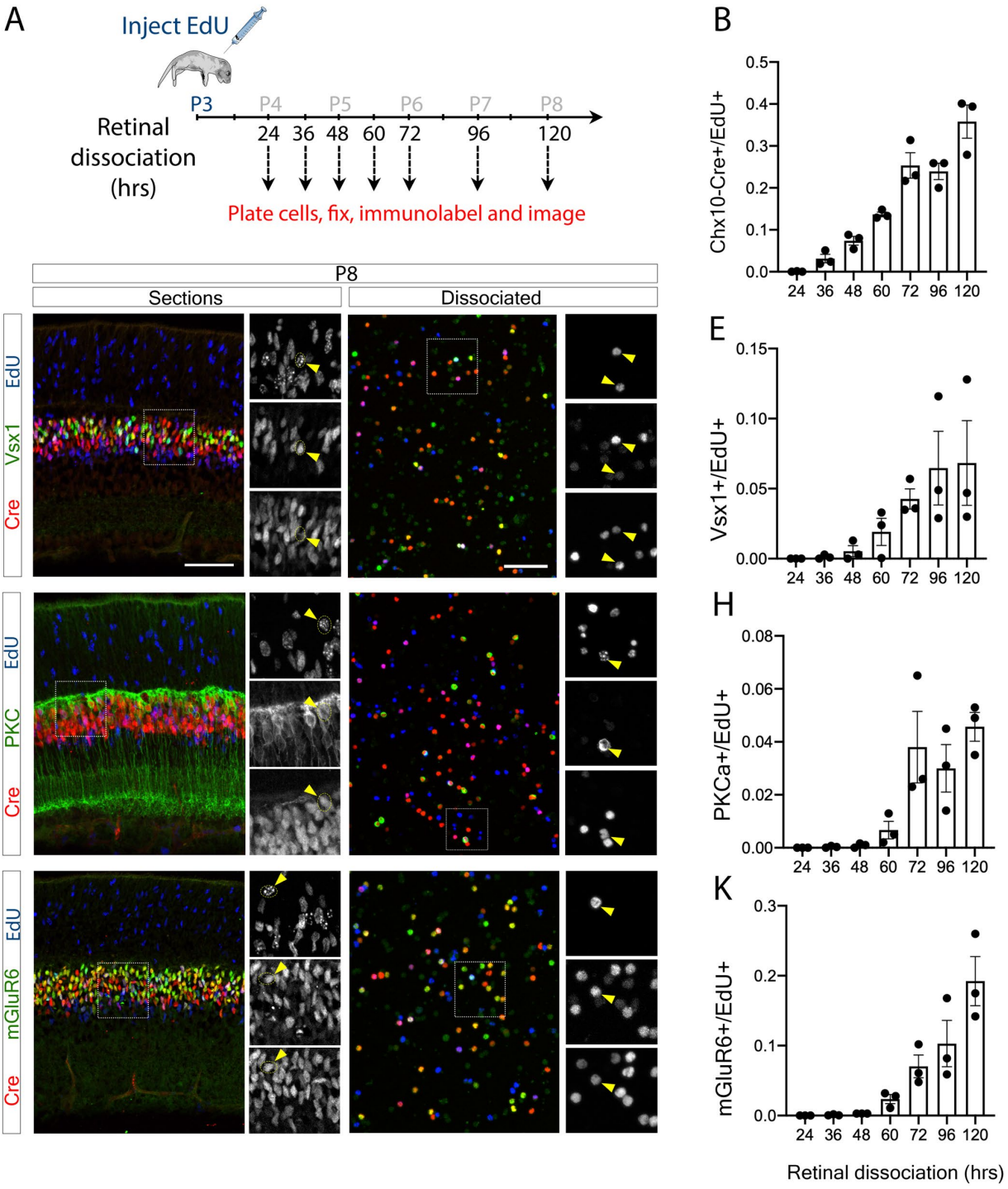

Figure 7 The onset of expression of bipolar cell markers after cell cycle exit

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Figure 7 The onset of expression of bipolar cell markers after cell cycle exit.
(A) Schematic of experimental procedure; P3 pups were injected with EdU at P3 followed by retinal dissociation, fixing and immunolabeling of cells at different time points after injection. **(B)** Proportion of dissociated retinal EdU+ cells positive for Cre immunolabeling (Vsx2-5.3-PRE-Cre) at different time-points after EdU injections. **(C, F, I)** P8 retinal sections immunolabeled against Cre (red), marker (green: mGluR6, PKCα or Vsx1) and EdU (blue). Right-side panels depict co-expression of all three markers (yellow arrows) on zoomed in regions. **(D, G, J)** P8 dissociated retinal cells immunolabeled against Cre (red), marker (green: mGluR6, PKCα or Vsx1) and EdU (blue). Right-side panels depict co-expression of all three markers (yellow arrows) on zoomed in regions. **(E, H, K)** Proportion of dissociated retinal EdU+ cells positive for Vsx1 (E), PKCα (H), and mGluR6 (K) at different timepoints after EdU injection. Total number of EdU+ cells counted was at least 1000 per marker. ONL: Outer nuclear layer, INL: Inner nuclear layer, GCL: Ganglion cell layer. Scale bar: 50μm. n=3 retinas per time-point. Error bars = SEM.

We next quantified the proportion of cells positive for either PKCα, Vsx1, or mGluR6 out of all Cre+ & EdU+ cells (Figure 8). Our results suggest all markers start appearing robustly after the initial expression of Cre. Initial expression of mGluR6 was seen 12 hours after the initial expression of Cre. An average of 3.5% ± 0.65% of Cre & EdU positive cells were positive for mGluR6 at this timepoint. There was a significant jump of mGluR6+ cells 24 hours after initial expression of Cre; an average of 17.9% ± 4.71% of Cre & EdU positive cells were positive for mGluR6. PKCα expression was first seen 24 hours after initial expression of Cre. An average of 4.83% ± 2.65% of Cre & EdU positive cells were PKCα+ at this timepoint. VSX1 expression was first seen 12 hours after initial Cre expression. An average of 5.63% ± 4.01% of Cre & EdU positive cells are positive for VSX1 at this timepoint. The average final proportion expression of all markers at P8 was 50.8% ± 5.67% for mGluR6, 12.5% ± 0.360% for PKCα, and 12.7% ± 2.94% for VSX1. These results suggest bipolar subtype specification occurs in a multi-step manner, along the presence of a postmitotic bipolar cell precursor intermediate cell 36 hrs after cell cycle exit before the expression of other bipolar cell markers (Figure 8 B).

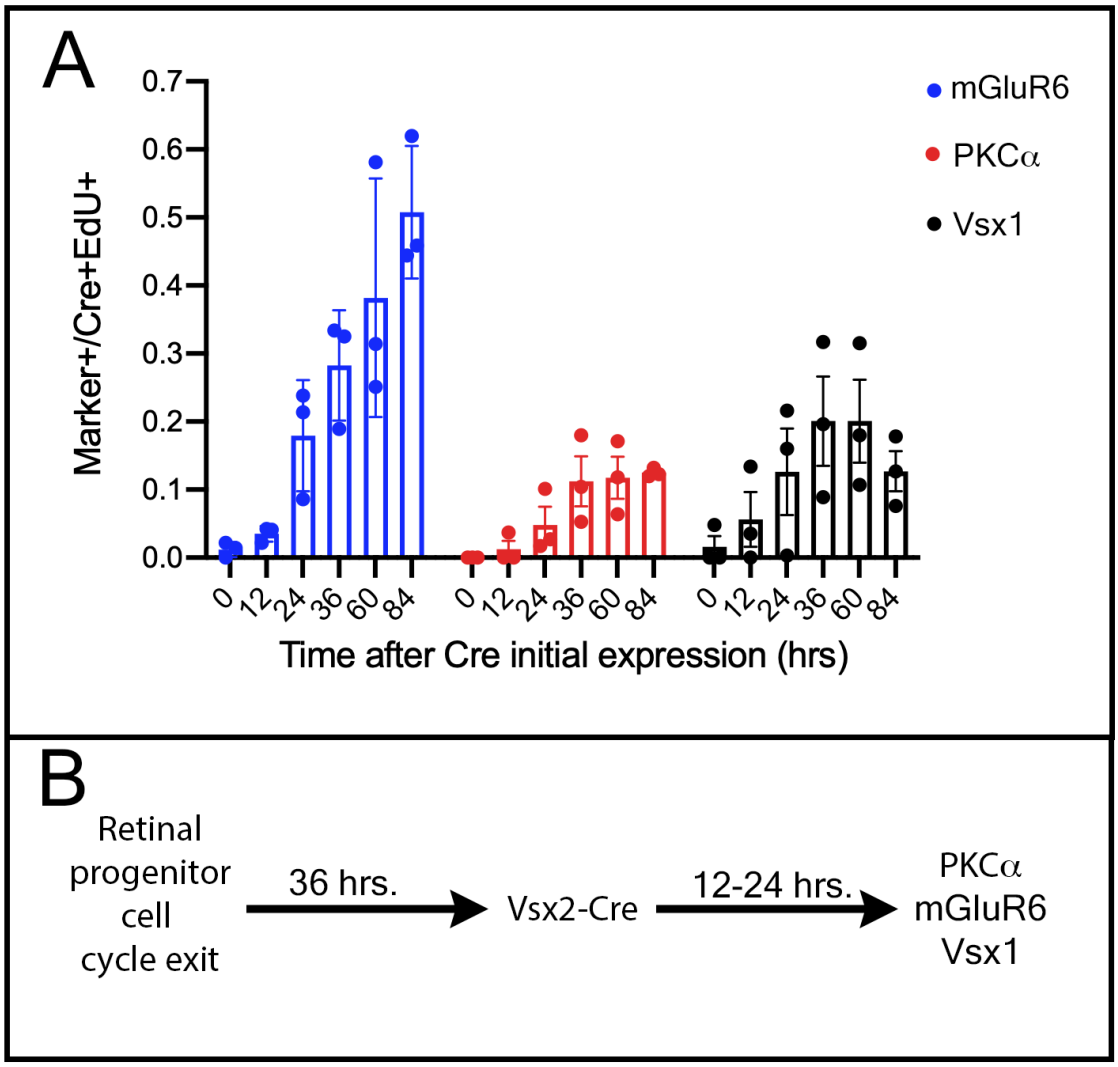

Figure 8 Bipolar cell markers are expressed within 12-24 hrs. after initial expression of Cre

(A) Initial expression of mGluR6 (blue), PKCα (red) and Vsx1 (black) relative to the initial expression of Cre+EdU+ in retinal dissociated cells injected with EdU at P3. **(B)** Summary model of results. Vsx2-Cre expression is first detected 36 hours after retinal progenitor cell cycle exit. 12-24 hrs later, the expression of the other BCs start to be seen. N=3 mice per timepoint. Error bars = SEM

3.2 Do cell-cell interactions play a role in bipolar cell specification?

3.2.1 Dissociated retinal cell culture as an experimental method

A robust experimental approach and assay is essential for making progress in science. We spent considerable time in thinking about how to experimentally examine cell-cell interactions during retinal development in a meaningful way and explored the use of retinal dissociation/cell culturing as an experimental approach. Dissociated retinal cell culture is a widely used tool in cell biology (Politi et al. 1988; Reh & Kijavin 1989; Seigel 1999), but like any other in vitro system, it has advantages and disadvantages. The main disadvantage of retinal dissociation is the loss of retinal architecture since the retinal structure is lost when cells are dissociated. This causes abnormal cell-contact, potential loss of specific phenotypes, altered gene expression and therefore questionable relevance to the mechanisms occurring in vivo. But there are strong advantages to retinal dissociation experiments: 1) high control of the culture environment: the researcher can control the culture conditions, such as content of the media, supplements, and incubator settings, additionally, pharmacological compounds can be used to upregulate/downregulate signaling pathways by addition to cell media, 2) cell number control: the number of cells cultured can be easily controlled by counting cells via a hemocytometer before plating, and 3) simple quantitative analysis: since dissociated cells are isolated from other tissue and often cultured as a monolayer, there is no background tissue/cells. This makes analysis of immunofluorescence and cell counting relatively simple (Zhang et al. 2002; Seigel 1999). Used in conjunction with in vivo methods, retinal dissociation culture is an ideal tool to study cell specification.

We decided to use retinal dissociation as our initial assay to explore the mechanisms of bipolar cell subtype specification. This method provides us with an isolated retinal cell population and high control of cell density, enabling us to study the effects of non-cell autonomous signals on subtype specification. Shortly after dissociation retinal cells lose their projections, but they can renew them and form physical interactions with each other (Figure 9). Quantification of marker co-expression is more accurate in dissociated cells than retinal sections (Figure 7, D, G, J).

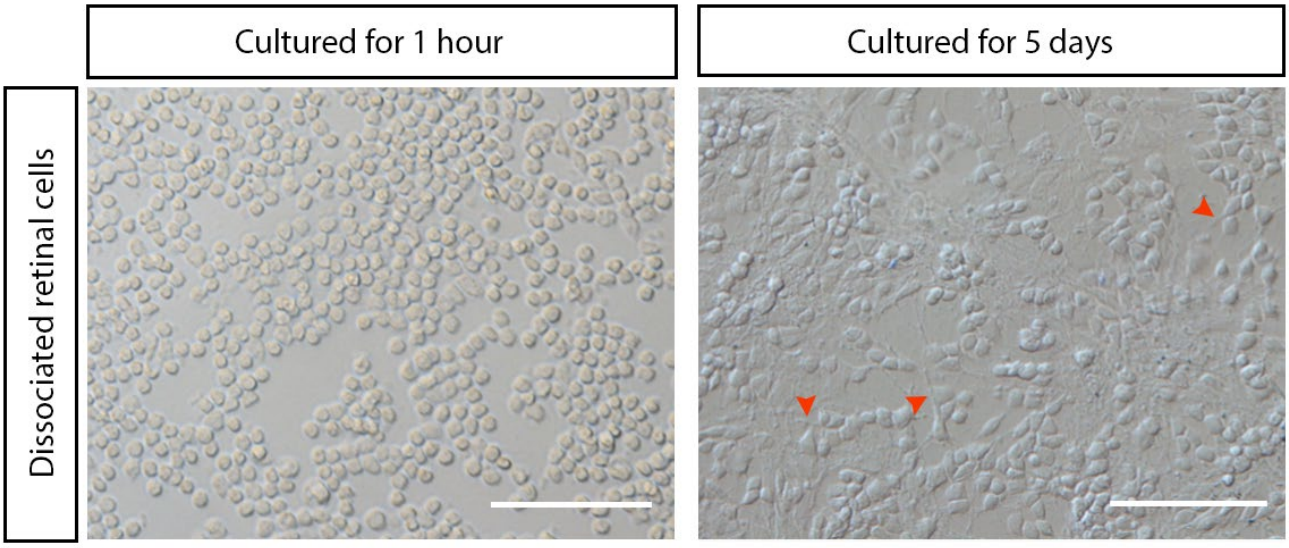

Figure 9 Dissociated retinal cells in culture physically interact with each other

Retinal cells from P3 mice were dissociated via papain incubation, triturated, and cultured for one hour (left panel) and 5 days (right panel) on 12 mm coverslips coated with mouse laminin and poly-D-lysine. Cell projections are lost after dissociation but are re-established in culture (red arrows). Scale bar = 50 µm.

3.2.2 Metabolic labeling of S-phase in retinal progenitors for accurate investigation of bipolar cell subtype specification in postmitotic cells

During retinal development, RPCs undergo numerous rounds of cell division until their final S-phase is achieved and they exit the cell cycle permanently. This event is defined as cell birth. The first method to identify progenitors in the developing mouse retina and estimate cell birth of different cell types was autoradiography of tritiated (3H) thymidine which is metabolically incorporated into the DNA of cells undergoing S-phase and thus allows the monitoring of DNA synthesis rates and cell proliferation (Young et al. 1985a, b; Hughes et al. 1958). However, due to its radioactive properties, this method has some harmful effects on cells such as DNA fragmentation, cell cycle arrest, chromosomal aberrations, and apoptosis induction,

all of which could seriously compromise research results (Hu et al. 2002). This prompted researchers to develop new methods to label progenitor cells.

Bromodeoxyuridine (BrdU) is a thymidine analogue that can be easily detected in cells via immunohistochemistry and offers various advantages when compared to 3H-thymidine (Gratzner 1982). Detection of proliferating cells by BrdU is faster and safer than 3H-thymdine since it is not a radioactive substance, and its detection can be done in thin sections of tissue unlike for autoradiography (Taupin 2006). Unfortunately, there are various drawbacks to this method as well. BrdU is highly toxic; injection of BrdU into pregnant rodents causes exencephaly, cleft palate, limb abnormalities and behavioral changes in the offspring (Bannigan & Langman 1979). Another important drawback of BrdU labeling is its method of detection. The monoclonal antibody used to detect BrdU binds to BrdU in a single-stranded DNA which means denaturing DNA via HCl treatment is necessary for its detection and this severely affects cell morphology and antigenicity, limiting the capacity to identify cell types (Taupin 2006).

Currently, the method of choice to label proliferating cells in the CNS is via incorporation of the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) into the DNA of cells undergoing S-phase. This method provides various advantages when compared to BrdU. Unlike BrdU, EdU detection does not require DNA denaturation, no antibodies are used, and instead relies on a chemical reaction called "click chemistry" which covalently binds a fluorescent azide group to the alkyne group of the EdU molecule (Chehrehasa et al. 2008). The azide is a much smaller molecule when compared with the BrdU antibody, which leads to EdU detection having a greater detection sensitivity due to increased cell permeability (Cavangh et al. 2011). In the retina, EdU is injected *in vivo,* and its detection can be performed along immunolabeling, allowing us to track the fate of RPCs at the time of injection by looking for co-expression of molecular markers and EdU after cell birth. Even though EdU is a better choice to label cell proliferation than BrdU, it still has some important cytotoxic effects. Prolonged exposure to EdU has been shown to cause cell cycle arrest, cell death and activation of cell stress responses *in vitro* (Kohlmeier et al. 2013).

