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Célia Márcia Azevedo Soares Fate of the earliest retinal ganglion cells during development of the visual system

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Universidade do Minho
Escola de Ciências da Saúde

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Tese de Doutoramento em Medicina

Trabalho efectuado sobre a orientação da
Professora Carol Mason
Columbia University in the City of New York

e da
Professora Joana Palha
Universidade do Minho

agosto de 2016

DECLARAÇÃO

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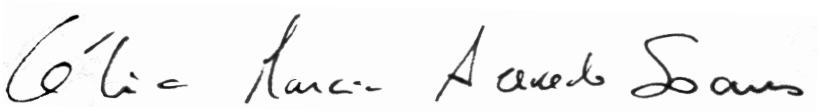

La vérité, c'est le langage qui dégage l'universel. Newton n'a point "découvert" une loi longtemps dissimulée à la façon d'une solution de rébus, Newton a effectué une opération créatrice. Il a fondé un langage d'homme qui pût exprimer à la fois la chute de la pomme dans un pré ou l'ascension du soleil. La vérité, ce n'est point ce qui se démontre, c'est ce qui simplifie.

Terre des hommes, Antoine de Saint-Exupéry, 1939

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Abstract

During development of the mammalian eye, the first retinal ganglion cells (RGCs) that extend to the brain are located in the dorsocentral retina, in the mouse, during mid-embryogenesis. These RGCs extend to either ipsilateral or contralateral targets, but the ipsilateral projections from the central retina do not survive into postnatal periods. The function and means of disappearance of the transient ipsilateral projection are not known. We have followed the course of this transient early ipsilateral RGC cohort, paying attention to how far they extend, whether they enter target regions in the brain and if so, which ones, and the time course of their disappearance. Several different techniques were tested for labeling the axonal projection from the central retina. While the use of a viral vector and a conditional Brn3b knock-out mouse presented difficulties in specifc labeling of RGCs and of ipsi- or contralateral projections, respectively, both the application of the lipophilic tracer DiI and *in utero* electroporation of GFP into central retina allowed the analysis of the growth and position of the ipsilateral and contralateral retinal projections. The proportion of ipsi- versus contralateral projections was calculated at E13.5 and 15.5 using DiI. *In utero* electroporation of E12.5 retina with GFP constructs was used to label axons prospectively into postnatal ages. Our results show that the earliest ipsilateral axons are segregated from the laterally-positioned contralateral axons in the optic tract. In agreement with previous reports, we found that the number of central retina ipsilateral RGCs declines after E16.5. Nonetheless, some ipsilateral axons from the central retina enter the superior colliculus (SC) and arborize minimally, but very few enter the dorsal lateral geniculate nucleus (dLGN). To understand whether caspases are involved in the disappearance of the ipsilateral projection from the central retina, immunohistochemistry experiments were performed but without conclusive results. To identify candidate genes expressed in ipsilateral vs contralateral RGC axons in the central retina at E13.5, a technique that combines retrograde labeling with DiI, applied retrogradely, with immunohistochemistry was developed. To date, no molecular marker was found that selectively labeled the transient ipsilaterally-projecting RGC axons from central retina. The results of this work and the methods developed will be useful to better understand the elimination of transient axonal projections, and their role in establishing neuronal circuits.

Sumário

Durante o desenvolvimento do olho, as primeiras células ganglionares da retina (CGR) que se extendem para o cérebro estão localizadas na retina dorsocentral dos murganhos. Apesar destas CGR projetarem tanto para alvos ipsilaterais como contralaterais, a projeção ipsilateral da retina central não sobrevive para o período pós-natal. A função e o contexto do desaparecimento da projeção transitória ipsilateral é desconhecida. Nesta tese, seguimos o percurso desta coorte de CGR transitória ipsilaterais, atendendo à sua extensão, a invasão de alvos, e desaparecimento. Foram testadas diferentes técnicas de marcação de projeções axonais da retina central. O uso de vetores víricos e de murganhos destituídos do gene Brn3b não marcou seletivamente uma coorte de CGR na retina central, enquanto as experiências com DiI e electroporação *in utero* da proteína fluorescente verde (PFV) permitiram a análise da projeção ipsilateral e contralateral da retina central, esta última de forma prospetiva. A proporção da projeção ipsi- vs contralateral marcada com DiI diminui do dia de gestação embrionária E13.5 ao E15.5. As experiências com DiI mostraram também a segregação lateral dos primeiros axónios ipsilaterais no trato óptico. A electroporação *in utero* de um plasmídeo de PFV foi usada para marcar os axónios em estadios temporais consecutivos até idades pós-natais. Em concordância com estudos anteriores verificámos um declínio acentuado do número de CGR ipsilaterais da retina central após E16.5. No entanto, alguns axónios ipsilaterais da retina central invadem o colículo superior e arborizam minimamente, com poucos axónios invadindo a componente dorsal do núcleo geniculado lateral com ramificações curtas. Para compreender se o desaparecimento da projeção ipsilateral da retina central está relacionada com a expressão axonal de caspases, foram realizadas análises imunohistoquímicas que não revelaram resultados conclusivos. Para testar a expressão diferencial de 'genes candidatos' nas CGR da retina central a E13.5, foi desenvolvida uma técnica que combina o uso do marcador biofílico DiI com imunohistoquímica. No entanto com esta técnica e em estudos anteriores não foram identificados marcadores moleculares que identifiquem exclusivamente a projeção transitória ipsilateral da retina central. Os resultados desta tese e os métodos desenvolvidos nela serão úteis para uma melhor compreensão da eliminação de projeções axonais transitórias com o objetivo final de compreender melhor a formação de circuitos neuronais.

Abbreviations List

4-HT: 4-hydroxy-tamoxifen

AP: alkaline phosphatase

BLBP: brain lipid binding protein

C: caudal

Caspases: cysteine-aspartic proteases

Cdh3: Cadherin 3

CKO: conditional knock-out

D: dorsal

DAB: diaminobenzidine

DCC: deleted in colorectal cancer

DiI: 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate

dLGN: dorsal lateral geniculate nucleus

Dox: doxycicline

DRD4: dopamine receptor D4

E: embryonic day

FACS: fluorescence-activated cell sorting

FGF: Fibroblast Growth Factor

GFAP: Glial fibrillary acidic protein

GFP: green fluorescente protein

GLAST: GLutamate ASpartate Transporter

IGL: intergeniculate leaflet

Isl: Islet

KO: knock-out

L: lateral

LGN: lateral geniculate nucleus

M: medial

mCherry: mammal Cherry

MO: morpholino

MPT: medial pre-tectal nucleus

MTN: medial terminal nucleus

NOT: nucleus of the optic tract

OC: optic chiasm

ON: optic nerve

OPT: olivary pretectal nucleus

OT: optic tract

Otx1: Orthodenticle Homeobox 1

P: postnatal day

Pax6: Paired box 6

PB: phosphate buffer

PBS: phosphate buffer saline

PCD: Programmed cell death

PFA: paraformaldehyde

PPT: posterior pre-tectal nucleus

R: rostral

RC2: radial cell antigen (nestin)