To determine the time-course of bipolar cell subtype specification and whether it requires non-cell autonomous factors, we designed a novel experimental approach to study the specification of bipolar subtypes by injecting EdU into postnatal mice, dissociating the retina at different timepoints after injection to target a temporally distinct population of postmitotic (or

progenitor) cells, and culturing the dissociated cells under different conditions such as high and low density until developmental maturity (Figure 10). In most neural developmental studies, EdU is either injected *in* vivo and analyzed directly in fixed tissue or added to culture media *in vitro.* Our approach is novel in that it allows normal development of EdU-labeled cells, *in vivo*, prior to retinal dissociation and culture, therefore enables one to ask both if, and when non-cell autonomous signals are required for bipolar subtype specification.

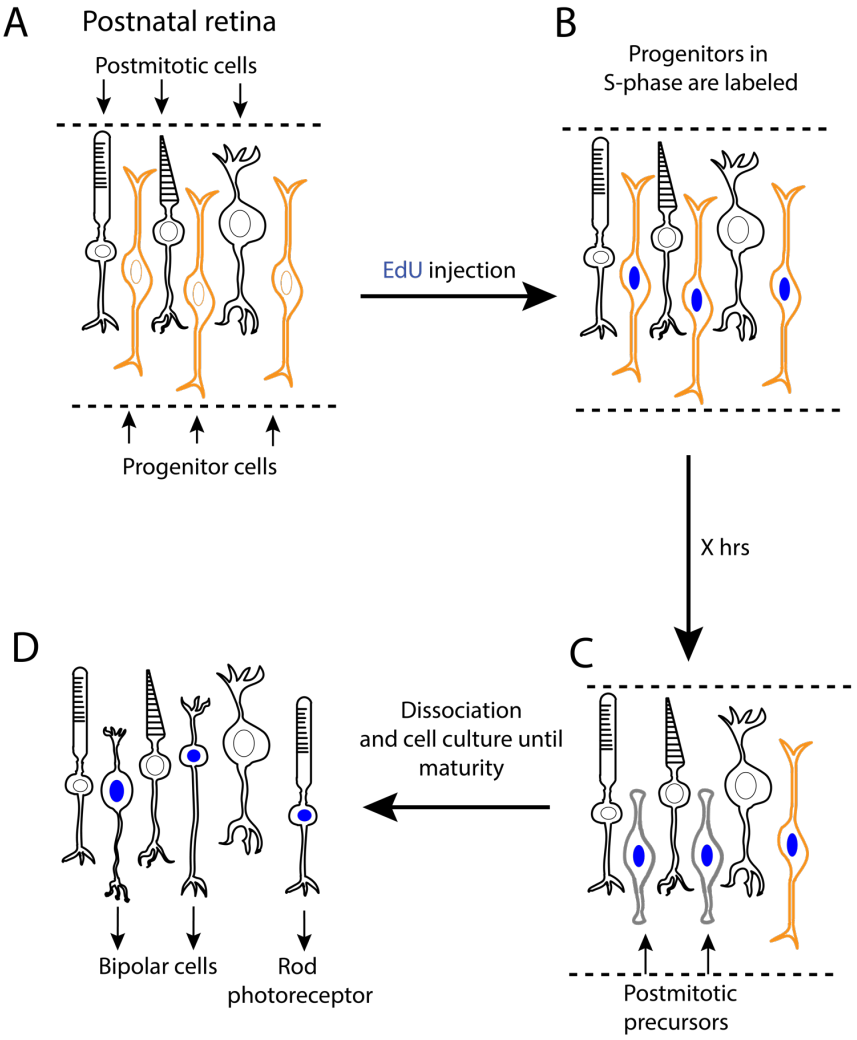

Figure 10 Metabolic labeling of EdU in postnatal retinal progenitor cells to track postmitotic cell fate specification in bipolar cells

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Figure 10 Metabolic labeling of EdU in postnatal retinal progenitor cells to track postmitotic cell fate specification in bipolar cells

(A) Postnatal retina is injected with EdU, there are both postmitotic retinal cells (black) and RPCs (yellow) present. **(B)** EdU is incorporated into the DNA of progenitors in S-phase, shown by blue nuclei. **(C)** "X" hours later, at which some of the previously labeled progenitors might be postmitotic precursor cells, the retina is dissociated and cultured until maturity under different experimental culture conditions. **(D)** Final cell maturation occurs in cell culture where now postmitotic cells retain the EdU previously injected, allowing for simple identification of cell types via immunohistochemistry.

EdU injection at P3 was chosen because it labels the highest possible number of RPCs that are fated to become BCs based on the temporal ordering of retinal cell birth. Retinal cell birth follows a chronological order with BCs being born in between P0 and P8, with the peak of cell birth happening at around P3-P4 (Young, 1985a; Figure 2). Additionally, the retina has a central-to-peripheral developmental gradient in which retinal development is ahead by 1-2 days in the central region (Rapport et al. 2004; Figure 11). At P3, the retina has a consistent distribution of RPCs across the central-to-peripheral gradient (Figure 11). For these reasons, P3 is the best timepoint to label the highest number of RPCs fated to become differentiated BCs in a developmentally unbiased spatial manner (Figure 11).

Notably, it must be mentioned that there are two very important assumptions made in these experiments. First, there are no thymidine analogues that exclusively label DNA during cell proliferation. 3H thymidine, BrdU and EdU all are incorporated into single-stranded DNA which means that these molecules will be incorporated into cells undergoing any cellular event involving DNA synthesis such as DNA repair and gene duplication. Since there is a large proportion of RPCs at P3 in the retina, we are assuming most of the cells that get EdU incorporated into their DNA are undergoing DNA synthesis due to cell proliferation.

The second important assumption being made is that bipolar cell subtype specification occurs at least partially postmitotically. My experimental design tests the effect of cell culture conditions on postmitotic cells. If cell fate specification occurs only prior to cell cycle exit (i.e., on RPCs), there would be no effect on bipolar subtype specification under different cell culture

conditions. There is evidence that some RPCs are biased towards generating specific cell types. For instance, lentivirus-mediated gene transfer to label RPCs and its progeny demonstrated clones with two horizontal cells (HCs) consistently contained a single HC subtype, suggesting cell fate specification occurred in RPCs and was inherited symmetrically by both daughter cells (Rompani & Cepko 2008). However, most research suggests postmitotic retinal cells are malleable to switch cell fate. For example, addition of ciliary neurotrophic factor (CNTF) to postnatal rat retinal explants resulted in a significant decrease in the number of postmitotic cells becoming rod photoreceptors, and an increase in bipolar, amacrine, and Muller glial cell numbers, suggesting cell fate specification occurs postmitotically (Ezzeddine et al. 1997). Additionally, viral tracing of retinal progenitor cells suggested Notch signaling is required in postmitotic cells to adapt the bipolar cell fate rather than the photoreceptor cell fate (Mizeracka et al. 2013).

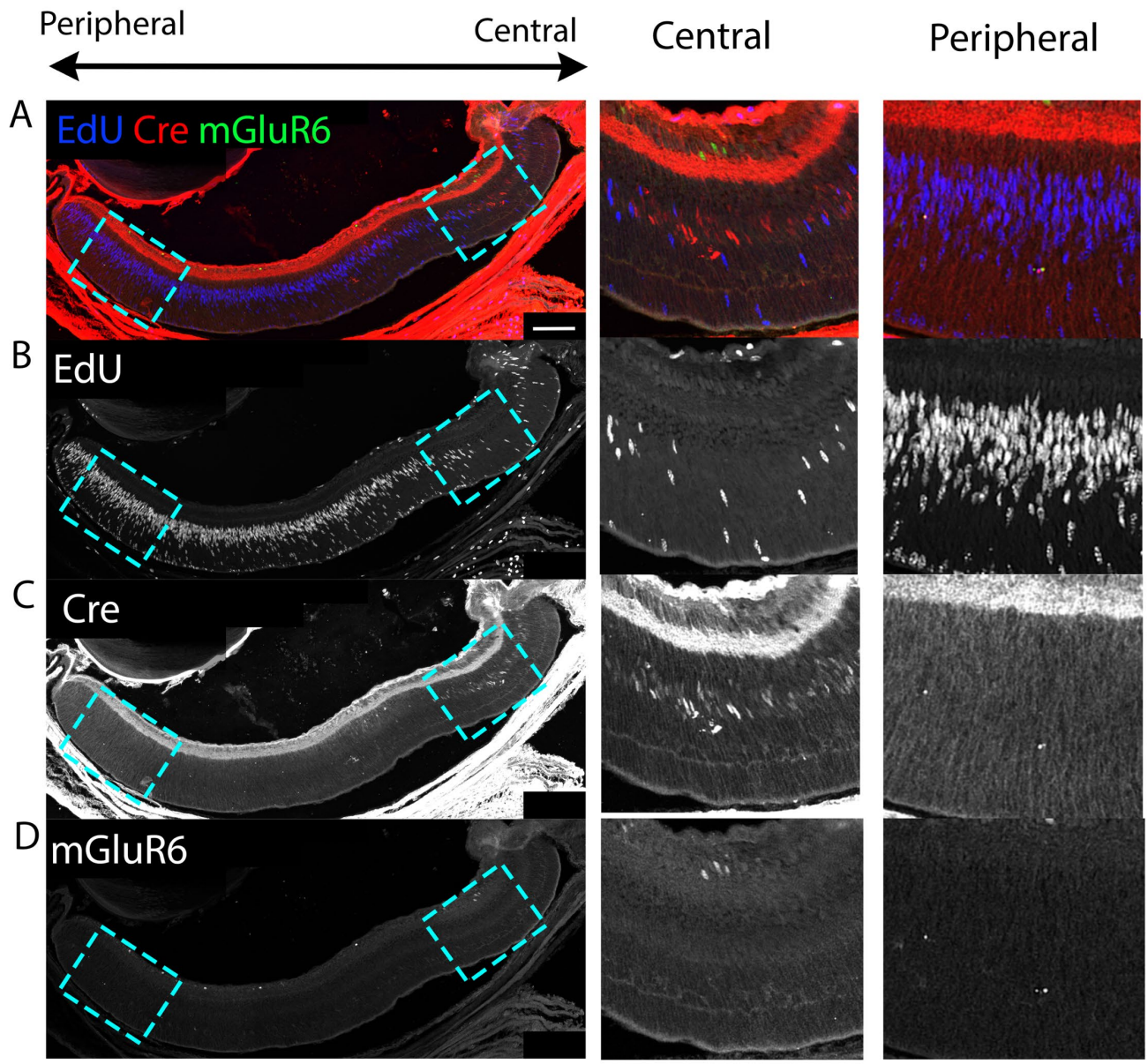

Figure 11 Retinal cell birth follows a central-to-peripheral developmental gradient

(A) Immunofluorescence of EdU-injected frozen P3 retinal sections expressing Cre, mGluR6 and EdU. **(B)** RPCs (EdU+ cells) are present throughout the central-to-peripheral spatial gradient except in the very central region (cyan circle). **(C)** Cre-expressing BCs can only be seen in the central-most region of the retina at this timepoint. **(D)** No mGluR6+ cells could be seen in the INL at this timepoint. Scale bar: 200 µm. Retinas were injected 1 hr. before harvesting.

3.2.3 Temporal requirement of cell contact on bipolar cell subtype specification

To explore the possibility of non-cell-autonomous mechanisms playing a role in the specification of bipolar subtypes, we chose to culture P3-EdU-injected dissociated retinal cells at different cell densities. As mentioned at the end of section 1.5.2.2 Extrinsic regulators of bipolar cell subtype development", there are many adhesion molecules involved in the synaptogenesis of bipolar cell dendrites and axons. There is evidence that the Notch and Sonic Hedgehog signaling pathways might be necessary for the specification of bipolar subtypes (Mizeracka et al. 2013; Wu 2017). We therefore hypothesized extrinsic mechanisms are involved in bipolar subtype specification. To test this, P3 mice were injected with EdU and their retinas dissociated 1, 24, and 48 hours after injection and cultured until "P8" or 120 hours after injection. At P3, most retinal progenitor cells (RPCs) only divide once, giving rise to rod photoreceptors (~70%), bipolar cells (~20%) and Muller glial cells (~10%) (Young, 1985a; Mizeracka et al. 2013).

The three timepoints for dissociation (1, 24 and 48 hrs. after injection) were chosen to examine the effect of cell density. At P3, the length of S-phase is 16 hours, G2 phase is 2.6 hours, and M-phase is 2.5 hours (Young 1985b). The 1-hour timepoint therefore tests only RPCs, since after only one hour after EdU incorporation, all progenitor cells will still be in the cell cycle. The 24-hour timepoint tests early post-mitotic cells since the majority EdU+ RPCs that were in their last round of division are out of the cell cycle at this point. The 48-hour timepoint tests late post-mitotic cells. Retinal cells were cultured at high (40,000 cells/12 mm coverslip) and low (1200 cells/12 mm coverslip) densities (Figure 13, B). If extrinsic mechanisms are required for the specification of BCs, one would predict a difference in the expression of subtype markers under different cell-density conditions.

The cell viability of retinal cells cultured at a low-density tends to be much lower than when cultured at high-density (Figure 12). To ensure the cell viability of our cultured retinal cells remained at an acceptable level (>80%) we performed various cell viability assays until an optimized papain incubation time and media improved the cell viability of low-density retinal cells cultured until developmental maturity (data not shown).

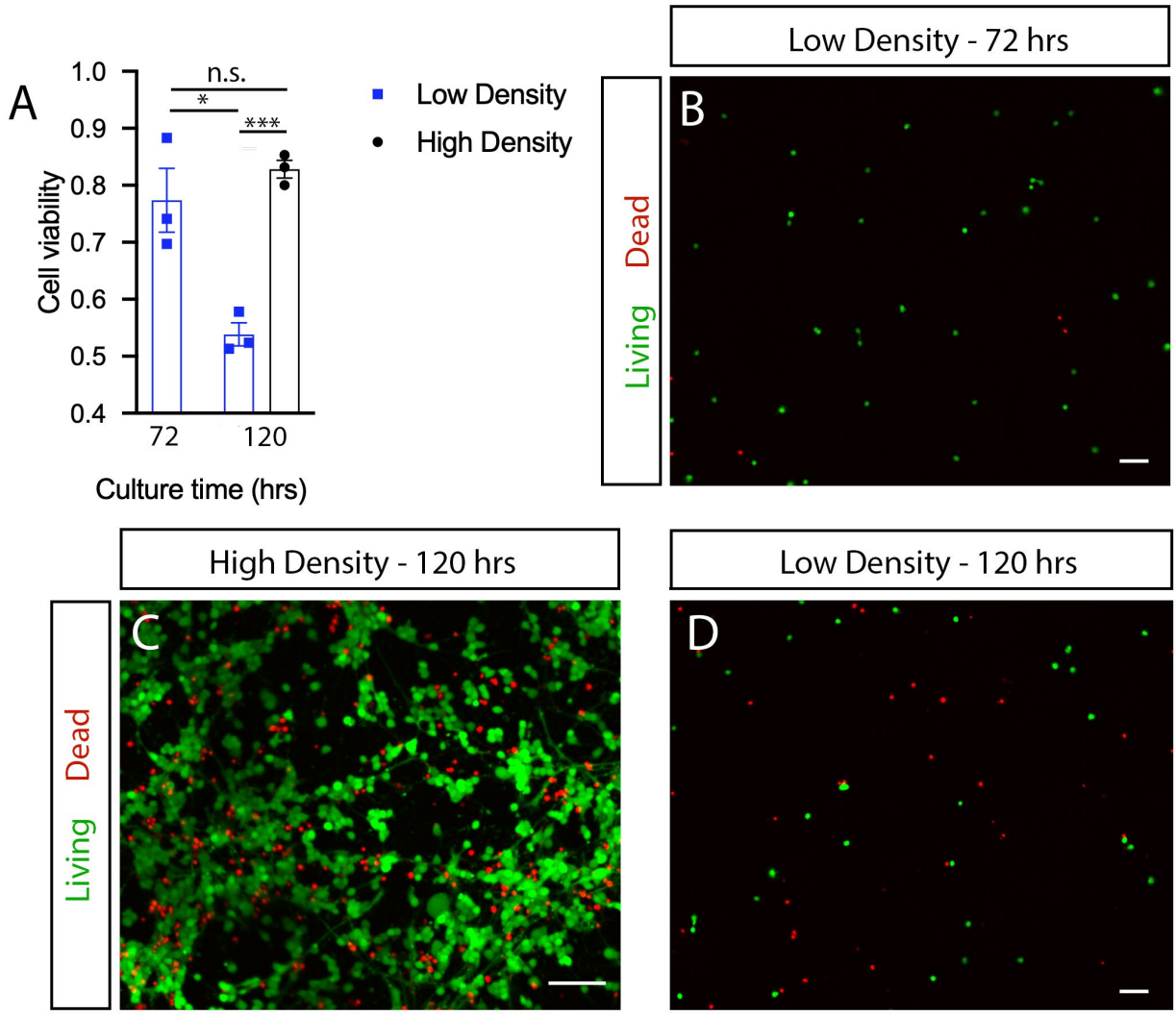

Figure 12 Cell viability assay via Calcein AM and EthD-1 homodimer fluorescent staining of cultured dissociated retinal cells

Cultured dissociated retinal cells were stained with fluorescent dyes Calcein AM (green, stains living cells) and EthD-1 homodimer (red, stains dead cells) after being cultured under different confluency conditions and for different times. **(A)** Quantification of cell viability (Living cell #/Total cell #). Cell viability significantly decreases when low-density dissociated retinal cells are cultured for 120 hours when compared to the high-density condition. Sample 10X image of low-density **(B, D)** and high-density **(C)** dissociated retinal cells stained with Calcein AM (green, living cells) and EthD-1 (red, dead cells) cultured for different times. N=3. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. Error bars = SEM. n.s. = not significant. Scale bars: 50 µm.

3.2.4 Cell contact is required for bipolar subtype specification

Using the experimental approach described in the previous sections, we quantified the proportion of P3-EdU-injected dissociated retinal cells expressing Cre, mGluR6, PKCα, and VSX1 at 1, 24 and 48 hours after injection, cultured until "P8" or 120 hours after injection, at high and low cell densities (Figure 13, Table 5). At the 1-hour timepoint the average proportion of EdU cells expressing Cre was 0.9% ± 0.2% at low-density and 3.3% ± 1.2% at high-density. This difference was statistically significant (Student's T-test, P = 0.002476, n = 5). At the 24-hour timepoint, the average proportion of EdU cells expressing cells was 2.8% ± 0.4% at low-density and 10.2% ± 1.6% at high-density. The difference was significant (Student's T-test, P = 0.002279, n =5). At the 48-hour timepoint the average proportion of EdU cells expressing Cre was 8.1% ± 1.2% at low-density and 11.6% ± 1.0% at high-density. No significant difference was found at this timepoint (Student's T-test, P > 0.05, n = 5). These results suggest that cell-cell contact is required 1-24 hrs after cell cycle exit for pan bipolar cell specification.

We then quantified the number of cells expressing mGluR6 out of all Cre+ & EdU+ cells (Figure 13, D; Table 5). At the 1-hour timepoint the average proportion of cells expressing mGluR6 was 0 at low-density and 4.3% ± 2.7% at high-density. The difference between these two conditions was not significant (Student's T-test, p > 0.05, n = 5). At the 24-hour timepoint the average proportion of mGluR6+ was 0 at low-density and 20.9% ± 5.7% at high-density. This difference was significant (Student's T-test, p = 0.006511, n = 5). Lastly for the 48-hour timepoint the average proportion of cells expressing mGluR6 out of all Cre & EdU cells was 10.2% ± 2.9% at low-density and 11.7% ± 1.5% at high-density. No significant difference was found between the two culture conditions (Student's T-test, p > 0.05, n = 5).

Quantification of cells expressing PKCα as a proportion of all Cre+ & EdU+ cells was performed (Figure 13, E; Table 5) revealed that at the 1-hour timepoint the proportion of cells expressing PKCα was 0 at low-density and 3.3% ± 0.14% at high-density. The difference between the two culture conditions was significant (Student's T-test, p = 0.047684, n = 5). At the 24-hour timepoint the average proportion of cells expressing PKCα was 0 at low-density and 4.6% ± 1.2% at high-density. This difference was statistically significant (Student's T-test, p = 0.005090, n = 5). Lastly for the 48-hour timepoint the average proportion of cells expressing PKCα was 6.4% ± 4.5% at low-density and 6.0% ± 1.7% at high-density. This difference was not significant (Student's T-test, p > 0.05, N = 5).

Quantification of cells expressing VSX1 as a proportion of Cre+ & EdU+ cells as well as Cre- & EdU+ cells was performed (Figure 13, F; Table 5). Cre- & EdU+ cells were added to the denominator because BCs expressing VSX1 have low expression of VSX2, which is often at the threshold for detection by immunolabeling and would result in VSX1+ VSX2-Cre-cells (Shekhar et al. 2016). For the 1-hour timepoint the average proportion of cells expressing VSX1 was 0 at low-density and 17.8% ± 4.1% at high-density. This difference was significant (Student's T-test, $p = 0.002378$, $n = 5$). At the 24-hour the average proportion of cells expressing VSX1 was 0 for low-density and 10.7% ± 2.0% for high-density. This difference was significant (Student's T-test, $p = 0.0007$, $n = 5$) At the 48-hour timepoint the average proportion of cells expressing VSX1 was 3.1% ± 2.0 for low-density, and 6.4% ± 1.3% for the high-density condition. The difference was not significant (Student's T-test, $p > 0.05$, $n = 5$).

In summary, the average proportion of both pan bipolar cell marker expression (Vsx2-Cre) and subtype-specific markers (mGluR6, PKCα, and VSX1) was much lower in low-density conditions than in high-density conditions at the 1- and 24-hour timepoint. These results suggest there is a critical timepoint somewhere in between 1 and 48 hrs after cell cycle exit in which postmitotic cells require cell-cell signaling for both pan-BC and bipolar-subtype specification. Additionally, due to the difference in between Vsx2-Cre expression and subtype-specific markers at both the 1 and 24 hr timepoints, these results suggest pan-bipolar specification and bipolar-subtype specification are two different mechanisms (Figure 15).

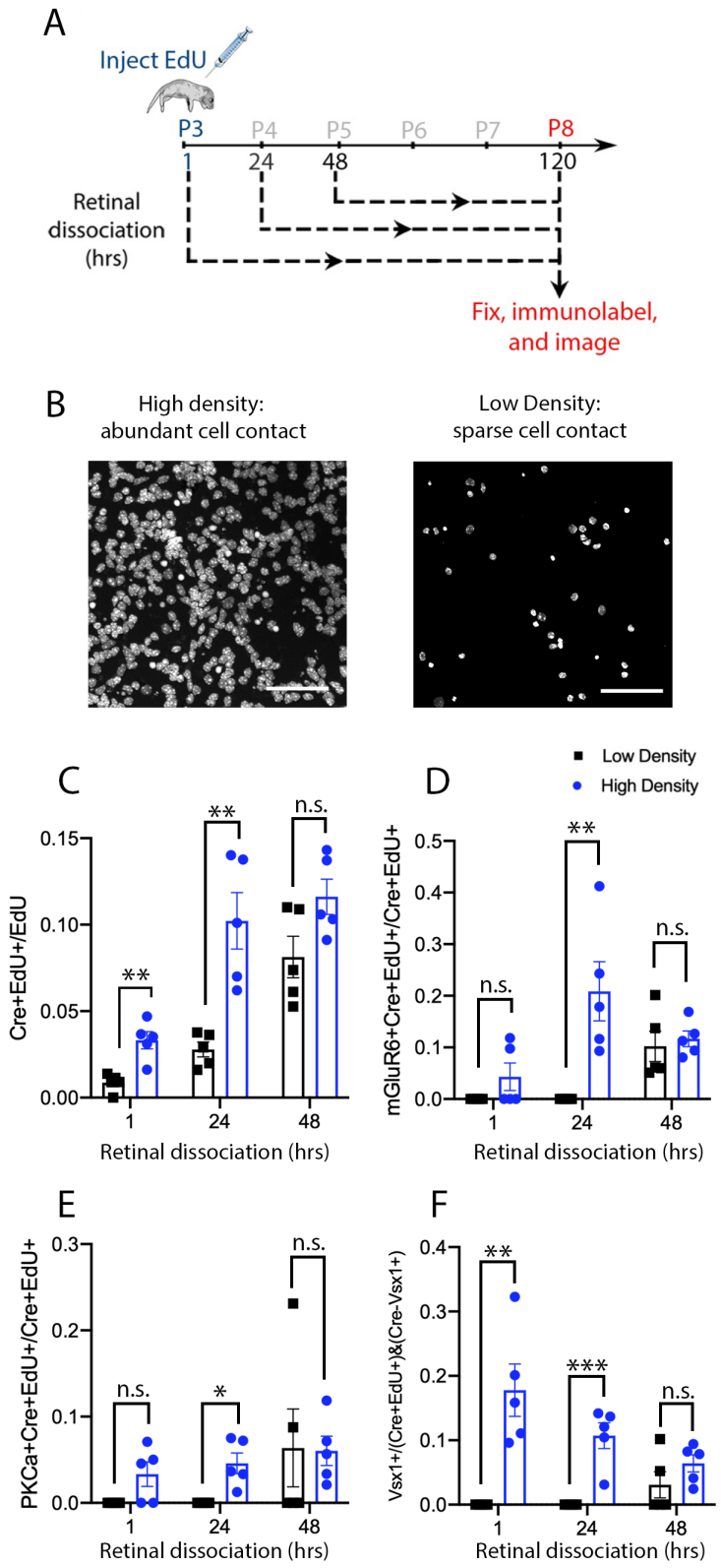

Figure 13 Cell contact is required for bipolar cell marker expression

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Figure 13 Cell contact is required for bipolar cell marker expression
(A) Schematic of experimental procedure. P3 mice were injected with EdU; retinal dissociation was performed 1, 24 and 48 hours after injection. Dissociated retinal cells were cultured until "P8" or 120 hours after injection. Cells were fixed, immunolabeled and analyzed. **(B)** DAPI stained representative images of the two experimental conditions used. High density condition shows abundant cell contact. In contrast, low density cell condition shows sparse cell contact. **(C-F)** Proportion of cells expressing Cre out of all EdU+ cells (C). Proportion of cells expressing mGluR6 (D) and PKCα (E) out of all Cre+ & EdU+ cells. And the proportion of cells expressing VSX1 out all Cre+ & EdU+ cells, as well as Cre-, EdU+ cells (F). n = 5 per time point. Error bars = SEM. Scale bars: 50 μm. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant.

3.2.5 The retinal environment is required for bipolar cell subtype specification

The extrinsic non-cell autonomous factors that are predicted to be present in the high-density condition, based on the experiments above, could be exclusive to the retinal environment, or they could be present in other cell types. There are hundreds of unique cell surface proteins and secreted molecules in the retina with the potential to have an effect in our high-density cultured cells (Visser et al. 2015). In trying to determine the nature of an extrinsic factor that promotes bipolar subtype development it would be helpful to know whether it involves a factor that is specific to the retinal environment. We chose to co-culture dissociated retinal cells with high-density human embryonic kidney 293T (HEK293T) cells to address this. HEK293T cells are a human-derived cell line widely used throughout biomedical research for their transfectability and cell culture growth feasibility (Graham et al. 1977; Stillman & Gluzman 1985).

The 24-hour timepoint was chosen for these experiments because it is when we observed the largest difference in marker expression between the low- and high-density conditions (Figure 13, C-F). Three conditions were assigned for these co-culture experiments: 1) Low-density dissociated cells alone (L.D.), 2) L.D. co-cultured with EdU-negative dissociated retinal cells (at high-density) from sibling mouse (Retina H.D.), and 3) L.D. co-cultured with high-density HEK293T cells (HEK293T H.D.). If a molecule(s) exclusive to the retinal environment is

responsible for the increase in pan bipolar specification, we would expect to see an increase in marker expression in condition 2) but not in condition 3).

We quantified the proportion of cells expressing Cre & EdU out of all EdU+ cells (Figure 14, B; Table 6). The average proportion of cells expressing Cre was 4.8% ± 0.89% for L.D., 10.8% ± 1.3% for Retina H.D. and 2.5% ± 0.88% for HEK293T H.D. The difference between the L.D. and Retina H.D. conditions was statistically significant (Student's T-test, $p = 0.0003$, $n = 5$). The difference between L.D. and HEK293T H.D. conditions was not significant (Student's T-test,>0.05, $n = 5$).

We quantified the proportion of cells expressing mGluR6, Cre and EdU out of all Cre+ & EdU+ cells (Figure 14, C; Table 6). The average proportion of cells expressing mGluR6 was 0.6% ± 0.6% for L.D., 10.5% ± 3.7% for Retina H.D., and 3.8% ± 3.8% for HEK293T H.D. The difference between the L.D. and Retina H.D. conditions was statistically significant (Student's T-test, $p = 0.0352$, $n = 5$). The difference between L.D. and HEK293T H.D. conditions was not significant (Student's T-test, $p > 0.05$, $n = 5$). Overall, these results suggest the retinal environment possess the right extrinsic signals that are not present in a non-neural cell line, to promote pan-BC and BC-subtype specification.

3.2.6 Summary

The role of non-cell autonomous signals on the specification of retinal bipolar cell subtypes is an understudied field. In these experiments, our aim was to determine whether extrinsic signals play a role in the specification of bipolar subtypes by dissociating EdU-injected retinal cells and culturing them at different cell densities until maturity. Our results suggest pan-bipolar specification requires a non-cell autonomous signal within 24-48 hrs after cell cycle exit (summarized in Figure 15). Furthermore, our experiments showed no cells expressing bipolar-subtype-specific markers at the 1 and 24 hr timepoints, suggesting the specification of subtypes requires non-cell autonomous signals that are independent of those involved in pan-bipolar specification. Lastly, our data suggests that by 48 hrs after cell cycle exit, bipolar cell subtype specification is mostly stablished since there was no difference in bipolar cell marker expression for the two different culture conditions at this timepoint. These experiments are interesting in that they reveal a novel bipolar cell type which we proposed is an undifferentiated postmitotic bipolar cell precursor intermediate. To our knowledge, this is the first experimental evidence for

the existence of such cell type *in vitro*. Finally, our co-culture experiments suggest the retinal environment has specific required molecular properties to allow the specification of bipolar subtypes.

Table 5 Total cell number – dissociated retinal cells cultured at high and low density.

	Cre+EdU+/EdU+		
Retinal dissociation (hrs)	1	24	48
Low Density	50/5911	297/9432	878/10821
High Density	256/7684	1637/16025	1521/13189
	Low Density: Cre+EdU+Marker+/Cre+EdU+		
Retinal dissociation (hrs)	1	24	48
mGluR6	0/18	0/121	32/310
PKCa	0/10	0/87	28/275
	High Density: Cre+EdU+Marker+/Cre+EdU+		
Retinal dissociation (hrs)	1	24	48
mGluR6	3/76	121/582	52/446
PKCa	3/103	28/604	28/459
	Vsx1+/(Cre+EdU+)&(Cre-Vsx1+)		
Retinal dissociation (hrs)	1	24	48
Low Density	0/22	0/89	10/317
High Density	17/93	52/483	41/641

Table 6 Total cell number – dissociated retinal cells co-cultured under different conditions.

Co-culture experiments			
	L.D.	Retina H.D.	HEK293T H.D.
Cre+EdU+/EdU+	69/1613	175/1682	38/1468
mGluR6+Cre+EdU+/Cre+EdU+	1/69	18/175	1/38

Table 7 Total cell number – dissociated retinal cells cultured with or without DAPT.

DAPT - 1 hr. post injection		
	DMSO	DAPT
Cre+EdU+/EdU+	439/8424	266/7428
mGluR6+Cre+EdU+/Cre+EdU+	31/138	16/87

PKCa+Cre+EdU+/Cre+EdU+	2/153	2/100
Vsx1+/(Cre+EdU+)&(Cre-Vsx1+)	58/162	38/89
DAPT - 24 hr. post injection		
	DMSO	DAPT
Cre+EdU/EdU+	2551/22868	2811/18684
mGluR6+Cre+EdU+/Cre+EdU+	303/894	103/437
PKCa+Cre+EdU+/Cre+EdU+	25/766	72/1377
Vsx1+/(Cre+EdU+)&(Cre-Vsx1+)	326/941	260/1112

Table 8 Total cell number – dissociated retinal cells cultured with Cyclopamine and/or DAPT.

24 hrs after EdU injection	DMSO	Cyclopamine	Cyclopamine & DAPT
Cre+EdU/EdU+	886/10737	730/12448	358/12091
mGluR6+Cre+EdU+/Cre+EdU+	37/231	33/222	7/89
PKCa+Cre+EdU+/Cre+EdU+	15/262	4/291	1/114
Vsx1+/(Cre+EdU+)&(Cre-Vsx1+)	73/412	47/241	32/163

Table 9 Total cell number – dissociated retinal cells cultured with Dll1&4-coated coverslips and/or Purmorphamine.