RPE: retinal pigment epithelium

rtTA: tetracycline transactivator

SC: superior colliculus

Sert: serotonin transporter

Shh: Sonic Hedgehog

SSEA: stage-specific embryonic antigen

TRE: tetracycline Response element

Trk: tyrosine receptor kinases

TTX: tetrodotoxin

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

V: ventral

vLGN: ventral lateral geniculate nucleus

VT: ventrotemporal

WGA: wheat germ agglutinin

wt: *wildtype*

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I. Introduction

A. Basic principles of neuroscience

Neurons are the basic units of the nervous system. They were observed by Christian Ehrenberg and Purkinje, even before Virchow established the definition of a cell, in 1855 (Lopez-Munoz et al., 2006). The basic concept of the neuron as an asymmetric polarized cell was observed in human tissue by Otto Friedrich Karl Deiters, who described that the nervous system has specialized cells with a prototypical structure - a cell body, with several cell extensions, and only one extension, a longer one, all of which developed and are maintained without the cell dividing. He named the longer one the axis-cylinder, while the rest, the "protoplasmic extensions", branch out extensively (Deiters and Guillery, 2013). This was a primitive description of what now we know to be the axon (the axis-cylinder) and dendrites (protoplasmatic extensions). But even Deiters did not conceptualize the correct definition of what a neurons is and how neurons connect with each other. Deiters supported the "reticular theory" postulated by German von Gerlach, that the nervous system was a diffuse protoplasmic network in the grey matter of the nerve centres (Guillery, 2005). This was a widely accepted theory supported by eminent scientists in the late XIX/ early XX century, such as Camilo Golgi. It was Ramon y Cajal with his "neuron theory" or "neuron doctrine" who established that the connections between neural cells were not a system of continuity but rather of contiguity (Lopez-Munoz et al., 2006). In 1888 in the first issue of the *Revista Trimestral de Histología Normal y Patológica*, Cajal described these specialized cells as individual cells, opposing the hypothesis supported by Camilo Golgi that the nervous system was a "reticular system" with cells connected by cytoplasmatic continuity (Sotelo, 2003). Despite the fact that most of the work of Ramon y Cajal was exclusively descriptive, Cajal combined his morphological observation with functional implications and postulated hypotheses about the unidirectional properties of information flow in neurons from dendrites to the cell body and then to the axon (Llinas, 2003). One famous example are the directional arrows that populate Cajal neuron's drawings.

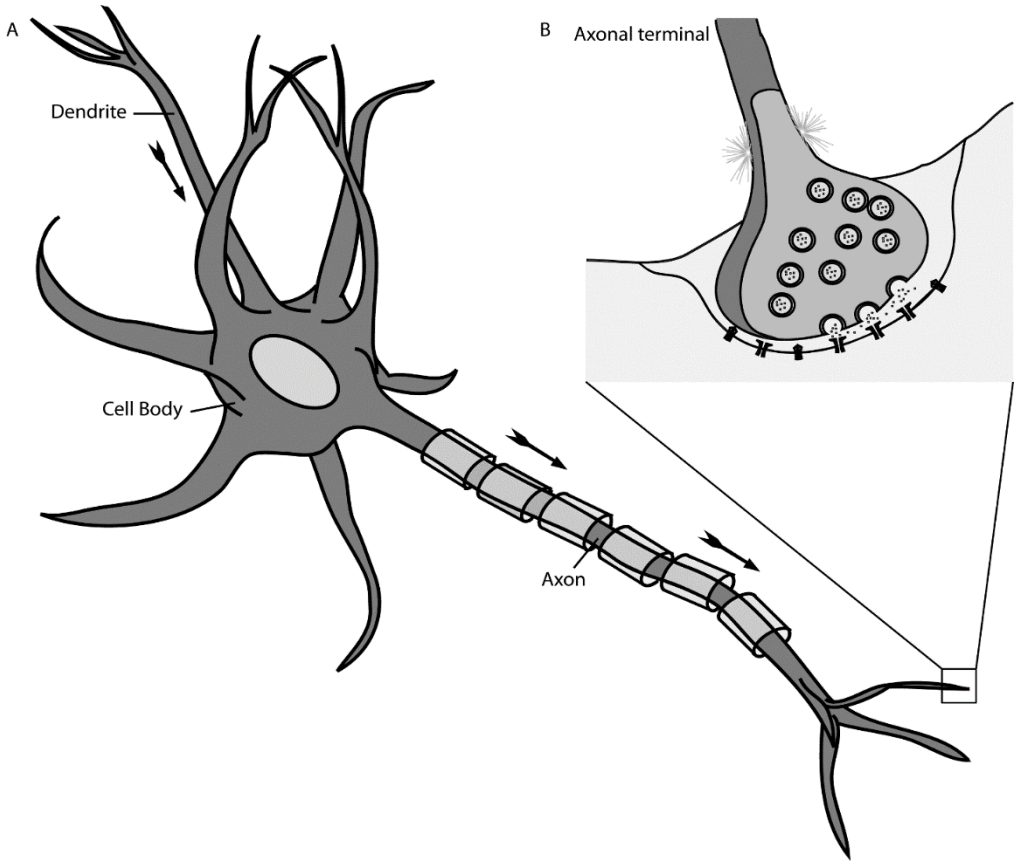


Figure I-1 Neuronal anatomy
Neuronal cells are composed of a cell body, a single extension called axons, and on multiple extension called dendrites. Information flows from the dendrites to the axon, with a highly specialized structure called the synapse connecting different neurons.

A few years later in 1891 Waldeyer would coin the term “neuron” (Figure I-1 A) as we use it now in Modern Neuroscience and in 1897 Sherrington would define that individual neurons are connected by a highly specialized structure called a synapse (Figure I-1B) (Lopez-Munoz et al., 2006). At these specialized cell structures the cell membrane of a presynaptic neuron is in close apposition to the cell membrane of a target neuron, the postsynaptic neuron, and information/electrical impulses flow from the presynaptic synaptic neuron to the postsynaptic neuron. The synaptic transmission can be chemical, with molecules called neurotransmitters being released from the presynaptic synaptic bouton and binding to receptors in the postsynaptic neuron and potentially initiating an electrical response and/or activating cellular

pathways. The synaptic boutons localized at the end of an axon are called axon terminals. Electrical impulses are conducted away from the cell body into the axon terminals that through chemical or electrical synapses transmit an impulse to a juxtaposed neuron through their dendrites.