24 hrs after EdU injection	DMSO	Dll1&4	Purmorphamine	Dll1&4, Purmorphamine
Cre+EdU/EdU+	1494/13848	1490/13959	1502/12886	2059/17810
mGluR6+Cre+EdU+/Cre+EdU+	193/816	187/558	115/658	333/1025
PKCa+Cre+EdU+/Cre+EdU+	9/377	24/424	11/290	14/235
Vsx1+/(Cre+EdU+)&(Cre-Vsx1+)	91/544	107/539	113/591	366/1366

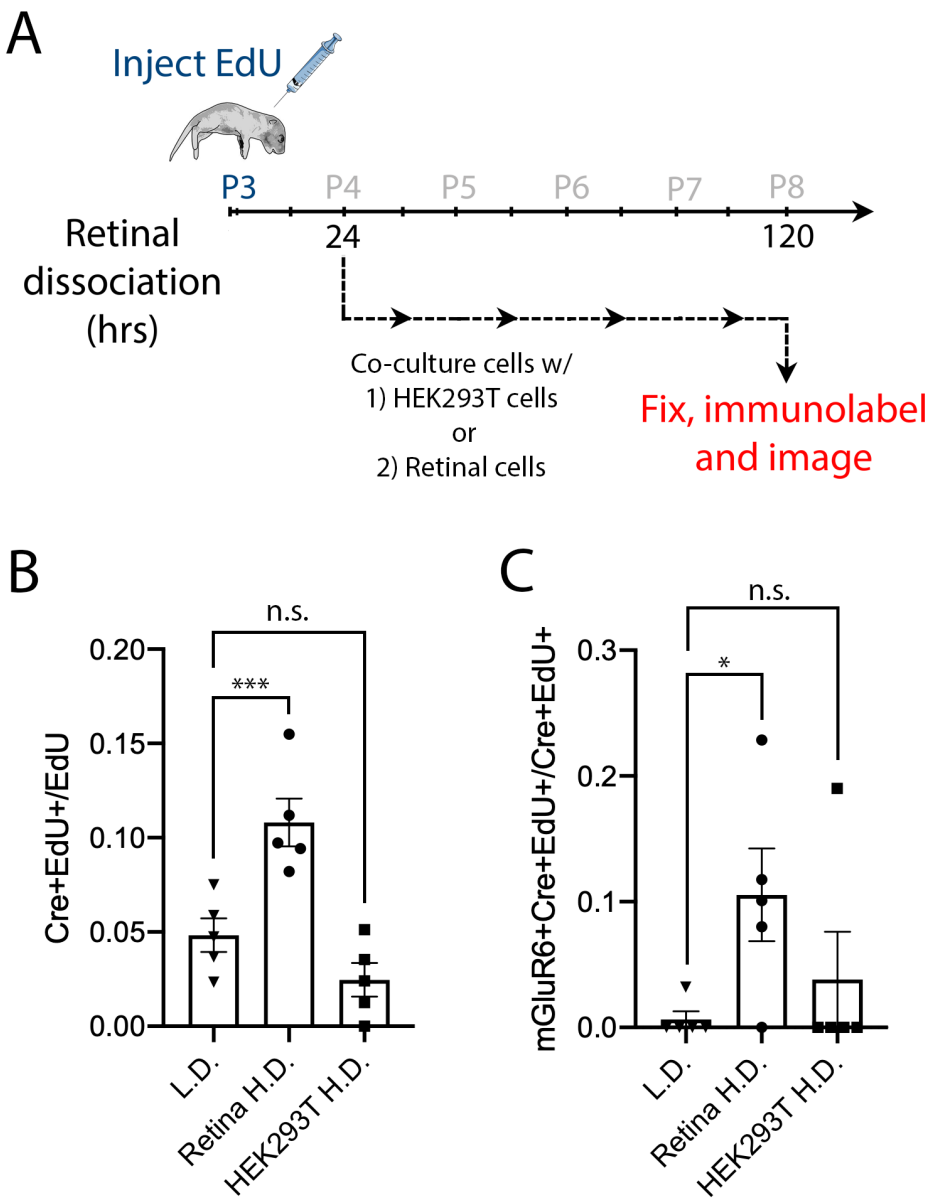

Figure 14 Retinal environment is required for bipolar cell subtype specification.

(A) Schematic of experimental procedure. P3 EdU-injected dissociated retinal cells were cultured at low density alone (L.D.), co-cultured with EdU-negative high-density dissociated retinal cells (Retina H.D.), and co-cultured with high-density HEK293T cells (HEK293T H.D.) **(B)** Quantification of the proportion of cells expressing Cre & EdU out of all EdU+ cells for the three different culture conditions. **(C)** Quantification of the proportion of cells expressing mGluR6, Cre & EdU out of all EdU+ cells for the three different conditions. N=5. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. Error bars = S.D. n.s. = not significant.

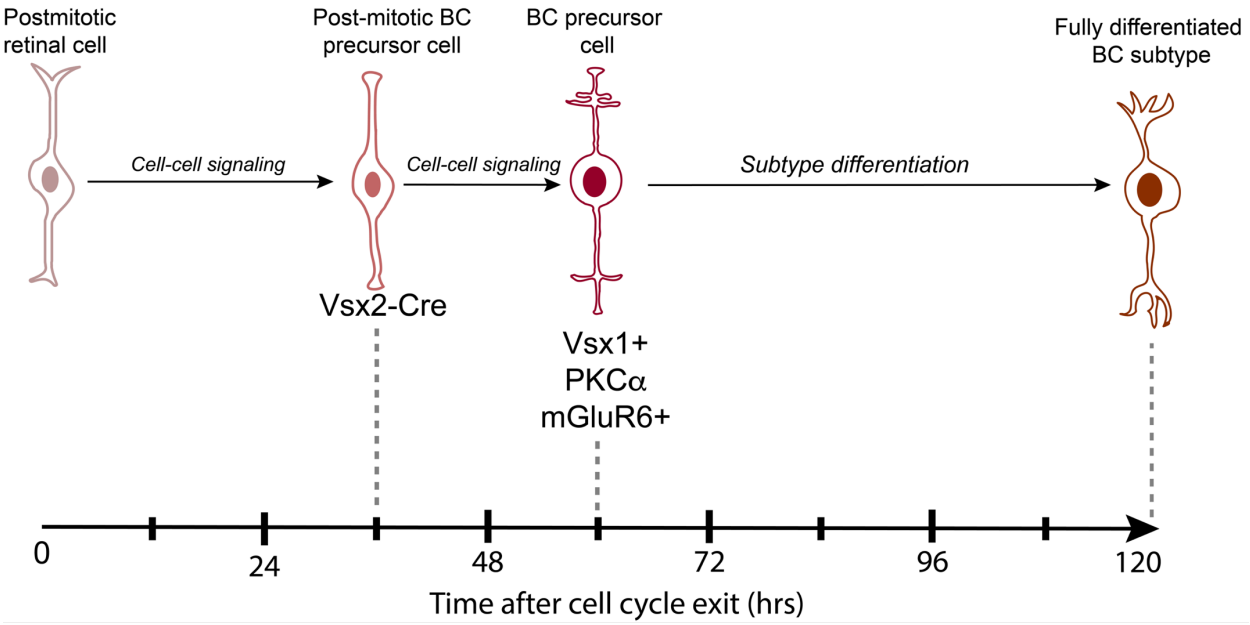

Figure 15 Cell signaling is required for bipolar cell subtype specification.

Summary model of results so-far. Vsx2-Cre expression is first seen 36 hours after cell cycle exit on a postmitotic bipolar cell precursor cell. Immunodetection of bipolar cell subtype-specific markers Vsx1, PKCα and mGluR6 is first seen ~60 hours after cell cycle exit in a bipolar cell precursor cell. Cell-cell signaling is required for the expression of all these markers. Further subtype differentiation occurs downstream of initial marker expression until subtype maturation.

Chapter 4 Notch and Shh signaling are partially required for bipolar cell subtype specification

4.0 Preface

Our results so far demonstrate that non-cell-autonomous mechanisms exclusive to the retinal environment are involved in pan and subtype specification of BCs, but the cellular mechanisms remain unknown. Our next aim was to investigate whether the Notch and/or the Sonic Hedgehog signaling pathways are involved. Their role in maintaining cell proliferation is well established (Jadhav et al. 2006; Hayes et al. 2007; Wang et al. 2005). But their function in retinal post-mitotic cells is controversial and understudied. We took advantage of various chemical compounds available to perform experiments involving the gain and loss of function of these pathways in vitro. The experimental approach was identical to our previous one: culturing P3-EdU-injected dissociated retinal cells at different timepoints after injection under different culture conditions.

4.1 Is the Notch signal transduction pathway involved in the specification of bipolar cell subtypes?

4.1.1 Notch signaling mechanism of action

The mechanism of action behind the canonical Notch cell-cell signaling pathway involves the extracellular domain of the ligands (*Delta, Jagged/Serrate and Lag)* expressed on one cell interacting with the extracellular domain of the Notch receptor on an adjacent cell (Figure 16). Work done in *C. elegans* and *Drosophila* showed that this ligand-receptor binding triggers the γ-secretase-mediated proteolytic cleavage of the intracellular domain of Notch (NICD) which then translocases to the nucleus to alter gene expression (Lieber et al. 1993; Struhl et al. 1993). In mammals, the transcription factor CBF1/RJBk is the major downstream nuclear effector of the Notch signaling pathway and activates the expression of bHLH transcription factors such as HES and HEY which in turn regulate other target genes that affect development (Bailey & Posakony 1995).

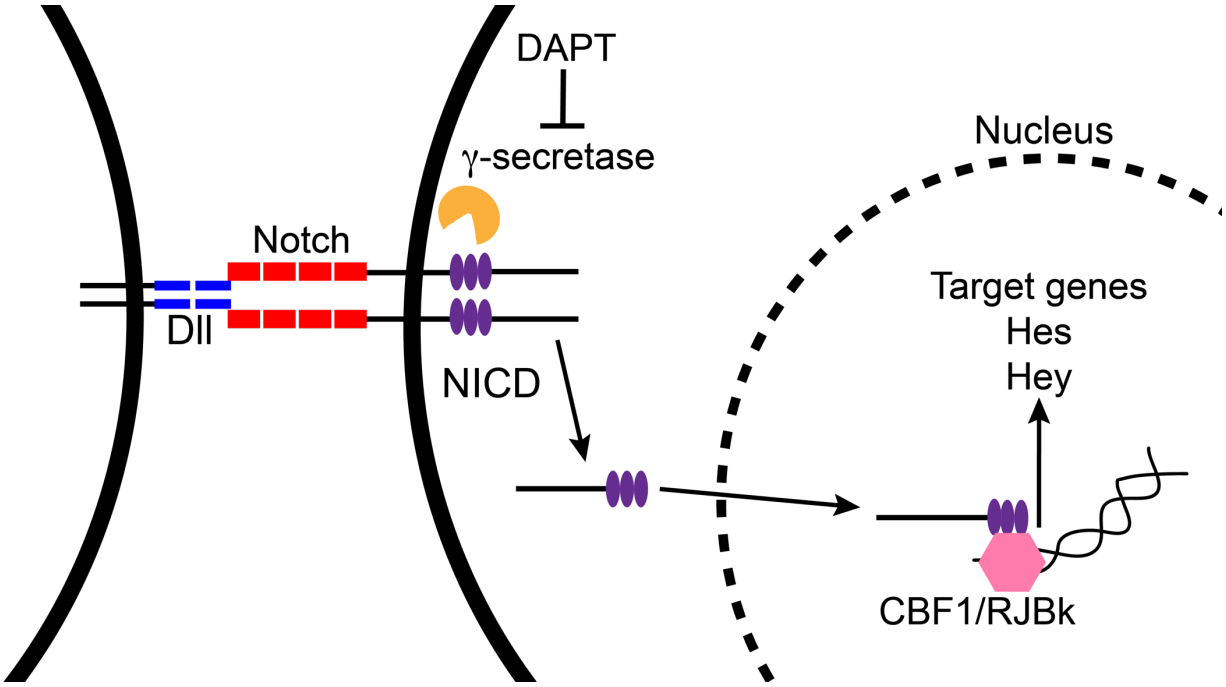

Figure 16 Notch-mediated cell signaling between adjacent cells

Notch-mediated cell signaling involves the Notch receptor interacting with its ligand Delta (Dll). Upon ligand-receptor activation, Notch Intracellular Domain (NICD) is created via proteolytic cleavage by γ-secretase, which is the target of Notch signaling inhibition via DAPT. NICD translocates to the nucleus where it activates the usually repressed transcription factor CBF1/RJBk which further regulates target genes.

4.1.2 Notch expression in the retina

In vertebrates, there are four different types of Notch receptors (Notch1-4) and two families of ligands (Delta and Jagged). The receptors *Notch1*, *Notch2, Notch3* and the ligands *Delta-1, Delta-3 ,Delta-4,* and *Jagged-2* are genes expressed in the developing mouse retina (Lindsell et al. 1996; Rocha et al. 2009; Ha et al. 2017; Bao & Cepko 1997; Zhu et al. 2013). *Notch1* and *Notch2* expression is retained in the adult retina exclusively in Muller glial cells (Zhu et al. 2013; Furukawa et al. 2000). Based on in situ hybridization (ISH) experiments, mRNA expression of Notch and its ligands in the retina is first detected at approximately embryonic day 14 (E14) and continues being expressed until P10 (Bao & Cepko 1997). Usually,

Notch signaling occurs reciprocally between RPCs but some Notch signaling components are also weakly expressed by retinal pigment epithelium cells and retinal ganglion cells; suggesting the possibility of an alternative Notch signaling mechanism (Rocha et al. 2009; Nelson et al. 2009; Ha et al. 2017).

4.1.3 The role of Notch signaling during retinal cell fate specification

The first hint at the role of Notch signaling in retinal development originates from studies performed in *Xenopus.* Transfection of an activated form of *Xotch* (the *Xenopus* version of Notch) into isolated retinal cells causes cells to retain RPC morphology, suggesting Notch plays a role in preventing cell differentiation (Dorsky et al. 1995). Retroviral transduction of activated *Notch1* in RPCs leads to expression of Muller glial (MG) cell markers, suggesting it is involved in MG specification (Furukawa et al. 2000). In the embryonic retina, NICD-mediated overexpression of Notch1 exclusively in RPCs via Cre recombinase promoted the specification of temporally appropriate RPCs, in contrast, when Notch1 was overexpressed in the postnatal retina, it led to an overproduction of glial-looking cells with abnormal gene expression (Jadhav et al. 2006). These results suggest Notch1 signaling has a role in maintaining RPC identity as well as MG specification.

The functional role during retinal development of Notch1 extends beyond maintaining a progenitor state and gliogenesis. In mice without *Hes1* (one of the Notch signaling effectors), there was a premature generation of rod photoreceptors and a loss of bipolar cells, indirectly suggesting Notch signaling might be involved in inhibiting the rod photoreceptor fate and specifying bipolar cells (Tomita et al. 1996b). Additionally, Notch1 inactivation in RPCs results in a dramatic increase in the number of cone photoreceptors and an increase in bipolar cell numbers, suggesting Notch1 signaling functions in cell fate determination in addition to its role in maintaining the progenitor state (Yaron et al. 2005; Jadhav et al. 2006). Exclusive inactivation of Notch1 in postnatal postmitotic retinal cells resulted in a higher proportion of rod photoreceptors and a decrease in the number of bipolar cells, suggesting Notch signaling might play a role in a rod versus bipolar cell fate decision process by inhibiting photoreceptor cell fate and/or promoting bipolar cell fate (Mizeracka et al. 2013).

4.2 Is the Hedgehog signal transduction pathway involved in the specification of bipolar cell subtypes?

4.2.1 Sonic Hedgehog signaling mechanism of action

In vertebrates, the canonical Hedgehog (Hh) signaling transduction pathway is initiated by the binding of Sonic Hedgehog (Shh) to the 12-transmembrane receptor Patched1 (Ptc1), releasing the inhibition of the 7-transmembrane protein Smoothened (Smo) and triggering the activation of the Gli transcription factors, the transcriptional effectors of Shh signaling (Ho et al. 2002, Figure 17). In the absence of Shh, Gli is supressed by the protein Sufu (Suppressor of fused homolog) by directly binding to the Gli transcription factors and anchoring them to the cytoplasm. Activated Smo prevents the degradation of Gli, and once activated, it translocates to the nucleus and functions as a transcription activator of target genes (PTCH1, CycD1, Myc, Bcl-2, NANOG, SOX2) involved in proliferation, apoptosis, suppression, and stem-cell renewal (Rimkus et al. 2016).

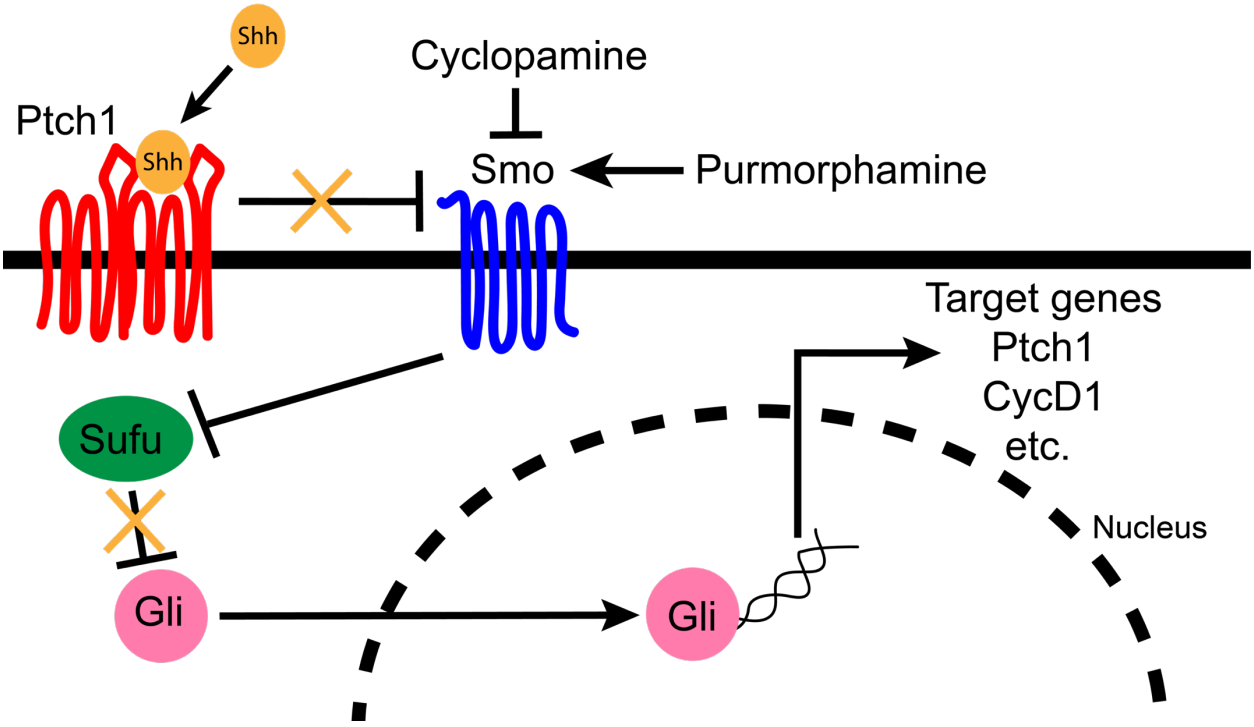

Figure 17 Hedgehog signaling pathway in vertebrates

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Figure 17 Hedgehog signaling pathway in vertebrates

The Hh ligand (Shh for vertebrates) binds to the 12-transmembrane receptor Ptch1, releasing the inhibition of the 7-transmembrane protein Smo which then inhibits Sufu, preventing it from sequestering Gli in the cytopplasm, which then translocates to the nucleus and works as a transcription activator to upregulate the expression of target genes involved in various developmental processes such as embryogenesis and tissue patterning. Inhibition of the Shh signaling pathway by cyclopamine occurs when it inhibits Smo, whereas activation of the Shh pathway occurs through the activation of Smo by purmorphamine.

4.2.2 Sonic Hedgehog expression in the retina

The components of the Hh signaling pathway are expressed in the mouse retina during development and in adults, and this has been documented thoroughly (Jensen & Wallace 1997; Wallace 2008). Both ISH and western blotting analyses have demonstrated Shh expression is detected as early as E14.5, throughout development, and maintained in the mature retina. By E17 onwards, ISH Shh signal is localized to cells in the developing ganglion cell layer, suggesting retinal ganglion cells (RGCs) are the main source of Shh in the retina. Ptc1+, Smo+, and Gli+ Shh-responding cells are RPCs located in the neuroblast layer during development and in the INL during postnatal retinal development (Jensen & Wallace 1997; Ringuette et al. 2016). In the adult retina Shh continues to be expressed in RGCs, and most of the Shh-responding cells are Muller glia cells based on immunohistochemical analysis (Wang et al. 2002). The function of this RGC to Muller glial cell interaction in the adult has not been fully explored yet. Additionally, electroporation experiments showed that the expression of Hh signaling downstream components are deactivated 36 hrs after cell cycle exit in postmitotic bipolar cells (Dee Wu, unpublished data, Chow lab), suggesting if Shh signaling plays a role on bipolar cell subtype specification, it must be taking place within 36 hrs after cell cycle exit.

4.2.3 The role of Sonic Hedgehog signaling in retinal cell fate specification

Hh signaling has a well-established role as a mitogen in RPC development in mouse retina, as mentioned in section 1.4.2.2 Extrinsic regulators of RPC competence", but it also has been implicated in retinal cell fate specification. (Waid & McLoon 1998; Wang et al. 2005;

Wallace 2008). Co-culture of younger retinal cells with older retinal cells demonstrated increasingly older retinal cells inhibited RGC production, suggesting there is a secreted factor inhibiting self-production (Waid & McLoon 1998). Shh is thought to be involved in this negative feedback mechanism; conditional Shh inactivation in mouse retinas increases RGC production and its overexpression results in the depletion of RGCs (Wang et al. 2005; Zhang & Yang 2001). Shh promotes the production of INL cells; ectopic cell-autonomous expression of SmoM2, a constitutively active form of Smo, results in an increase in the number of bipolar cells, amacrine and Muller glial cells (Yu et al. 2006). Additionally, conditional inactivation of Shh leads to a higher rate of photoreceptor differentiation (Yu et al. 2006). Shh signaling could also influence bipolar cell subtype specification in establishing rod vs cone subtypes. Examination of mice with depleted numbers of RGCs showed a significant decrease in the number of cone bipolar cells and no effect on rod bipolar cells, suggesting Shh could play a role in the specification of bipolar cell subtypes (Bai et al. 2014). However, whether these observed phenotypes are a consequence of its mitogenic role in RPCs or a more specific role in cell fate (e.g. an effect on postmitotic cells) remains unknown.

4.3 Loss of Notch signaling 1 hr after cell cycle exit decreases pan bipolar cell specification

DAPT (*N*-[*N*-(3,5 -diflurophenacetyl)-1-alanyl]-*s*-phenylglycinet-butyl ester) is a chemical compound that indirectly blocks Notch activity by preventing the formation of the Notch intracellular domain (NICD, a critical component of the pathway) by inhibiting γ-secretase (Geling et al. 2002, Figure 16). It has been used in retinal development research as a supplement to retinal explants in culture (Nelson et al. 2006; Hayes et al 2007; Nakamura & Chiba 2007; Ringuette et al. 2016). We used DAPT to observe the effect of blocking Notch activity in high-density cultured dissociated cells. If the Notch signaling pathway is required for pan or bipolar subtype specification, we would predict to see a decrease in bipolar cell marker expression under the presence of DAPT. P3 EdU-injected mice were cultured at high-density 1 hour after injection until bipolar cell maturity (120 hours after injection) with or without DAPT (Figure 18, A). Although Notch signaling in postmitotic cells has been implicated in the bipolar versus photoreceptor cell fate decision (Mizeracka et al. 2013), its potential postmitotic role in bipolar cell subtype specification is unknown. We quantified the proportion of cells expressing bipolar

cell markers when dissociated retinal cells were cultured with or without 10 μM of DAPT, 1 hour after EdU injection at P3. 10 μM of DMSO was used as a vehicle control. The proportion of dissociated retinal cells expressing Cre out of all EdU+ cells was 5.03% ± 0.26% with DMSO and 3.32% ± 0.36% with DAPT (Figure 18, B; Table 7). The difference between these two culture conditions was significant (Paired Student's T-test, p = 0.0160, n = 5). The proportion of cells expressing mGluR6 out of all Cre+ & EdU+ cells was 19.39% ± 3.80% with DMSO and 22.33% ± 3.96% DAPT (Figure 18, C; Table 7). This difference was not significant (Paired Student's T-test, p = 0.1514, n = 5). The proportion of cells expressing PKCα out of all Cre+ & EdU+ cells was 1.14% ± 0.71% with DMSO and 1.9% ± 1.9% with DAPT (Figure 18, D; Table 7). This difference was not significant (Paired Student's T-test, p = 0.6201, n = 5). The proportion of cells expressing VSX1 out of (Cre+&EdU+ and Cre-&EdU+) cells was 35.54% ± 2.55% with DMSO and 42.8 ± 3.41% (Figure 18, E, Table 7). This difference was not significant (Paired Student's T-test, p = 0.2572, n = 5). Overall, these results suggest the loss of Notch one hour after cell cycle exit decreases pan bipolar specification, in agreement with published findings, but does not play a role in bipolar subtype specification.

4.4 Loss of Notch signaling 24 hrs after cell cycle exit increases pan bipolar cell specification

In the previous experiment, DAPT inhibition of Notch was present for both RPCs and post-mitotic cells. To examine the role of Notch signaling specifically in early postmitotic cell bipolar cell precursors, we dissociated P3-EdU-injected retinas 24 hrs after EdU injection. If Notch signaling is required post-mitotically for either pan-BC or subtype specification, we would predict a change in bipolar cell subtype marker expression when cells are treated with DAPT.

The proportion of cells expressing Cre out of all EdU+ cells was 11.50% ± 1.08% with DMSO and 15.24% ± 0.92% with DAPT (Figure 19, B; Table 7). This difference was small and unexpected but statistically significant (Paired Student's T-test, p = 0.0087). The proportion of cells expressing mGluR6 out of all Cre+ & EdU+ cells 33.87% ± 3.86% with DMSO and 23.44% ± 4.11% with DAPT (Figure 19, C; Table 7). This difference was not significant (Paired T-test, p = 0.0597, n = 5). The proportion of cells expressing PKCα out of all Cre+ & EdU+ cells was 3.30% ± 0.84% with DMSO and 5.21% ± 0.36% with DAPT (Figure 19, D; Table 7). This difference was not significant (Paired T-test, p = 0.0869, n = 5). The proportion of cells

expressing VSX1 out of (Cre+&EdU+ and Cre-&EdU+) cells was 34.44% ± 4.55% with DMSO and 23.33% ± 5.79% with DAPT (Figure 19, E; Table 7). This difference was not significant (Paired T-test, p = 0.1848, n = 5). Overall, these results suggest the loss of Notch in early post-mitotic cells increases pan bipolar cell specification but has no effect on bipolar cell subtype specification. However, our data trends suggest the loss of Notch signaling could still have an effect 24 hrs after cell cycle exit on the expression of all three bipolar cell subtype markers since the variability was high, and there are outliers (Figure 19, C, D, E). This is further discussed in section 5.3.

The increase seen in the proportion of Cre+EdU+ out of all EdU+ cells is unexpected since published findings suggest Notch is required postmitotically for bipolar cell specification by decreasing photoreceptor specification (Mizeracka et al. 2013). One would then expect the loss of Notch 24 hrs after exiting cell cycle to decrease the number of pan BCs, not increase. However, it is important to keep in mind that Vsx2-Cre expression does not always imply bipolar specification, especially given the known role of *Vsx1* on repressing *Vsx2.* This is further discussed in section 5.3 The role of Notch and Shh signaling on bipolar cell specification".

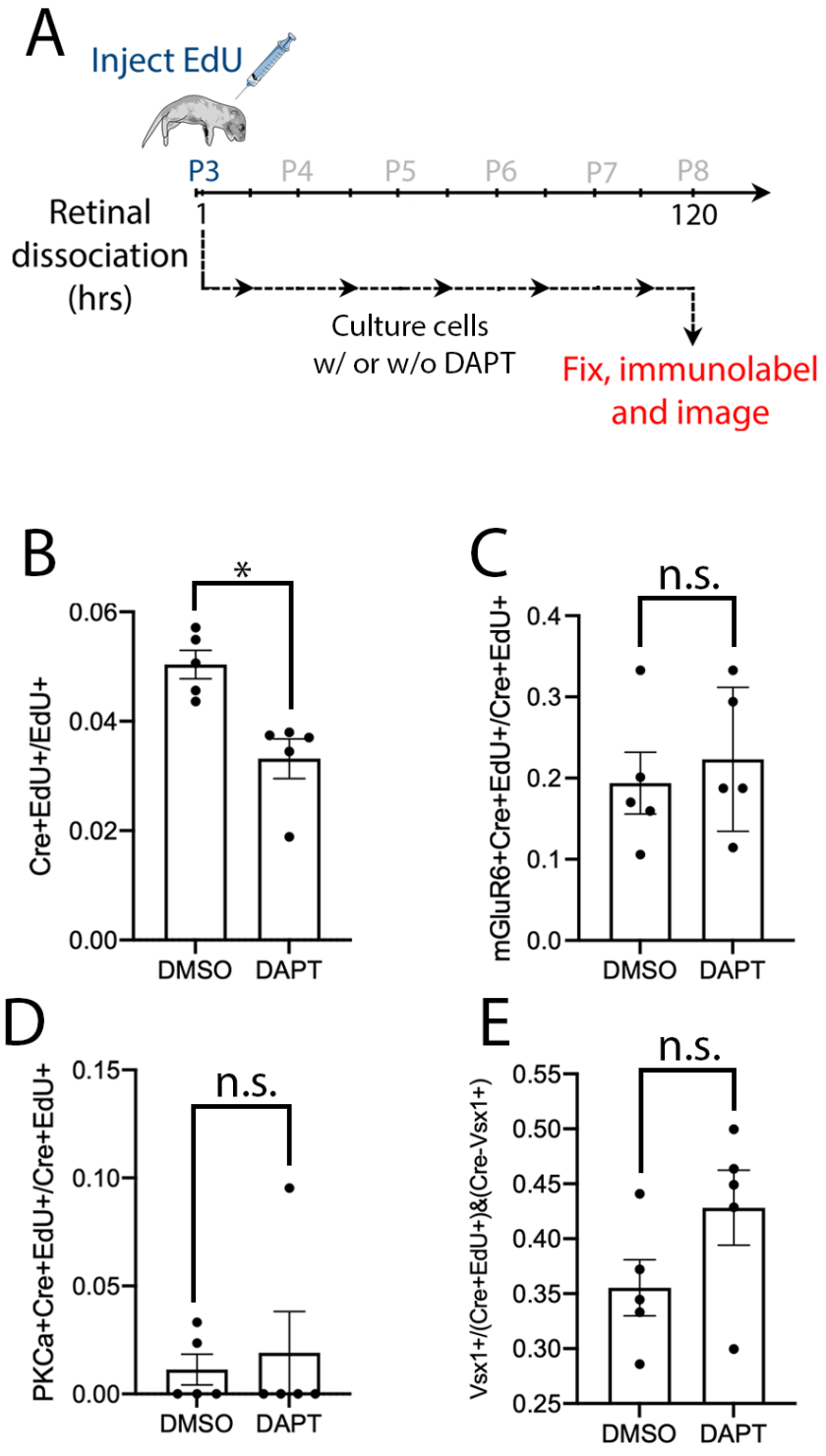

Figure 18 Loss of Notch 1 hr after cell cycle exit reduces pan bipolar cell specification

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Figure 18 Loss of Notch 1 hr after cell cycle exit reduces pan bipolar cell specification **(A)** Schematic of experimental procedure. P3 EdU-injected mice were dissociated 1 hour after injection and cultured at high-density with or without 10 μM of DAPT until "P8" (120 hours after injection). Dissociated cells were then fixed, immunolabeled and analyzed. **(B)** The proportion of cells expressing Cre and EdU out of all EdU+ cells under different culture conditions: DMSO (vehicle control) and DAPT. The proportion of cells expressing mGluR6 **(C)** and PKCα **(D)** out of all Cre+ & EdU+ cells under different culture conditions. **(E)** The proportion of cells expressing Vsx1 out of all Cre+ & EdU+ cells and Cre- EdU+ cells. N=5. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. n.s. = not significant. Error bars = SEM.

4.5 Loss of Notch and Shh signaling decreases pan bipolar cell specification in early post-mitotic cells

Our next goal was to inhibit both the Notch and Shh signaling to see if they have a specific or a redundant effect in bipolar subtype specification. We decided to use the steroidal alkaloid cyclopamine to block Shh signaling. Cyclopamine blocks Shh signaling by inhibiting the receptor Smoothened, a critical component of the Shh pathway (Chen et al. 2002; Figure 17). We treated dissociated retinal cells with 10 μM of cyclopamine and/or 10 μM of DAPT to observe the effects on bipolar subtype marker expression. Current evidence suggests Shh signaling plays a role in specifying cone bipolar cells (Bai et al. 2014; Wu 2017). However, whether this is an effect on progenitor cells or in post-mitotic cells remains controversial. To address this, P3-EdU-injected retinas were dissociated 24 hours after injection (to restrict effect seen to postmitotic cells) and cultured until "P8" (120 hours after injection). The proportion of cells expressing Cre & EdU out of all EdU+ cells was 8.30% ± 1.2% for DMSO treatment (control), 5.90% ± 1.00% for cyclopamine-only treatment and 3.00% ± 0.40% for cyclopamine and DAPT treatment (Figure 20, C). The only significant difference was between the cyclopamine and DAPT treated retinal cells (Paired Student's T-Test, $p = 0.0211$, $n = 5$). The proportion of cells expressing mGluR6, PKCα and VSX1 was not significant among all three culture conditions. These results suggest combined Shh and Notch signaling together are required for postmitotic cells 24 hrs after cell cycle exit for pan bipolar cell specification.