After defining a neuron the next step is to review the way these specialized cells connect with each other during development, in order to ultimately consider how neural circuits are responsible for higher functions such as vision or human behavior. This is one of the most prominent questions in modern neuroscience and has been the focus of many scientists, although it is out of the realm of this thesis.

B. The development of neural connections

The functionality of neural circuits depends on the establishment of precise stereotyped connections between neurons. Most neural circuits are established and refined during specific time periods during nervous system development. A neuron must send projections (dendrites or axons) to a target and form synapses with another neuron or other cell type, such as the neuromuscular junction where a neuron's axon targets and connects with muscle cells. The process of projecting to a target and establishing a synaptic connection is controlled by multiple processes dependent on transcription factors, adhesion molecules and neural activity (Luo and O'Leary, 2005; McLaughlin and O'Leary, 2005). An interplay of intrinsic and extrinsic factors influences the determination of the neural identity of a progenitor cell and the differentiation of the neural progenitor into a specific neural cell type. Gradients of factors and spatial organization also play a role in determining a neuronal cell identity. From early development neural cells present polarity with one extension being determined as an axon and others as dendrites (Ruthel and Hollenbeck, 2003). Axons and dendrites extend away from the cell body in a specialized cell structure called a growth cone located at the tip of the growing axon. Neural outgrowth is dependent on trophic factors, environment molecular cues, physical properties, adhesion molecules, signaling pathways and cytoskeleton dynamics (Raper and Mason, 2010). Molecular gradients, guidance cues, interaction with guidepost cells and axon-axon interactions provide directional guidance information to the extending axons. Next, axons

must recognize a target, innervate it and extend axonal branches in the target. At the target axons form synapses with other cells, usually on the cells' dendrites. During the development of neural connections, an excessive number of neurons, branches and synapses are formed. Later in a process of refinement of the neural circuit the neurons, projections and synapses that are not appropriate to the functional circuit are eliminated.

Electrical activity plays a crucial role in the structural and functional refinement of neural connections not only during the initial phase of development but also throughout an organism's lifetime (Ackman and Crair, 2014; Blankenship and Feller, 2010; Katz and Shatz, 1996). One crucial theory that relates circuit formation to activity is the Hebbian Theory postulated by Donald Hebb in 1949 in the book "The Organization of Behavior". In this book Hebb proposed: "Any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other." This hypothesis has been summarized in the principle "Cells that fire together, wire together". The Hebbian Theory or Hebbian learning established a basic principle for neural network assembly with activity modulating the structure and functional refinement of organized networks of neural connections not only in development but throughout an organism's lifetime. More recently, some authors have unraveled the mechanisms of circuit refinement by activity, identifying the activity-dependent regulation of the binding between pre-synaptic and post-synaptic adhesion molecules through the activity of local proteolytic activity on the post-synaptic bud (Peixoto et al., 2012). Nevertheless, while the role of activity in the refinement of the neural circuits is important, it is not sufficient to define and refine a neural circuit. Additional mechanisms such as the expression of adhesion molecules, establishment of molecular gradients and competition for the neurotrophic factors, are also crucial for the establishment of a neural circuit (Feldheim and O'Leary, 2010; Kaneko et al., 2008; Spalding et al., 2004) and the description of their role in development of the retinal projection will be expanded in further sections of this theses.

C. Transient neural projections

The development of neural circuits requires the formation of neural projections but not all projections persist to later stages of development and are eliminated. Of interest some

projections do not form randomly, but rather have a stereotyped anatomy that is consistent between members of the same species or even between evolutionarily-related species. The presence of these stereotyped transient projections suggests an underlying a mechanism for their formation that is consistent between individuals in the same species and not the merely the result of randomized processes. Nevertheless it is still unclear whether these stereotyped projections have a biological function, particularly in the early development of neural circuits, or rather the result of a developmental environment still undergoing refinement.
While the dynamics of the retinal projections through development will be described in subsequent chapters, several other examples of transient projections in neural development will be described here in different models and species.

D. Examples of transient axonal projections

Transient postmamillary component of the rat fornix

Stanfield et al. used anterograde and retrograde tracing techniques (Fast Blue and wheat germ agglutinin (WGA) conjugated to horseradish peroxidase) to study the development of the fornix in albino rats (Stanfield et al., 1987). Neurons from subicular complex of the hippocampal region project axons to the mammillary nuclei through the fornix, one or two days prior to birth. Before the fornix axons innervate the mammillary nuclei, a prominent group of fornix neurons project beyond these nuclei, overshooting their final target. These postmamillary axons continue to grow to the midbrain and pontine tegmentum during the first week after birth while sending elaborated collaterals to the mamillary nuclei. One to two weeks later the postmamillary component of the fornix projection becomes progressively smaller until it is eliminated whereas the projections to the mammillary nuclei remain and arborize. Since most of the cells of origin of the postmamillary component of the fornix survive during the period of elimination of the postmamillary component of the fornix, it has been proposed that the axons of the fornix that innervate and form arborizations within the mamillary nuclei, originate as collaterals from axonal projections that overshoot the final target during development, instead of representing the projections of neurons that die during development. While the mammillary projections survive into the adult circuit, the distal postmamillary component is subsequently

eliminated. The formation of a transient postmamillary component of the fornix during development has also been described in cats (Nauta, 1958).

Transient axon branching in layer 5 cortical neurons

During the development of axonal projections, an axon might branch to multiple targets in its pathway and subsequently eliminate the branches that do not project to targets appropriate for their function. An example of this strategy is the subcortical projection of layer 5 neurons in mice. Layer 5 neurons extend a primary axon along a stereotyped pathway through the subcortical cortex into the spinal cord. After this initial axonal extension to the spinal cord, the primary axon forms collateral branches into different subcortical targets. Subsequent to this period of extensive branching, the collateral branches are selectively eliminated. The elimination is correlated with the functional role of layer 5 neurons. For instance, the layer 5 neurons from the motor cortical area eliminate their collaterals unrelated to motor function, such as the collaterals that transiently project to visual subcortical areas (Stanfield and O'Leary, 1985). A similar process was described in hamsters (O'Leary and Stanfield, 1986). It is not clear whether this axon collateral elimination occurs by degeneration or other processes such as axonal retraction.

The precise molecular mechanism of collateral elimination in the layer 5 neurons, whether by degeneration or other process such as axonal retraction, remains elusive. Other authors suggest that Otx1 is associated with this process. Otx1 is a homeodomain transcription factor that is expressed in mouse subcortically projecting layer 5 neurons, but not in the neuronal population that projects intracortically (Weimann et al., 1999). This protein undergoes a nuclear translocation from the cytoplasm coincident with the period of refinement and elimination of layer 5 neuron collaterals. Weimann et al. observed the failure of the layer 5 visual cortex neurons to eliminate the normally transient projections to the spinal cord in the Otx1 KO mice, a target not appropriate to their function. These observations suggest that the Otx1 transcriptionally regulates genes responsible for the dynamics of axonal pruning and elimination. These findings in layer 5 cortical neurons support the general hypothesis that the elimination of transient axonal projection might be transcriptionally regulated.