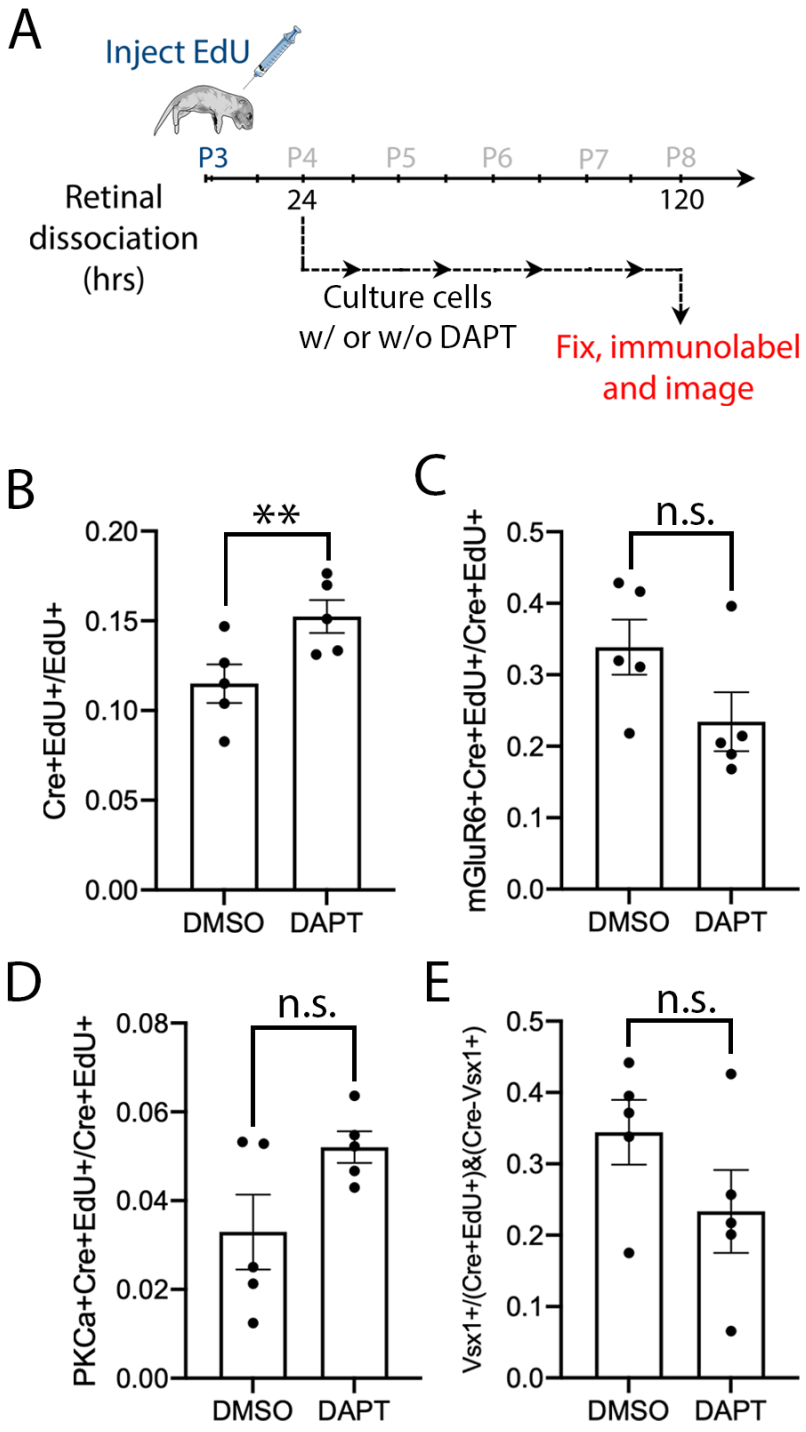

Figure 19 Loss of Notch signaling in early post-mitotic cells increases pan bipolar cell specification

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Figure 19 Loss of Notch signaling in early post-mitotic cells increases pan bipolar cell specification

(A) Schematic of experimental procedure. P3 EdU-injected mice were dissociated 24 hours after injection and cultured at high-density with or without 10 μM of DAPT until "P8" (120 hours after injection). Dissociated cells were then fixed, immunolabeled and analyzed. **(B)** The proportion of cells expressing Cre and EdU out of all EdU+ cells under different culture conditions: DMSO (vehicle control) and DAPT. The proportion of cells expressing mGluR6 **(C)** and PKCα **(D)** out of all Cre+ & EdU+ positive cells under different culture conditions. **(E)** The proportion of cells expressing Vsx1 out of all Cre+ & EdU+ cells and Cre-, EdU+ cells. N=5. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. n.s. = not significant. Error bars = SEM

4.6 Activation of Notch and Shh in early post-mitotic cells increases VSX1 expression.

To determine whether the activation of Notch and Shh increases bipolar cell subtype specification, we cultured P3-EdU dissociated retinal cells 24 hours injection until P8 (120 hours after injection). *Notch1* is the most prominent Notch receptor in the postnatal retina and its main ligands are *Delta-like 1* (*Dll1)*, *Dll4 and Jagged-2* (Bao & Cepko 1997). During retinal development, these genes are expressed by RPCs and activate the Notch signaling pathway via cell-cell contact among neighboring progenitors (Lindsell et al. 1996; Rocha et al. 2009; Ha et al. 2017). However, their role in post-mitotic cells is unclear. We decided to use coverslips coated with recombinant versions of the proteins DLL1 and DLL4 to activate Notch signaling in vitro. Purmorphamine is a purine derivative that activates the receptor Smoothend, a critical component of the Shh pathway that is normally repressed, therefore activating the signaling pathway (Sinha & Chen 2006; Figure 17). We decided to treat retinal cells with 10μM of purmorphamine and/or 5μg/mL of DLL1&4 to activate the Shh and the Notch pathway alone or simultaneously to see if bipolar cell subtype marker expression is affected in post-mitotic cells. There was no difference in Cre, mGluR6 and PKCα expression among the different experimental conditions (Figure 21, C, D, E). However, the proportion of VSX1+ cells out of [(Cre+EdU+) and (Cre-EdU+)] was 16.70% ± 1.9% for DMSO (control) and 26.80% ± 2.1%, this difference was significant (Student's p-test, $p = 0.0067$, $n = 10$). This result suggests these

pathways play a role in either VSX1 expression or bipolar cell subtype specification (Figure 21, F).

4.7 Summary

The objective of these experiments was to investigate the role of the Notch and the Shh signaling transduction pathway on the specification of bipolar cell subtypes. Our retinal dissociation/cell culture experimental approach allowed us to accurately isolate EdU-injected postmitotic retinal cells and test the effect of the loss/gain of these signaling pathways on the expression of bipolar subtype markers. Our results showed that simultaneous activation of the Notch and Shh signaling pathway 24 hrs after cell cycle exit increased the numbers of *Vsx1+* cells, suggesting these pathways play a role in the specification of bipolar cell subtypes 2,6 and 7 (Figure 21). Simultaneous inhibition of Notch and Shh decreased pan bipolar specification but had no effect on bipolar subtype specification (Figure 20). Unexpectedly and contrary to our Notch and Shh inhibition experiments, the removal of Notch signaling 24 hrs after cell cycle exit resulted in a small, yet statistically significant increase in the number of pan bipolar cells BCs (Figure 19). Altogether, these results suggest there is an important non-cell-autonomous component involving the Notch and Shh signaling in the specification of bipolar cell subtypes.

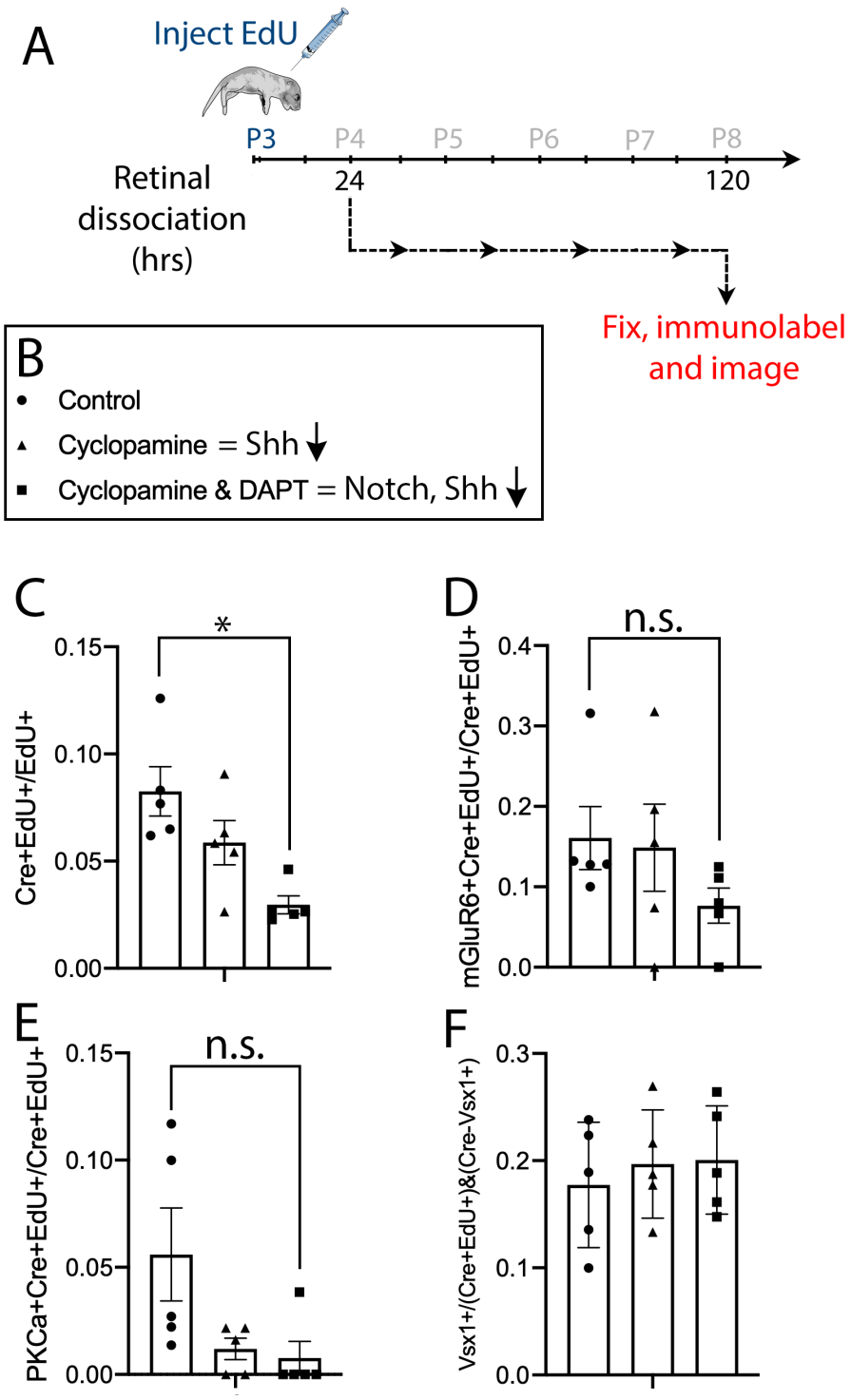

Figure 20 Loss of Notch and Shh signaling decreases pan bipolar cell specification in post-mitotic cells.

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Figure 20 Loss of Notch and Shh signaling decreases pan bipolar cell specification in post-mitotic cells.

(A) Schematic of experimental procedure. P3 EdU-injected mice were dissociated 24 hours after injection and cultured at high-density under different conditions until "P8" (120 hours after injection). Dissociated cells were then fixed, immunolabeled and analyzed. **(B)** List of culture conditions, their function, and key for bar graphs. **(C)** The proportion of cells expressing Cre and EdU out of all EdU+ cells under different culture conditions. Proportion of cells expressing mGluR6 **(D)** and PKCα **(E)** out of all Cre+ & EdU+ cells under different culture conditions. **(F)** The proportion of cells expressing Vsx1 out of all Cre+ & EdU+ cells and Cre-& EdU+ cells under different culture conditions. N= at least 5. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. n.s. = not significant. Error bars = SEM

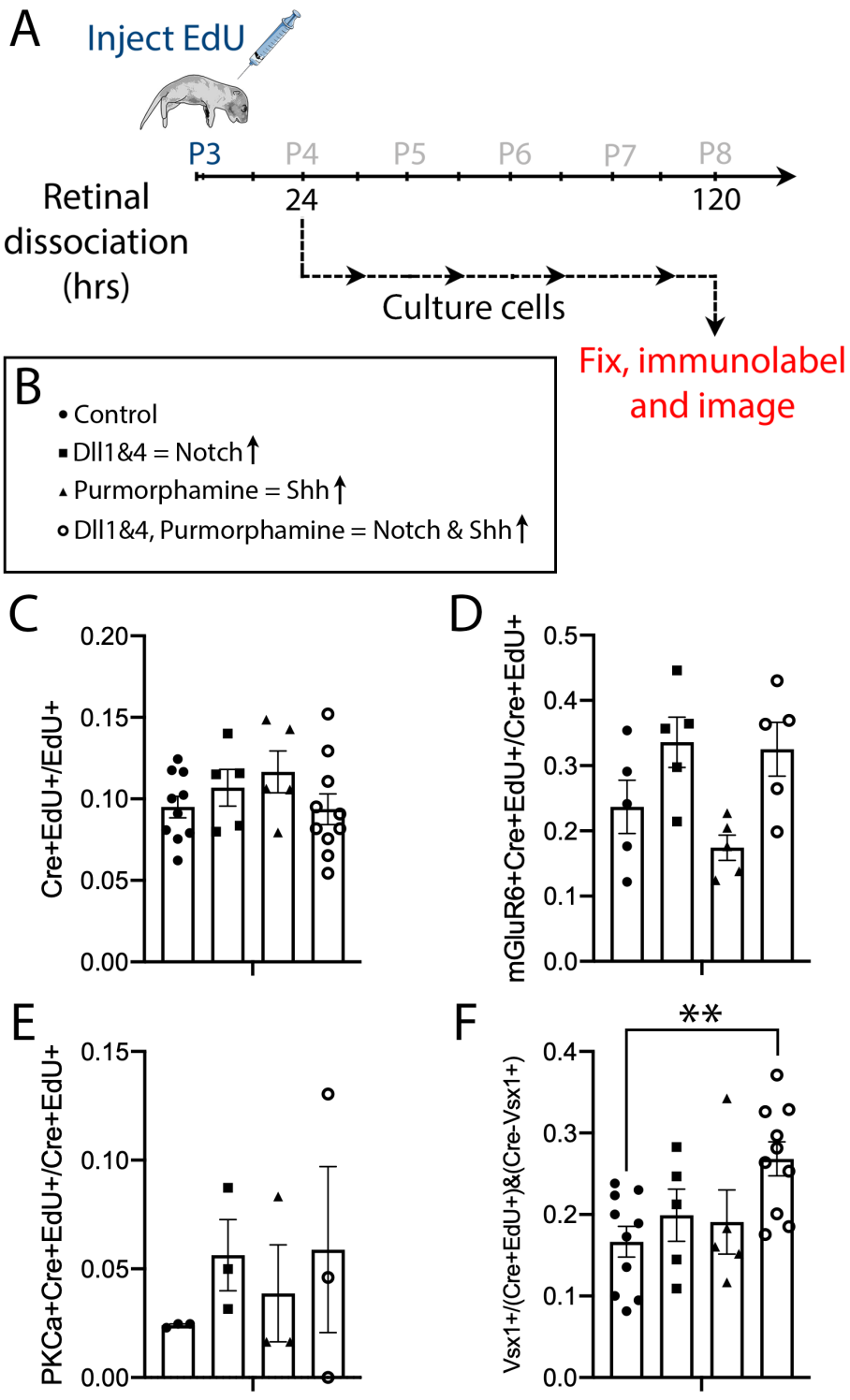

Figure 21 Activation of Notch and Shh signaling increases Vsx1 expression

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Figure 21 Activation of Notch and Shh signaling increases Vsx1 expression

(A) Schematic of experimental procedure. P3 EdU-injected mice were dissociated 24 hours after injection and cultured at high-density under different conditions until "P8" (120 hours after injection). Dissociated cells were then fixed, immunolabeled and analyzed. **(B)** List of culture conditions, their function, and key for bar graphs. **(C)** The proportion of cells expressing Cre and EdU out of all EdU+ cells under different culture conditions. Proportion of cells expressing mGluR6 **(D)** and PKCα **(E)** out of all Cre+ & EdU+ cells under different culture conditions. **(F)** The proportion of cells expressing Vsx1 out of all Cre+ & EdU+ cells and Cre- & EdU+ cells under different culture conditions. N= at least 5. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$. n.s. = not significant. Error bars = S.D.

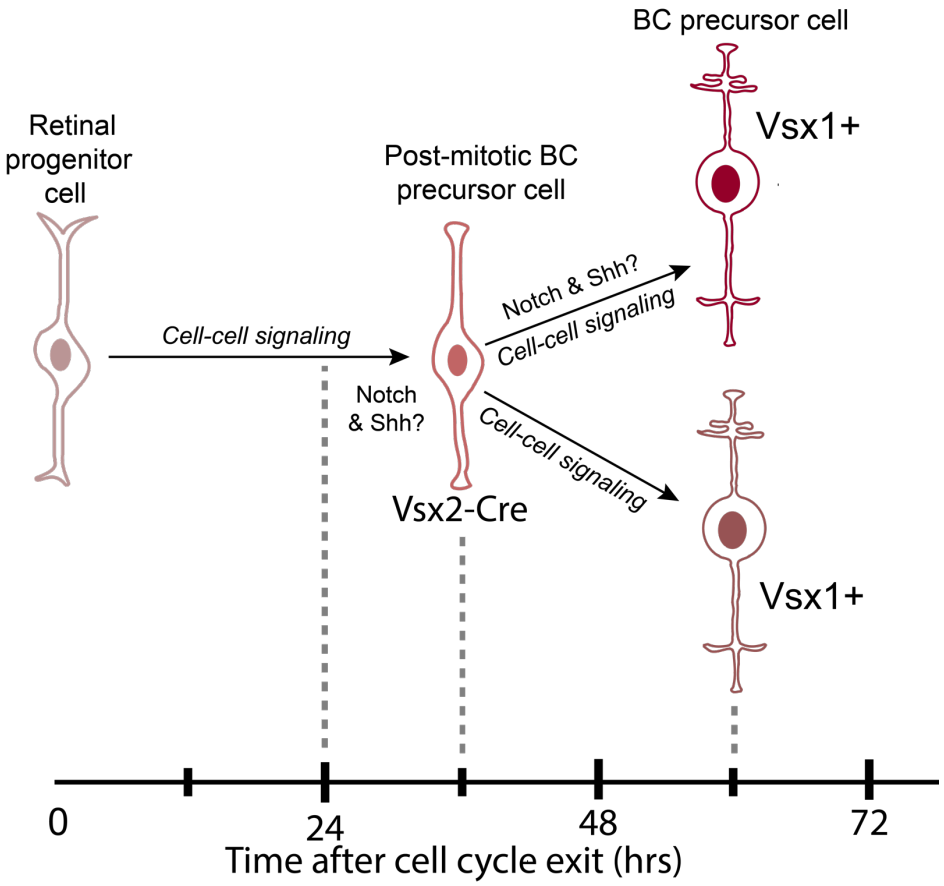

Figure 22 Summary of results

The mechanisms underlying bipolar cell subtype specification involve cell-cell signaling and potential Notch and Shh signaling. A post-mitotic bipolar cell precursor intermediate cell is first identifiable via Vsx2-Cre immunolabeling against Cre 36 hours after cell cycle exit. Bipolar cell markers mGluR6, PKCα and VSX1 are first seen by immunolabeling 48-60 hrs after cell cycle exit. Both mechanisms require cell-cell signaling. Notch and Shh signaling might be required 24 hours after cell cycle exit for Vsx2-Cre expression, and for VSX1 expression in post-mitotic cells.