E. Mechanisms of elimination of transient neurons and neural projections

Programmed cell death during development

Programmed cell death (PCD) is the death of a cell by an intracellular molecular program, that could be an apoptotic or autophagic, leading to the elimination of a cell. PCD is a natural process during neural development that occurs in both proliferating and post-mitotic neural cells (Yamaguchi and Miura, 2015). PCD elimination of neuronal cells during development is a way to refine the number of neurons in the nervous system and neuronal projections. In the retina, studies have shown the role of PCD in the elimination of retinal neurons projecting to inappropriate topographical sites in developing avian visual systems (Clarke, 1992). To support these ideas, in *Drosophila* inhibition of the apoptotic cascade during development prevents the regression of inappropriate neural projections in different systems (Buss et al., 2006).

Caspases and the elimination of projections

Developing neurons can eliminate or retract inappropriate projections without going through PCD. A long list of molecules are involved in PCD, but one of the most studied are caspases. Caspases (cysteine-aspartic proteases) are a family of protease enzymes with an important role in apoptosis. It is possible to divide the apoptotic caspase family into two types: the initiator and the effector. Initiator caspases, such as Caspase 2, -8, -9 and -10, cleave the inactive pro-forms of the effector caspases, and activate them. On the other hand, effector caspases, such as Caspase 3, -6 and -7, cleave other protein substrates to trigger the apoptotic cascade that result in the death of a cell (McIlwain et al., 2013). The expression of caspases in the cell body has been studied for years as a mechanism of elimination of neurons, but recently a new role for the caspase family has been proposed during the development of the neural circuits: caspases play a role in the refinement of the neural projections through their local activity within axons, as opposed to the cell body. In this thesis we will focus on the role of caspases in the elimination of axonal projection and not on their role in PCD.
The local activation of caspases within specific neural projections is involved in axonal degeneration, arborization, and dendrite pruning in normal development and disease (Campbell and Okamoto, 2013; Nikolaev et al., 2009; Schoenmann et al., 2010; Simon et al.,

2012). Caspase-3 and Caspase-6 knockout mice present a delay in the natural developmental pruning of inappropriate RGC axonal ramifications in the superior colliculus which suggests that both Caspase-3 and Caspase-6 are involved in the axon degeneration that occurs normally during development (Simon et al., 2012). *In vitro* studies suggest that the local activation of Caspase-3, even at low levels, activates Caspase-6 and that the latter acts as an effector for the elimination of inappropriate axonal projections (Simon et al., 2012). In *Xenopus*, the local interaction between caspase6 and Slit-Robo seems to play a role in arborization through the local increase in microtubule instability that would facilitate the formation of axonal ramifications (Campbell and Okamoto, 2013).

F. Visual system

In this and the following sections, the model system used to study transient neuronal projections, the visual system, will be described, from anatomical and developmental perspectives.

The retina

The retina is a light sensitive layered tissue located at the back of the eye. The retina senses light and initiates a cascade of chemical and electrical processes that ultimately transmits visual and non-visual information to the brain. Different types of highly specialized neurons are involved in this process. In adult rodents, these neurons are organized into distinct layers (Badea et al., 2009b). Their position from the basal (closest to the lens) to apical sides is:

(1) Ganglion cell layer, containing the RGC cell bodies and axons. These are the axons that project from the retina to the brain.
(2) Inner plexiform layer, containing the synapses between the bipolar cell axons and the dendrites of the ganglion and amacrine cells.
(3) Inner nuclear layer containing the cell bodies of the horizontal, bipolar and amacrine cells;
(4) Outer plexiform layer, containing the synapses between the photoreceptors and the bipolar cells dendrites.
(5) Outer nuclear layer, containing the cell bodies of the photoreceptors, rods and cones.

(6) Rods and cones layer, containing the photoreceptor Inner and Outer Segments.
(7) Pigment epithelium containing retinal pigment epithelial (RPE) cells. This epithelium has an important role in light absorption, spatial buffering of ions, visual cycle factors, and phagocytosis of the outer segment of photoreceptors.

Müller glia or Müller cells are the principal glial cells of the retina spanning across the entire thickness of the neural retina. These cells modulate neuronal activity by controlling the extracellular milieu, provide mechanical support of the neural retina and have a relation of metabolic symbiosis with the retinal neurons (Bringmann et al., 2006).

Each neural cell type in the retina can be divided in distinct morphological, functional and molecular subtypes (Sanes and Masland, 2015).

Early development of the eye and retina

The vertebrate eye originates from different embryonic tissues: the retina and the epithelial layers of the iris and ciliary body originate from the anterior neural plate (Fig. III-2 orange and yellow), while the lens and cornea are derived from the surface ectoderm (Fig. III-2 green) (Graw, 2010). The coordinated activation of transcription factors and inductive signals at the correct time and position ensures the correct development of eye components. The expression of eye-field transcription factors define the area in the anterior neural plate where the eye field will be positioned. The group of eye-field transcription factors includes homeobox genes such as Pax6, Six3, Six6, and Lhx2.

Following eye field formation, the neuroepithelium of the ventral forebrain invaginates, resulting in the formation of two bilateral optic vesicles connected to the brain's ventricles by the optic stalk. The neural retina develops from the inner layer of the invaginated optic cup, and the retinal pigment epithelium (RPE) is derived from the outer layer. The space between these two layers is called the sub-retinal space. In mice, the sub-retinal space is connected to the brain ventricles until E11.5-E12.5 (Fuhrmann, 2010; Mann, 1964).

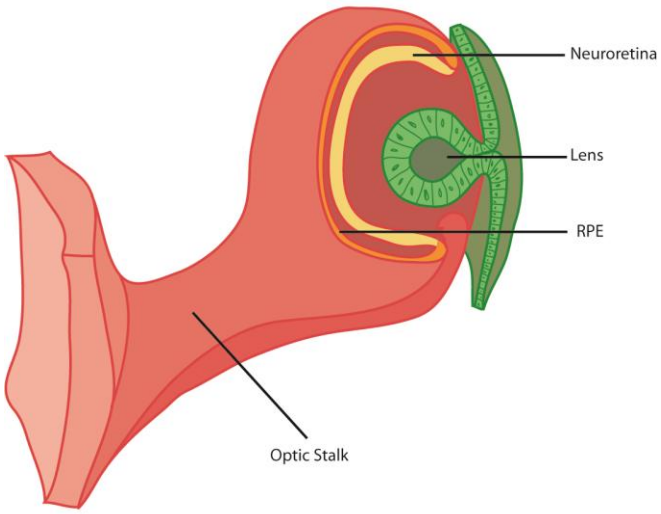


Figure I-2 Development of the optic cup from the anterior neural plate in orange and yellow and from the surface ectoderm in green.
Based on Ida Mann, 1964.