Chapter 5 Discussion

The broad aim of my master's thesis study was to explore the mechanisms underlying the specification of the fifteen murine retinal bipolar cell subtypes. I developed a novel retinal dissociation cell culture approach using EdU-labeling of retinal progenitor cells to explore different aspects of bipolar cell subtype development. This approach enabled me to examine the model for a stepwise progression of bipolar cell subtype specification. My approach also allowed me to determine if the Notch and Hh signaling pathways played any role in bipolar cell subtype specification in postmitotic cells. My findings: (1) support the idea that bipolar cell subtype specification follows a multi-step mode, (2) provide evidence there is a critical cell-contact dependent period for bipolar cell subtype specification within 48 hrs after cell cycle exit, and (3) suggest that the Notch and the Hh signaling pathways play a role in bipolar cell subtype specification during this period. Here, I discuss my findings, interpret my results, and compare them with relevant literature.

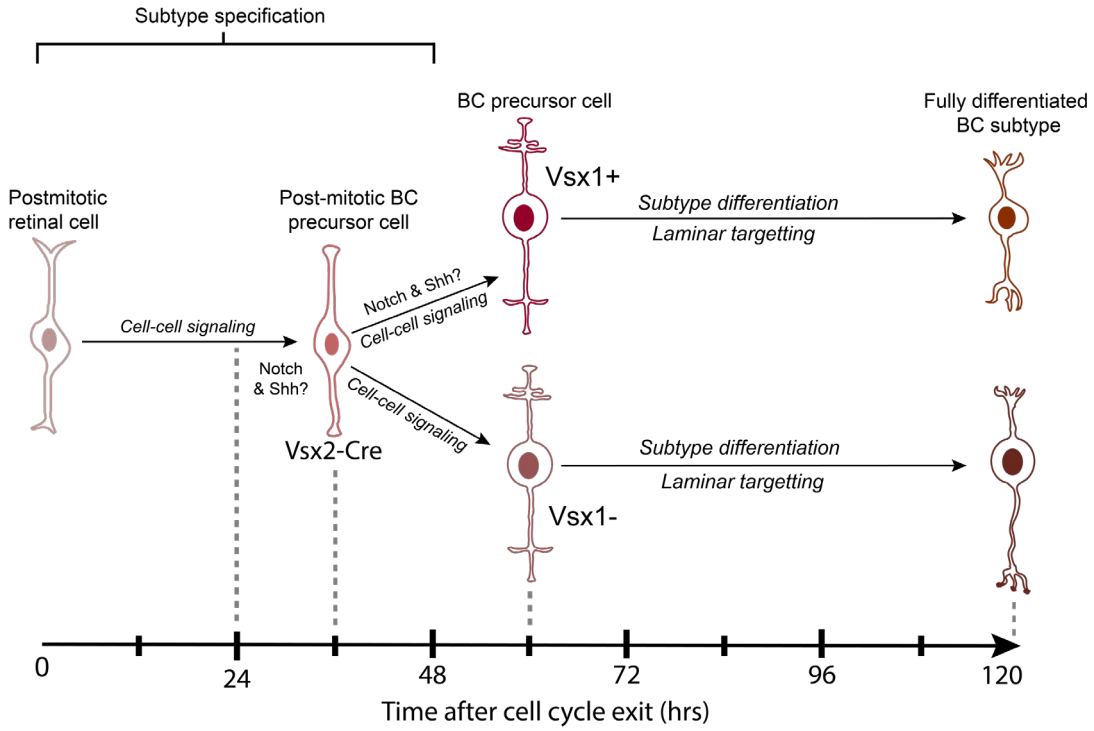

Figure 23 Summary of results: bipolar cell subtype specification involves cell-cell signaling and potential Notch and Shh signaling.

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Figure 23 Summary of results: bipolar cell subtype specification involves cell-cell signaling and potential Notch and Shh signaling.

Postmitotic bipolar cell precursor cell is first identifiable via Vsx2-Cre immunolabeling. It appears between 24 and 36 hours after cell cycle exit. Bipolar cell subtype-specific markers mGluR6, PKCα, and VSX1 appear after Vsx2-cre and are detected by immunolabeling at 48-60 hrs after cell cycle exit. Both mechanisms require cell-cell signaling. My data suggests Notch and Shh signaling are required in postmitotic cells 24 hours after cell cycle exit for Vsx2-Cre expression, and for VSX1 expression. By 48 hrs after cell cycle exit, bipolar cell subtype fate determination is at least partially complete.

5.1 The onset of expression of bipolar cell markers after cell cycle exit

5.1.1 Bipolar cell subtype specification as a multi-step mechanism

My first objective was to examine the onset of expression of four bipolar cell molecular markers after cell cycle exit to see whether the mechanism behind bipolar cell subtype specification occurs in a direct or a multi-step manner. Mice were injected at P3, the peak of bipolar cell birth, with the thymidine analogue EdU to label all proliferating RPCs (Figure 5). My results show that Vsx2-Cre, a pan bipolar cell marker, is first detected by immunolabeling 36 hrs after injection of EdU, whereas mGluR6 (ON bipolar cells), PKCα (rod bipolar cells), and Vsx1 (subtypes 2, 6 and 7 bipolar cells) are all detected 48-60 hrs after EdU injection (Figure 7).

Even though all three bipolar cell markers analyzed appeared at approximately the same time, the proportion of each at different timepoints is not consistent. Notably, at 60 hours after EdU injection, the average proportion of Cre+ and EdU+ cells expressing mGluR6 and VSX1 were somewhat comparable (17.93% ± 4.72% and 12.63% ± 6.38%, respectively). Whereas at the 120-hour timepoint (or 84 hrs. after initial Cre expression) the average proportion of mGluR6+ cells (50.80% ± 5.67%) was closer to its total proportion in the retina (78%) and considerably higher than the proportion of VSX1+ cells (12.70% ± 2.94%) (Figure 8, A). This observation suggests VSX1 is expressed in postmitotic cells prior to mGluR6. Over 60% of VSX1+ cells are ON BCs, meaning they co-express mGluR6 (Robert L. Chow, unpublished data, Chow Lab). If we assume VSX1 expression equals subtype specification and mGluR6 expression equals ON subtype specification, these results suggest ON subtype specification

occurs after VSX1 expression. This is an expected possibility, given that *Vsx1* is a transcription factor that regulates the expression of genes involved in the differentiation of bipolar cells (Chow et al. 2004; Shi et al. 2011) whereas mGluR6 is a metabotropic receptor involved with the function of ON bipolar cells rather than their differentiation. Interestingly, *Vsx2* (Vsx2-Cre) still precedes the expression of *Vsx1* (Figure 7, B, E) and a previous study from our lab showed it also preceded the expression of *Bhlhb5* (Nickerson et al. 2011), which is another transcription factor involved in the differentiation of bipolar cell subtypes. Altogether, these results suggest there might be a temporal order in the expression of transcription factors regulating bipolar cell subtype development. This reinforces the possibility of a multi-step model for bipolar cell specification. Future studies must involve exploring the initial expression of other important transcription factors involved in bipolar cell subtype development such as *Bhlhb4, Prdm8, Irx5/6,* and *Isl1* relative to each other.

5.1.2 A Bipolar Cell post-mitotic precursor

Neuronal subtype differentiation does not necessarily occur precisely at the time of cell cycle exit and is likely a gradual transitional process. For example, in the cortex, newly born neurons continue to drive gene expression from a neural progenitor promoter days after cell cycle exit, suggesting that achieving a final cellular fate is a slow process. (De Anda et al. 2016). Additionally, these postmitotic neurons have the capacity to reprogram into different subtypes for a few days via ectopic expression of a transcription factor (De la Rossa et al. 2013; Rouaux & Arlotta 2013). These results suggest cortical post-mitotic precursors have a high degree of cell fate plasticity after cell cycle exit for a short period of time. Similar results are observed in the retina; RGCs do not become identifiable subtypes until a week after cell cycle exit (Shekhar et al. 2022). Past research from our lab showed the expression of the bipolar-specific transgene *Vsx2-5.3-PRE-Cre* (Vsx2-Cre) occurs before the expression of various bipolar cell subtype markers and is expressed in some photoreceptors, suggesting a cell class fate switch from bipolar to photoreceptor (Nickerson et al. 2011). This observation also suggests there could be a post-mitotic bipolar cell precursor intermediate cell prior to subtype differentiation.

My results support the possibility of a post-mitotic bipolar cell precursor intermediate cell prior to subtype differentiation. Vsx2-Cre is detectable by immunolabeling 36 hours after cell cycle exit, whereas the subtype markers mGluR6, PKCα and VSX1 are detected 48-60 hours

after S-phase. While this result in consistent with a multi-step model for bipolar cell subtype differentiation, it is not possible to draw strong conclusions based on immunolabeling of a limited number of markers. Protein expression is only correlative and does not necessarily mean a cell subtype is fully differentiated. A thorough cell transcriptomic analysis examining the initial expression of all known bipolar subtype genes would provide more convincing data to support a multi-step model. Nevertheless, my findings argue against a direct model of subtype differentiation and suggest the transition from a post-mitotic precursor cell to a fully differentiated bipolar cell subtype could be a plastic, multi-step process.

5.2 Cell contact is required for both pan- and subtype- bipolar cell specification

The role of intrinsic mechanisms in the specification, differentiation and survival of various bipolar cell subtypes is well established, but the role of extrinsic mechanisms remains understudied. My next objective was to examine the role of cell-cell interactions in bipolar cell subtype specification by culturing EdU-injected dissociated retinal cells at high-density (abundant cell contact) or low-density (scarce cell contact) until maturity (Figure 13). This experimental approach allowed us to analyze the role of cell contact on the expression of various bipolar cell markers in post-mitotic cells. To our knowledge, this is the first study to focus on the role of non-cell autonomous mechanisms on bipolar cell subtype specification in post-mitotic cells. My results revealed cell contact is required for the expression of the pan bipolar cell marker VSX2-Cre in RPCs and in early post-mitotic cells. The proportion of cells co-expressing Cre and EdU out of all EdU+ cells was significantly lower when dissociated retinal cells were cultured at low density 1 and 24 hours after injection of EdU (Figure 13, C). Additionally, cell contact also seemed to be required for the expression of other bipolar cell subtype markers, specifically in early post-mitotic cells as shown by the 24-hour timepoint difference between the two culture conditions (Figure 13, D-F). These results suggest there are extrinsic mechanisms mediating the specification of pan bipolar and bipolar cell subtype specification, and that by 48 hrs after cell cycle exit, these mechanisms are no longer required to maintain a specified state.

In contrast with our results, an in vitro lineage-tracing study using long-term video microscopy demonstrated isolated embryonic RPCs can give rise to the major cell types in an intrinsic, stochastic manner (Gomes et al. 2011). In this study, when E20 (Embryonic day 20) RPCs were cultured for 9-15 days at a low-density, the percentage of the differentiated cell types

resembled what is seen in vivo at P0. Cell types were identified by morphology and immunohistochemistry. The authors concluded retinal cell birth at this stage follows a stochastic mode of division in which each RPC has a statistical bias to give rise to a cell type based on their total abundance in the retina. This suggests RPCs have the potential to specify into their specific major cell types, including bipolar cells, without the need of any environmental cues. This stands in contrast with our results in which the proportion of EdU+ cells expressing the bipolar cell marker VSX2-Cre was significantly lower when cells were cultured at a low-density. There are a few possible reasons for this discrepancy.

Firstly, the method of cell type identification in the Gomes study was different from ours. They identified RPCs visually and bipolar cells based on the presence of *Isl1* immunolabeling along with cell morphological analysis whereas we performed immunolabeling against the bipolar cell specific marker VSX2-Cre and relied on EdU to identify all RPCs undergoing S-phase and did not perform immunolabeling against *Isl1.* It is possible the expression of *Isl1* in post-mitotic cells does not require cell contact. This could be addressed in future experiments by performing *Isl1* immunolabeling on our cultured dissociated retinal cells.

Secondly, the Gomes study did not quantify the proportion of RPCs that successfully differentiated out of all RPCs observed since no method to identify progenitors was conducted. Our study quantified the proportion of cells expressing VSX2-Cre by looking at the proportion of all EdU+ cells. When EdU-injected retinal cells are dissociated 1 hour after injection and cultured at low-density, we still see a small proportion of cells expressing the pan bipolar cell marker VSX2-Cre (Figure 13, C). Altogether, this suggests the requirement for cell contact for pan bipolar cell specification is not absolute, meaning some cells are still able to differentiate successfully but the majority cannot. This observation also hints at the possibility that RPCs could have an intrinsic potential to specify into bipolar cell subtypes but require extrinsic signals for this mechanism to be activated.

Lastly, the experimental design of our assay is different enough so that it could explain the difference in our results from the Gomes study without affecting our conclusions. The timepoint chosen for their experiments was different than ours. They used embryonic RPCs whereas our experiments were conducted on early postnatal RPCs. It is possible that extrinsic mechanisms play a unique developmental role in the postnatal stage but not in the embryonic stage. More importantly, in their experiment RPCs are isolated from other nearby cells but not

from each other after cell division. Daughter cells can still interact with each other and thus possibly extrinsically affect their specification, especially since all the RPCs analyzed in the published study involve two cell divisions which means there could have been an important interaction between two RPCs. In our experiments, EdU-labeled RPCs are dissociated after DNA replication which results in the endogenous cell contact between any existing daughter cells being lost at the time of dissociation. The role of cell contact between daughter cells on cell specification is well documented. In the sensory organ precursor lineage of *Drosophila*, extrinsic Notch-ligand interactions between daughter cells in asymmetric cell divisions help establish cell fate (Hawkins & Garriga 1998). The signaling mechanisms and cell fate determinants in daughter cells have not been explored in the vertebrate retina, but there could be important extrinsic mechanisms at play between these cells affecting cell specification.

Perhaps the most striking result from our retinal dissociation cell culturing experiments is the lack of bipolar cell subtype marker expression in the 1 and 24-hour timepoints (Figure 13, D-F). This stands in contrast with the lack of difference of marker expression at the 48-hour timepoint. These results suggest cell contact is required for the expression of these markers 1-48 hours after cell cycle exit which could mean extrinsic signals are involved in bipolar cell subtype specification and differentiation. Not many other studies have focused on the role of extrinsic signals on the specification and the differentiation of bipolar cell subtypes. However, there are a few important caveats to consider when looking at our results.

It goes without saying development in vitro is drastically different from in vivo development. The removal of the retinal architecture after dissociation means some molecules for general maintaining cells healthy such as growth factors are possibly lost. It is possible that the lack of bipolar cell marker expression in the low-density condition is not due to extrinsic signals but rather due to a developmental delay. Unfortunately, cell viability starts to decrease significantly the longer retinal cells are cultured for and thus longer culturing times are problematic (Figure 12, data not shown). However, we argue against this possibility due to two important observations:

1) If the lack of subtype markers is due to a developmental delay due to the loss of important growth factors or other molecules required to maintain cell proliferation and prevent development delay, the co-culture of retinal dissociated cells along HEK293T cells should have rescued the proportion of marker expression, but this is not the case (Figure 14, B, C). This

suggests the lack of marker expression in low-density cultures is not due to the lack of general cell maintenance growth factors and molecules but rather due to the loss of cell contact from other retinal cells.

2) A developmental delay would mean cultured retinal cells dissociated 48 hours after EdU injection should still show signs of a delay. However, this is not the case, there is no difference in the proportion of cells positive for marker expression out of all Cre+ & EdU+ cells at the 48-hour timepoint for low vs high density culture conditions. (Figure 13, D-F). Nevertheless, it is possible that there is a critical developmental timepoint susceptible to delay 1-24 hours after cell cycle exit. Future studies could focus on improving the viability of retinal cells for longer culture times and ensure developmental delay is not a contributing factor to our results.

5.2.1 The retinal environment is required for bipolar cell specification

My results suggest the retinal environment has the appropriate molecular composition for bipolar cell specification to occur. The proportion of cells expressing bipolar cell markers was significantly higher when EdU-injected, dissociated retinal cells were co-cultured 24 hours after injection with high-density EdU- dissociated retinal cells from a litter mate than when co-cultured with HEK293T cells or at low-density (Figure 14). This apparent "rescue" could be due to multiple potential interactions with other cell types. At the time of dissociation, there are 6 different retinal cell types present in the retina: photoreceptors (both cone and rod), retinal ganglion cells, amacrine cells, horizontal cells, RPCs, and previously born bipolar cells. Morphological and immunohistochemical analysis has confirmed the presence of all these cell types in our cultured dissociated cells other than RGCs, since common antibodies used to identify RGCs did not yield reliable immunolabeling results on our dissociated retinal cells (data not shown). Deletion of RGCs and amacrine cells at P0-P1 does not seem to disturb the formation of the ON and OFF layers, suggesting these cells are not involved in bipolar cell specification (Gunhan-Agar et al. 2000; Gunhan et al. 2002). However, the effect on bipolar cell subtype numbers was not quantified, and since the RGCs and amacrine cells were removed somewhat late during development (~P0), previously secreted molecules by these cells could have remained in the retinal environment and influence bipolar cell specification. Further research is required to unequivocally conclude RGCs and amacrine cells have no role on bipolar

cell specification, and our results suggest it is a strong possibility. Confirming the presence or absence of RGCs and amacrine cells in our dissociated retinal cells is a crucial future experiment. Additionally, isolation of a retinal cell type via immuno-panning followed by dissociated retinal cell culture is an excellent future experiment to directly test the role of these cell types on bipolar cell specification.

Even though various adhesion molecules are required for the correct formation of bipolar cell dendritic and axonal arborization (Dunn & Wong 2012; Lee et al. 2011; Sanes & Zipursky 2020), there is no evidence of these molecules playing a role in the specification of bipolar cell subtypes thus far. Both homotypic and heterophilic molecular interactions are involved in synaptogenesis of bipolar cells. For example, removal of most rod bipolar cells results in an abnormal expansion of the dendritic field of the remaining rod bipolar cells, suggesting homotypic signals between these cells are responsible for constraining their dendrites (Johnson et al. 2017). *Cdh8* and *Cdh9* are required for the laminar restriction of bipolar cell subtypes 2 and 5, respectively (BC2s and BC5s). Overexpression of Cdh9 by electroporation in OFF BC2s results in abnormal axonal arborization resembling that of ON BC5s, in contrast, when Cdh8 is overexpressed in ON BC5s, its axonal arbor resembles that of OFF BC2s (Duan et al. 2014). Interestingly, this apparent switch in morphological features between these two subtypes did not seem to accompany a switch in subtype identity, since OFF BC2s and ON BC5s remained negative and positive, respectively, for *Isl1,* an ON BC marker. Additionally, time-lapse recording of *Grm6-GFP* developing ON BCs from P3 until P19 discovered that their axonal and dendritic projections originate from basal and apical neuroepithelial-like processes, and that their axonal arbors are fist restricted to their corresponding INL depth at around P5, which happens to match the time at which final bipolar cell subtype specification occurs (Morgan et al. 2006). This suggests there could be critical cell-cell interactions between RGCs or amacrine cells and bipolar cells involved in the synaptogenesis as well as the specification of ON BCs. However, it is important to note the transgenic mouse line used was already expressing *Grm6* (mGluR6) and therefore could have been already a specified bipolar cell. Altogether, these results suggest bipolar cell subtype laminar targeting occurs downstream of bipolar cell subtype specification.

In conclusion, we propose non-cell autonomous mechanisms occurring upstream of other laminar organization events play an important role in bipolar cell subtype specification. Additionally, due to the different results seen at the 1- and 24-hour timepoints regarding Cre

expression and subtype marker expression, our results suggest pan bipolar cell specification and bipolar cell subtype specification are two independent mechanisms with both requiring cell-cell signaling. Furthermore, our co-culture experiments demonstrate the retinal environment possess the right molecular properties for bipolar cell subtype specification. To our knowledge, this is the first study to test the role of cell contact on post-mitotic retinal cells. By injecting EdU and culturing dissociated retinal cells 1,24 or 48 hours after injection, we selectively cultured EdU+ at different time points after cell cycle exit and tested the effect of a high vs low cell density environment on bipolar cell development. Our experimental design is a valuable tool to conduct experiments with post-mitotic cells that could be used in future research.

5.3 The role of Notch and Shh signaling on bipolar cell specification

The role of the Notch signaling pathway on inhibiting the photoreceptor fate and promoting the specification of bipolar cells in RPCs is well characterized (Jadhav et al. 2006; Yaron et al. 2006). However, its specific role as an extrinsic cell signaling mechanism on the specification of bipolar cell subtypes has not been studied much, with only one current study suggesting it plays a role in post-mitotic cells (Mizeracka et al. 2013). In this study, they designed an experiment to isolate the effect of the loss of Notch1 to postmitotic cells: they co-infected retinas with a replication-incompetent LIA-Cre gamma-retrovirus and Cre-dependent *Notch1*-conditional knockout (N1-CKO) at P3. Due to the nature of these viruses, it is not expressed by the initially infected RPC and only one daughter cell inherits the virus. Therefore, mature cells derived from terminal divisions (postmitotic when infected) in the adult retina can be identified by the lack of LIA-Cre+ cell clones nearby when analyzed at P21. Their results showed the loss of Notch1 in postmitotic cells reduced the number of bipolar cells and increased the number of rod photoreceptors, suggesting Notch1 is required for the postmitotic specification of bipolar cells and inhibiting the rod photoreceptor cell fate. In contrast, when I cultured dissociated cells 24 hrs after injection with EdU with the Notch inhibitor DAPT, there is an increase in the number of bipolar Vsx2-Cre bipolar cells. This discrepancy can be explained due to critical experimental differences. In the Mizeracka study, the retina was left intact, whereas in my experiments, retinal cells were dissociated and since we obtained different results, it is reasonable to assume that the dissociated retinal cells were exposed to different environmental signals that could have interacted with Notch signaling and change its true effect on postmitotic

cells. Alternatively, Notch signaling could be exclusively required within 24 hrs after cell cycle exit. Future experiments could address this possibility by dissociating retinal cells ~12 hrs after injection of EdU with DAPT until maturity.

When Notch signaling was inhibited via DAPT in dissociated retinal cells cultured 1 hour after EdU injection until P8, we observed a decrease in pan bipolar cell specification (Figure 18, B). This is an expected result since RPCs injected with EdU one hour prior to dissociation are still in the progenitor phase (Yaron et al. 2006). Unfortunately, we were not able to quantify the proportion of photoreceptor cells in our experiments due to the lack of a reliable marker, but future studies could focus on achieving this and repeating this experiment. It would be interesting to see if the observed loss of pan bipolar cell specification occurs while there is an increase in photoreceptor cell numbers. Interestingly, the loss of Notch signaling 1 hour after cell cycle exit had no significant effect on bipolar cell subtype marker expression, which suggests Notch is not required post-mitotically at this timepoint (Figure 18, C-E).

When dissociated retinal cells were cultured with DAPT 24 hours after injection of EdU until P8, the proportion of Vsx2-Cre expressing cells increased (Figure 19, B). This is an unexpected and conflicting result to our previous experiment showing a decrease of Vsx2-Cre cells after inhibiting Notch signaling 1 hr after cell cycle exit. It suggests Notch could have an opposite effect by inhibiting pan bipolar cell specification on post-mitotic cells. However, this result could also be an indirect result from the loss of Notch signaling since *Vsx1* is a known repressor of *Vsx2* (Clark et al. 2008; Shekhar et al. 2016) and the number of VSX1+ cells decreased in this experiment (Figure 19, E). Therefore, the increase in Vsx2-Cre+ cells could be due to an indirect upregulation of *Vsx2* due to the loss of repression from *Vsx1*. This would also explain the trend in the increase in PKCα+ cells (Figure 19, D). This possibility is further validated by the increase in VSX1+ expression when Notch and Shh are simultaneously activated (Figure 21, E). The loss of Notch signaling 24 hours post cell cycle exit did not significantly affect bipolar cell subtype marker expression, but it showed interesting trends (Figure 19, C-E): even though the number of Vsx2-Cre+ cells increased, the number of mGluR6+ cells and VSX1+ cells slightly decreased whereas PKCα+ cells increased. The lack of statistical significance in these results could be due to the presence of outliers (e.g., Figure 19, C and E). These trends further exemplify how pan bipolar cell specification and bipolar cell subtype specification are two independent mechanisms since the expression of bipolar subtype

markers did not have a uniform response. In summary, these results suggest Notch signaling could play a role in the specification of bipolar cell subtypes and an indirect inhibiting role on pan bipolar cell specification 24 hours after cycle exit.

Previous studies have demonstrated mechanistic similarities in the role of the Shh signaling and Notch signaling pathways in retinal development. They both seem to be required for the specification of bipolar cells over photoreceptors (Yaron et al. 2006; Jadhav et al. 2006; Yu et al. 2006). However, this phenotype may be indirect and due to an effect on RPCs and not specific to bipolar cells since the specification of other INL cell types such as amacrine cells and Muller glial cells is also affected (Yu et al. 2006). There is also other indirect evidence that Shh signaling is required for cone bipolar specification. Retinas lacking RGCs, the main source of Shh, display a severe reduction in the number of cone BCs and no effect on rod BCs numbers (Bai et al. 2014). In addition, activation of Shh signaling in postnatal RPCs results in an increase in cone bipolar cell numbers and no effect on rod bipolar cell numbers (Wu 2017). These results suggest Shh signaling could have a specific effect on cone bipolar cell subtype specification in addition to its mitogenic effect on RPCs. But whether this is an effect on RPCs or post-mitotic cells remains unclear. Previous lab data from our lab in which Shh signaling was activated via Cre-mediated Smoothened activation in our VSX2-Cre mice line showed no difference in the number of cone and rod bipolar cell subtypes (Wu 2017). And electroporation of a Cre-dependent, Hh signaling reporter construct into Vsx2-Cre mice showed deactivation of the Hh signaling at the time of Vsx2-Cre initial expression (Dee Wu, unpublished data, Chow lab). But because the onset of Cre activation in post-mitotic cells occurs at around 36 hours after cell cycle exit (Nickerson et al. 2011, Figure 7, B), Shh signaling could still have a post-mitotic effect 1-36 hours after cell cycle exit. In the current study, we tested this possibility by culturing dissociated retinal cells 24 hours after cell cycle exit with the presence of an inhibitor or an agonist for Shh signaling. Also, due to its very similar role, the co-inhibition or co-activation of the Notch signaling pathway were tested as well.

Our results show co-activation of the Hh and the Notch signaling pathway increased VSX1 expression in dissociated retinal cells cultured 24 hours after injection until P8 (Figure 21, F). This is an interesting result somewhat consistent with previous observations, this result suggests the increase in cone bipolar cell numbers seen in previous experiments could be due to an increase in OFF cone bipolar cell specification, since 40% of VSX1+ cells are OFF cone

bipolar cells (Robert L. Chow, unpublished data, Chow Lab). An unlikely yet possible alternative explanation could be that Shh and Notch signaling activation increase the expression of VSX1 without affecting cone bipolar cell specification. Future studies could address this by conducting additional immunohistochemistry analysis to confirm the identity of these VSX1+ cells as OFF cone bipolar cells. Interestingly, when the opposite experiment was performed and Hh and Notch signaling were co-inhibited, we do not observe a decrease in VSX1 expression as would be expected if Notch and Shh were responsible for VSX1+ expression (Figure 20, F). This suggests the observed increase in VSX1 expression is not a direct result from Notch and Shh co-activation but is rather a downstream consequence with other molecular regulators at play. Altogether, these results suggest Notch and Shh play an important role in the expression of VSX1 in bipolar cells and therefore potentially in the specification of subtypes 2,6 and 7.

An interesting study conducted on zebrafish showed *Vsx1*+ postmitotic bipolar cells can be re-specified into amacrine cells by ectopic Notch signaling (Engerer et al. 2021). In zebrafish, *Vsx1* is expressed by progenitor cells, bipolar cells and amacrine cells. Notch inhibition via DAPT resulted in a ~50% decrease in the number of *Vsx1*+ amacrine cells. Conversely, Notch overexpression via addition of Notch Intracellular Domain (NICD) resulted in a 9-fold increase in the number of *Vsx1*+ amacrine cells. Time-lapse recording showing the reprogramming of bipolar cells into amacrine cells happening *in vivo* and the lack of difference in the proliferation rate of progenitors suggested this was a postmitotic effect. Even though there are critical differences between zebrafish and mammals, these results provide evidence that Notch signaling has the potential to influence cell fate postmitotically and could explain the increase in VSX1+ cells seen when Notch and Hh signaling were co-activated (Figure 21, F)

Inhibition of both the Shh and Notch signaling pathways decreased the expression of VSX2-Cre in dissociated retinal cells cultured 24 hours after injection until P8 (Figure 20, C). This result suggests the presence of both signaling pathways are required for the specification of pan bipolar cells, which is consistent with published findings. However, when the opposite experiment was conducted and both the Notch and Hh pathways are activated, there was no increase in VSX2-Cre expression (Figure 21, C). This conflicting result could be due to the mitogenic effects of Shh and Notch on RPCs. The loss of these signaling pathways on RPCs could potentially be preventing them to re-enter the cycle and therefore decrease the total cell number of VSX2-Cre+ cells. The reason why the reverse phenotype is not observed could be due

to the culture environment missing additional signals present in the endogenous retina that are also playing a role along Shh and Notch signaling increasing pan bipolar cell numbers.

Notably, we obtained different results when Notch signaling was inhibited by itself (Figure 19) than when it was inhibited along Shh signaling 24 hrs after cell cycle exit (Figure 20). As summarized above, when only Notch was inhibited with DAPT, the proportion of Vsx2-Cre increased and trends showed a decrease in mGluR+ and VSX1+ cells, and an increase in PKCα+ cells (Figure 19, B, C, D). We believe this phenotype is the result of the loss of *Vsx1* inhibition of *Vsx2*. In contrast, co-inhibition of Notch and Shh signaling resulted in a decrease in Vsx2-Cre+ cells, and trends showed a decrease of both mGluR6+ cells and PKCα+ cells, and no difference in VSX1+ cells (Figure 20). The discrepancy in Vsx2-Cre+ numbers could be explained by fact that there are normal numbers of VSX1+ cells during co-inhibition, and therefore *Vsx1* inhibition of *Vsx2* occurs at normal levels, allowing the direct role of Notch and Shh on Vsx2-Cre+ cell numbers to be observed.

Interactions between the Notch and Hh signaling pathways in retinal development have been investigated. Disruption of Notch signaling induces downregulation of the Gli proteins (the pathway activators of Shh) in RPCs, suggesting Notch signaling functions upstream of Hh signaling during retinal development (Ringuette et al. 2016). This interaction would explain the lack of an effect on the expression of bipolar cell markers when only Hh signaling was activated via purmorphamine, since the lack of Gli proteins due to lack of Notch signaling would render activation of the Hh pathway in these cells functionless (Figure 21). In conclusion, our results suggest Notch and Hh signaling play a role in the specification of bipolar cells, specifically on the expression of VSX1, and thus potentially on the specification of subtypes 2, 6 and 7. However, the variability and inconsistent trends in our results suggest that there are other molecular regulators at play during the postmitotic specification of bipolar cells that need to be investigated.

5.5 Summary, future direction, and significance

The present study highlights the importance of cell-cell signaling, the role of the retinal environment and the involvement of the Shh and Notch signal transduction pathways on the specification of bipolar cell subtypes. By using the thymidine analogue EdU as an RPC marker and culturing dissociated retinal cells at low and high cell densities, we were able to temporally

distinguish different cellular stages after cell cycle exit and analyze the effect of cell-cell signaling on the expression of four bipolar cell markers. Our results found evidence that non-cell autonomous mechanisms are required for the specification of pan bipolar and bipolar cell subtype within the first 48 hours after cell cycle exit. Furthermore, the results were different for pan bipolar and bipolar subtype marker expression, suggesting the specification of these two cells occurs via different mechanisms. The results of our co-culture experiments suggest the retinal environment is required for the specification of bipolar cells. Additionally, our results suggest Notch or Shh signaling alone have no effect on bipolar cell subtype specification, but together, their activity has a dual effect that increases the specification of VSX1+ cell subtypes.

There are multiple future experiments that should be considered based on our results. First, several refinements could be made to our experimental approach which was initially limited in scope to make our analysis feasible. Our approach could be broadened to address some of the questions that arose in our study. In addition to the initial expression of four different bipolar cell subtype markers, there are many other markers that should be analyzed. These include markers for bipolar subtypes, Muller glial cells, amacrine cells, retinal ganglion cells (RGCs) and early photoreceptor markers. Immunolabeling of these cell types would allow us to identify the fate of the EdU+, VSX2-Cre- cells seen. It would be interesting to test whether there is a postmitotic cell fate switch from bipolar cell to a rod photoreceptor or to other retinal cells happening in vitro. The initial expression of these markers after cell cycle exit would further provide insight into the mechanism of bipolar cell subtype specification. Also, to improve the validity of our results, repeating our experiments with an increased cell culture time would be a valuable experiment. If developmental delay is not an issue, the results should remain the same. This could be done by improving the viability of dissociated cells in culture by optimizing the cell media

An important next step in our research will be to determine the nature of the extrinsic factors that appear to be mediating bipolar cell subtype differentiation that our data suggests are acting within the first 48 hours after cell cycle exit. One possibility is that other retinal cell types such as RGCs and amacrine cells (the main postsynaptic partners of bipolar cells, Figure 2) are the source of these extrinsic factors. There is extensive research demonstrating cell adhesion molecules play an important role in synaptogenesis, but the role of these molecules on bipolar cell subtype specification has not been addressed (Dunn & Wong 2012; Lee et al. 2011; Sanes &

Zipursky 2020). A first step might be to determine whether RGCs and/or amacrine cells are sufficient to promote bipolar cell subtype specification. This could be done by isolating amacrine cells and retinal ganglion cells using previously published immuno-panning protocols (Winzeler & Wang 2013; Park et al. 2020) and performing co-culture experiments like those in Figure 14. These experiments could be combined with cDNA library screening to identify the molecular proteins affecting bipolar cell subtype specification.

My results provide the first experimental piece of evidence to suggest the existence of a postmitotic neural subtype precursor intermediate cell in the vertebrate retina. This suggests that similar mechanisms could be taking place in other areas of the central nervous system. While past research has focused on the development of general cell classes, my experiments focused on the specification of neural cell subtypes, and my results are the first to suggest there is a distinct mechanism for pan-bipolar and bipolar subtype specification and that this mechanism follows a multi-step process involving a plastic postmitotic precursor intermediate cell. Additionally, my results implicate the Notch and Hedgehog transduction pathways as important players in this mechanism.

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