New findings using tridimensional embryonic or induced pluripotent stem-cell cultures have shown that the optic cup morphogenesis is a self-organizing process. These 'retina in a dish' cultures were able to replicate most of the dynamics of invagination of the optic cup in vitro as further processes in retinal development (Eiraku et al., 2011; Zhong et al., 2014).
Multiple molecules are expressed in the optic cup contributing to morphogenesis, patterning, cell proliferation, cell specification and differentiation: Pax6 is a gene crucial for multiple stages of eye development. It is required for eye morphogenesis, patterning, activation of tissue-specific genes, neuronal cells specification and differentiation, and interaction with other regulatory pathways (Shaham et al., 2012).
FGF signaling has a role in neurogenesis in the retina of non-mammalian vertebrates, such as chick and fish. The onset of neurogenesis in the retina is triggered by FGF that generates the first neurons: the RGCs in the central retina (Martinez-Morales et al., 2005). Retinal neurogenesis starts in the inner layer of the optic cup in the central retina (Drager, 1985) through symmetric and asymmetrical cell division of progenitors with subsquent interkinetic cell migration in the progenitor layer (Eiraku et al., 2011). These post-mitotic RGCs express and

release Shh, further propagating the differentiation wave for the eye (Esteve and Bovolenta, 2006). In mice, the disruption of FGF signaling leads to the failure of the optic fissure to close, forming a coloboma; a decline in progenitor's cell proliferation in the temporal retina, and a reduction of the number of RGCs, most evident in the temporal retina in non-albino mice (Chen et al., 2013).

G. Development of retinal ganglion cells

RGCs are neurons located in the basal side of the retina. RGCs are the first neurons to differentiate in the retina, forming a three-layered retina with a ganglion cell layer, a progenitor layer and the RPE, in mouse. These neurons are born in an organized centrifugal pattern with the first RGCs being born in the dorsocentral (DC) retina at E10.5 and with additional RGCs being born in the more peripheral retina until near the birth (Drager, 1985).
RGCs receive input from bipolar and amacrine neurons that receive input from photoreceptors on the apical side of the retina. RGCs receive both image forming and non-imaging forming information within the retinal circuit. A special subset of RGCs are able to perceive light and are called intrinsic photosensitive RGCs (LeGates et al., 2014; Mure et al., 2016).
Math5 is a basic helix-loop-helix transcription factor transiently expressed in retinal progenitors during early histogenesis of the retina and important for establishing retinal progenitor cell competence for a RGC fate (Mu et al., 2005). The exact role of Math5 in RGC specification is still being studied and updated with the use of new techniques (Brzezinski et al., 2012).

Genes expressed in retinal ganglion cells during development

a) Brn3b

Brn3b is a POU-homeodomain transcription factor that acts downstream of Math5 and it is expressed in the early post-mitotic RGCs (Pan et al., 2008). Brn3b is necessary for RGC terminal differentiation, axon outgrowth, and cell survival (Erkman et al., 2000; Shi et al., 2013). Nevertheless, in Brn3 KO mice RGCs are capable of being generated but 80% undergo apoptosis before birth (Gan et al., 1999). Brn3b is a common marker used to identify and label RGCs.

b) Islet1

Islet1 (Isl1) is a LIM-homeodomain transcription factor expressed in RGCs later than Brn3b, and also important for the determination of RGC cell fate (Pan et al., 2008). The deletion of Isl1 leads to the apoptosis of near 67% of the RGCs (Pan et al., 2008). Isl1 is another common marker for identification and labeling post-mitotic RGCs in mammals and non-mammals (Pittman et al., 2008). However, the available Isl1 antibodies do not have optimal specificity, so we have used an antibody that was produced by the Jessell lab (Columbia. University) that is specific for Isl1 but also recognizes Isl2 and thus is referred to as the Isl1/2 antibody. This has better technical performance and has been used in the present studies to identify RGCs during development.

H. The retinal pathway

The axons of RGCs connect the retina to the brain. RGC axons project outward from both eyes through a cylindrical nerve, the optic nerve (ON). Both optic nerves meet at the ventral midline in a structure called the optic chiasm (OC). Caudal to the optic chiasm the RGC axons project to their targets through a tract running superficially on the thalamus and midbrain called the optic tract (OT). From the OT, the RGCs project to their targets in the thalamus and midbrain, with the exception of the RGCs that project into the suprachiasmatic nucleus, located dorsally to the OC.

In the next sections of this thesis, the development of the projections from the eye to the brain will be described.

Guidance of retinal ganglion cells within the retina

RGCs organized in the most superficial retina extend their axons into the optic fiber layer at the inner surface of the retina. Here they grow in a highly direct, radial fashion towards their exit point from the eye at the center of the retina, at the optic disc, to the brain. When growing axons reach the optic nerve head, their growth cone assumes a different morphology than when they are growing in straight paths such as the optic nerve or tract. Then when they reach other decision points or new environments, such as at the optic chiasm or their targets growth

cones greatly increase in size, surface area, and morphological complexity, tipped with multiple filopodia and lamellipodia. Such increasing complexity of growth cone morphology is associated with changes in direction and extension, usually at "decision points" or intermediate targets (Bovolenta and Mason, 1987; Godement et al., 1994; Holt, 1989; Hutson and Chien, 2002). Multiple molecules are implicated in the retinal projection towards the optic disc in the basal lamina, such as chondroitin sulfate proteoglycans that are repulsive and prevent axons from growing to the peripheral retina, receptor tyrosine phosphatases, proteins of the Ig superfamily, Netrin1, and Slit–Robo signaling (Erskine and Herrera, 2007, 2014). Also, the Shh high central–low peripheral gradient in the retina has an important role in the promoting growth towards the optic disk (Kolpak et al., 2005). Intra-retinal fasciculation of the RGCs axons in bundles is also an important mechanism to guide the RGCs axons toward the optic disk, and when disturbed intra-retinal pathfinding errors occur (Marcos et al., 2015; Muhleisen et al., 2006). The interaction between netrin1 and deleted in colorectal cancer (DCC) is also important for RGC axon exit through the optic disc, since the RGC axons of mice lacking either netrin-1 or DCC project correctly to the optic disc but are unable to exit the eye (Deiner et al., 1997). Other factors, such has EphBs, BMP receptor 1B and NrCAM also play a role in the targeting of RGC axons to the optic disc and exit from the eye (Erskine and Herrera, 2007).

From the retina to the optic chiasm

The first RGC axons reach the optic nerve at E13.0 (Colello and Guillery, 1990, 1992). At this early stage (E14-15), the ipsilateral RGC axons are present in virtually all fascicles of the developing optic nerve, intermingling with the contralateral RGC axons. At later stages of development (E16-17), although ipsilateral RGC axons pass predominantly within the temporal part of the stalk, they remain intermingled with contralateral axons. A significant number of ipsilateral RGC axons also lie within the nasal part of the optic stalk (Colello and Guillery, 1990). The RGC axons occupy most of the optic nerve but as they approach the optic chiasm their growth cones come to lie predominantly close to the pial surface (Colello and Guillery, 1992).

Multiple factors along this pathway keep the RGC axons at the correct position inside the optic nerve pathway. For instance, Sema5A seems to have a role in helping ensheath the retinal

pathway probably through its inhibitory response in the context of L1, laminin, or netrin 1 signaling, while Shh expression at the chiasm border defines a restricted pathway within the ventral midline guiding the progression of RGC axons (Trousse et al., 2001). Furthermore, the Vax1 KO mice present alterations in this pattern of expression of Shh as a barrier around the optic nerve in the ventral diencephalon and their RGC axons form whorls of fibers at the distal end of the optic nerve (Bertuzzi et al., 1999; Hallonet et al., 1999).

The optic chiasm – a decussation point for ipsilateral and contralateral axons

RGC axons exit the optic stalks into the ventral diencephalon and either grow ipsilaterally or contralaterally to form an X shaped pathway at the optic chiasm (OC). Multiple molecules are expressed in the ventral diencephalon such as: Pax2, Vax1, Nkx2.1, Nkx2.2, Dlx2, FoxD1, Six3, Shh, and others, some of which might contribute to the formation of the optic chiasm (Marcus et al., 1999), but the exact role of these patterns of transcription factor expression is still elusive.

The region where the optic chiasm is formed consists of two major cell types, encountered by growing RGC axons: (1) Radial glia cells at the base of the third ventricle extend processes that drape the midline of the chiasm that express glial markers such as RC2, BLBP, and GLAST (but not GFAP) during the period of RGC axon growth, from E12 to P0; and (2) SSEA-positive early born neurons that develop caudally to the chiasm and extend to the ventral midline (Petros et al., 2008). As axons interact with these cells, they are thought to react to the molecules on the surfaces of these cells that provide directives to cross or avoid and turn to the same side of the brain to form the optic tracts.

Development of the ipsilateral and contralateral retinal projections in mice

During the development of the mouse visual circuit, RGCs located in the ventrotemporal (VT) crescent of the retina project to the ipsilateral side of the brain while RGCs outside this crescent project contralaterally at E14.5 to E16.5. After E16.5, contralateral RGCs also project from the VT crescent. RGC axons diverge ipsilaterally or contralaterally at the ventral midline of the brain in an x-shaped structure called the optic chiasm (Petros et al., 2008). The molecular mechanism that determines the cell fate of ipsilateral and contralateral RGCs and the adhesion molecules

that regulate the dichotomous decision of 'to cross' or 'not to cross' the ventral midline have been partially described (see further sections in this thesis). However, during the early phase of eye development, from E10.5-13.5, the first RGCs to be born are clustered in the DC retina in a mixed population of both contra- and ipsilaterally-projecting RGCs (Colello and Guillery, 1990; Drager, 1985; Guillery et al., 1995). These DC RGCs project to the optic nerve and are the first axons reach the optic chiasm between E12.5 and E13.0 (Marcus and Mason, 1995). The first ipsilateral RGCs to project to the optic tract (OT) take a more lateral course in the OC while the ipsilateral RGC axons from the VT interact with the medial area of the OC making an almost 90° turn when they project to the OT. The behavior of ipsilateral and contralateral axons at the OC observed with live imaging consisted of different rhythms of axonal progression at different ages. Sretavan and Reichardt observed in time-lapse studies *ex vivo* at E13.5, that the ipsilaterally projecting RGC axons make turning decisions without pausing over 10-20 min, whereas contralaterally projecting axons occasionally pause before crossing the midline (Sretavan and Reichardt, 1993). Godement et al (1994) also performed *ex vivo* live imaging of the RGC axons projection both in early growing fibers (E13-14) and later-growing fibers (E15-16). Most of their description focused on the later-growing ipsilateral RGC axons labeled from the VT retina. These authors observed the 'saltatory' nature of the RGCs axonal growing with both ipsilateral and contralateral RGC axons pausing for a longer time at the ventral midline (30min-6h) at E15-17. The shape of the axonal growth cones varied with the rhythm of progression with the 'advancing' axons presenting an arrow-like growth cone and the pausing axons presenting a more complex morphology (Godement et al., 1994; Mason and Wang, 1997). The authors suggested that this more lateral course and the continuous progressive growth of ipsilateral RGCs at the OC could justify why the DC retina RGCs are the first to reach the OT, before their contralateral counterparts. Interestingly, these ipsilateral RGCs that arise from the central retina are transient (Colello and Guillery, 1990; Petros et al., 2008).

Little is known about the fate, projection and molecular profile of this population of transient ipsilateral RGC axons from the central retina. The purpose of this thesis is to approach some of the questions that remain in the field on the neurodevelopment of this transient visual projection.

Transient retino-retinal pathway

Instead of projecting from the optic nerve to the chiasm and then to optic tract, some RGC axons take an alternative pathway after the optic chiasm and project to the contralateral optic nerve. These retino-retinal projections are also transient and are present in the normal development of the rodent retinal projections, disappearing before birth (Bunt and Lund, 1981).

The development of the human and primate visual projections

In humans, the eyes are positioned more frontally located when compared with other species such as fish, birds and rodents. During development, the human eyes recapitulate the evolution of the visual angles through species, as described by Ida Mann (Mann, 1964).

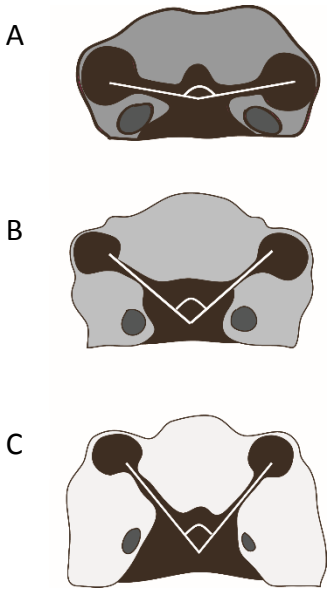


Figure I-3 The orientation angle between the eyes decreases through the human embryonic development.
Ida Mann described in her book "The development of the human eye" (1964) the variation of the binocular angle during human embryonic development, with the angle between the eyes decreasing from a more lateral eye position in early ages (A) to a full frontal position in adult (C).

In adult humans, the ipsilaterally projecting RGCs occupy ~40% of the temporal retina (Petros et al., 2008), but it is not clear what is the percentage of ipsilateral and contralateral RGCs projecting from the retina during the embryological human development, recapitulating the

increase of ipsilateral cells RGCs with the frontalization of the visual field observed throughout evolution (Mann, 1964).

The fiber organization in the human optic chiasm differs from rodents and other species in particular in the organization of the ipsilateral RGC axons. In most species the ipsilateral RGC axons reach the optic chiasm midline and turn to the ipsilateral optic tract, while in humans the ipsilateral RGC axons from the ventrotemporal and nasotemporal retina remain in a more lateral position at the chiasm (Neveu and Jeffery, 2007). Marsupials share with humans this characteristic more lateral position of the ipsilateral RGC axons at the optic chiasm (Neveu et al., 2006).

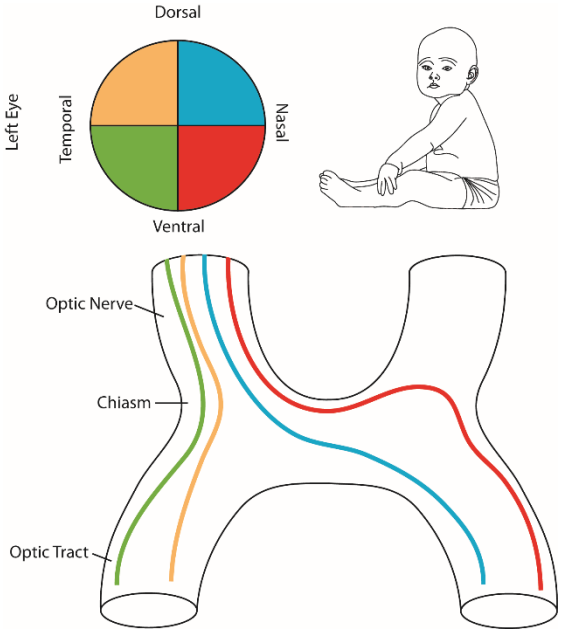


Figure I-4 Visual projections to the chiasm in humans
The human chiasm differs from the mouse chiasm. While the mouse ipsilaterally projecting RGCs interact with the medial most chiasm, in humans these retinal ganglion cell axons keep a more lateral position in the chiasm. Based in Netter Atlas of Human Anatomy, Flashcard 2E, Elsevier.

The access to human embryonic samples to study the anatomy of the optic projections is limited. Many studies on the developmental anatomy of the visual pathway in primates come from data from monkeys, such as the rhesus monkey, first using the Marchi technique of lesioning a retinal area and study the subsequent axonal degenerative process to understand

the retinal projections (Hoyt and Luis, 1962) and later using tracers such as horseradish peroxidase (HRP) or DiI (Meissirel and Chalupa, 1994).

The size of the ipsilateral retinal projection to the brain varies among species

Adult fish, birds and pre-metamorphic frogs have only contralateral projections from the retina to the brain visual targets (Petros et al., 2008). Nevertheless, amphibians such as *Xenopus laevis* (African clawed frog), *Rana pipiens* (Northern leopard frog) and *Rana esculenta* (edible frog) develop ipsilaterally projecting RGCs after metamorphosis in a process that is dependent on thyroid hormone (Hoskins, 1986). While these are similar members of the Class Amphibia and Order Anura there are some variations in their eye position that translates in different position of the population of ipsilateral RGCs in the retina: *X. laevis* have their eyes at the top of their heads and more ventral ipsilateral RGCs in the retina, while the eyes in *R. pipiens* and *R. esculenta* are more laterally positioned and the ipsilateral RGCs are more temporally positioned (Hoskins and Grobstein, 1985).

Mice also have a reduced area in the ventrotemporal retina projecting ipsilaterally, in adults, occupying ~3-5% of the retina, and intermingled with the contralaterally projecting RGCs population in the retina (Guillery et al., 1995). Other mammals, such as cats and ferrets, and marsupial have a broader ventrotemporal retina area projecting ipsilaterally to the brain, with ~12%-15% of RGCs projecting ipsilaterally. In primates the ipsilateral/contralateral RGCs proportion is 50/50 (Petros et al., 2008).

The frontalization of the eye position correlation with an increased proportion of the ipsilateral component has been associated with an adaption that increases depth perception as the eyes move to a more frontal position (Petros et al., 2008). Two theories support the adaptive value of the primate frontal eye-field: (1) the convergent eye field improves the depth perception of objects and (2) gives a more accurate visual control of the hands (Larsson, 2013).

After the optic chiasm, RGC axons travel along the lateral surface of the neuroepithelium in the diencephalon, forming the optic tract (OT). The organization of the RGC axons in the OT will be described in further sections of this thesis.

Molecular determinants of RGC laterality

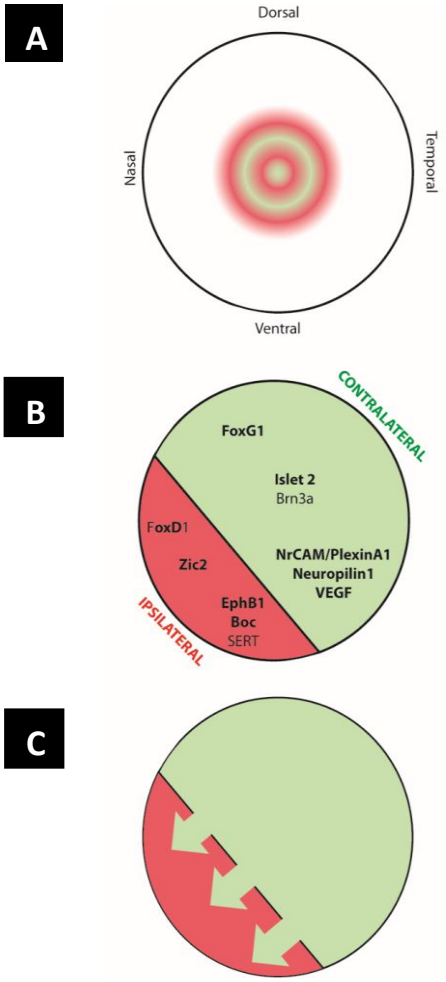


Figure I-5 Molecular determinants of laterality
A-Retina at E11.5 when the first RGCs are born in the central retina in both ipsilaterally (red) and contralaterally (green) projecting RGCs intermingled in the dorsocentral retina. B- At E14.5 the retina is divided in two molecular domains one expressing genes associated with the fate of RGC that project ipsilaterally in the ventrotemporal retina (VT), and RGCs that project contralaterally. C -After E17.5 the genes that determinate the fate of contralateral RGCs are also expressed in the VT area.

Multiple molecules are involved in the establishment of the binocular visual projections. Early in development of the eye, transcription factors in the retina establish territories that later will define the areas occupied by RGCs that project contralaterally or ipsilaterally. Other transcription factors act as master regulators that trigger a cascade that upregulates or

downregulates the expression factors such as adhesion molecules or regulators of activity-dependent refinement. These factors when expressed either in ipsilateral or contralateral RGCs define their identity, laterality and final refinement at an appropriate area at the target.

Patterning in the retina with regard to laterality of projection - Fox genes

In mouse, Foxg1 (BF-1) and Foxd1 (BF-2) winged helix transcription factors are expressed in complementary territories in the early development of the retina (Hatini et al., 1994), defining the nasal and temporal areas of the retina. These two transcription factors have been suggested to play a role in the determination of the ipsilateral and contralateral territories in the retina and activating molecular pathways that establish the ipsilateral or contralateral projections.

Foxd1 is expressed in the temporal retina around the time that the first ganglion cells are born (E11.5) and in the ventral diencephalon during the formation of the optic chiasm. Foxd1 acts upstream of the genetic program associated with the ipsilateral RGC identity and midline crossing: Zic2 and EphB1 (Herrera et al., 2004), and topographical position of ipsilateral RGCs at the rostral superior colliculus (Carreres et al., 2011) (see in later sections). In contrast, Foxg1 is expressed in the nasal retina and ventral diencephalon (Marcus et al., 1999) and it has been suggested to contribute to the determination of the contralateral RGCs territory. Foxg1 knock-out mice present an increase in the ipsilateral retinal projection to the brain suggesting that Foxg1 represses the ipsilateral pathway (Pratt et al., 2004; Tian et al., 2008). The exact downstream pathway of Foxg1 is still under study (Fotaki et al., 2013). One hypothesis is that Foxd1 and Foxg1 interactions establish complimentary territories in the retina, one of the functions being to set up the ipsilateral and contralateral RGC projections.

In zebrafish, it was shown that the opposing signaling of ventral Shh and dorsal Fgf precedes and determines a FoxG1/FoxD1 opposing gradient (Hernandez-Bejarano et al., 2015). The opposing Shh and FgF signaling as an initiator of nasotemporal patterning has not been studied in mice, in our knowledge.

Contralateral retinal ganglion cells

a) Transcription Factor Islet2

Islet2 (Isl2) is a LIM homeodomain transcription factor expressed in the developing retina exclusively in contralateral RGCs, but only in about 30% of this population. Using a Isl2 tau-lacZ knock-in mouse, Pak at al., show that contralaterally projecting RGCs were affected, and only in the VT retina (Pak et al., 2004). Isl2 is expressed in a non-overlapping manner with the ipsilateral RGCs marker Zic2, in late-born RGCs that project contralaterally from VT retina. Isl2 KO mice have an increased ipsilateral retinal projection as a consequence of an increased number of ipsilateral RGCs projecting from the VT retina where later-projecting contralateral RGCs should be specified. These results support the hypothesis that even with the expression of Isl2 in most the contralateral RGCs in the retina, the expression of this transcription factor is important for the laterality of the later born contralaterally projecting RGCs within the VT retina (Pak et al., 2004).

b) Transcription Factor Brn3a

Brn3a is a POU-domain transcription factor. Like Isl2, Brn3a is expressed in a non-overlapping way with the ipsilateral RGC marker, Zic2, being expressed mostly in contralateral RGCs. Evidence shows that Brn3a is not functionally involved in the laterality of contralateral RGCs, since known mediators of the laterality of RGC axons are not re-patterned in the absence of Brn3a (Quina et al., 2005; Shi et al., 2013).

c) Adhesion Molecules: NrCAM, PlexinA1 and Sema6D

NrCAM is a member of the L1 family of cell adhesion molecules, selectively present on RGC axons and absent from the soma (Zelina et al., 2005). NrCAM is expressed in RGCs in retinal areas that project contralaterally and it is critical for the guidance of late-born RGCs from the ventrotemporal retina, promoting their growth at the optic chiasm. NrCAM is also expressed at the optic chiasm (Williams et al., 2006). It is unknown whether the early central retina ipsilateral RGCs axons also express NrCAM. In addition to NrCAM, Plexin A1 is also expressed in all contralateral RGCs. NrCAM and PlexinA1 in contralateral RGC axons interact at the optic chiasm with three molecules: Semaphorin6D (Sema6D) and NrCAM expressed on midline radial

glia; and Plexin-A1 on chiasm neurons. When confronted with semaphorin alone (in HEK cells), RGCs from contralateral regions of the retina are inhibited in their growth, but NrCAM, along with Plexin-A1 converts this inhibition to growth promotion. The triple combination/interaction of these three molecules (NrCAM/PlexinA1/Sema6D) is crucial *in vivo* for contralateral RGCs axons to fasciculate and to cross the midline at the optic chiasm (Kuwajima et al., 2012).

d) Neuropilin1/VEGF

A second, parallel system appears to function for midline crossing: Neuropilin1 is a transmembrane glycoprotein that acts as a co-receptor for a number of extracellular ligands and it is expressed in contralateral RGCs. Neuropilin1 has been demonstrated to interact with VEGF164 at the mice optic chiasm to provide growth-promoting and chemoattractive signals, facilitating the crossing of contralateral RGC axons to the opposite side of the brain. VEGF164 is a neuropilin-binding isoform of the classical vascular growth factor VEGF-A (Erskine et al., 2011).

Ipsilateral retinal ganglion cells

a) Transcription factor: Zic2

Zic2 is a member of the Zic family of zinc finger transcription factors (Zic1–5) which play an important role in early embryonic neural patterning and body midline formation (Merzdorf, 2007; Nagai et al., 1997; Pan et al., 2011), with genetic variants in ZIC1, ZIC2 and ZIC3 being described to increase the risk of neural tube defects in humans (Klootwijk et al., 2004). ZIC2 mutations are among the best known mutations associated with holoprosencephaly (Brown et al., 1998).

Zic2 is expressed early in the optic vesicle and stalk during the eyecup development but downregulated to lower levels by E10.5. Later from E14.5 to E17.5, Zic2 is transiently expressed in high levels in the ventrotemporal retina RGCs; and during the development of the ciliary margin zone (Nagai et al., 1997).

A series of experiments by Herrera et al. showed that Zic2 specifies the ipsilaterality of RGCs and it is necessary and sufficient to regulate RGC axon repulsion by cues at the optic chiasm midline. This transcription factor is only transiently expressed in ipsilateral RGCs, and Zic2

expression at the RGCs nucleus is downregulated after the axons tips cross the midline (Herrera et al., 2003).

Zic2 expression in RGCs is tightly correlated with the size of the ipsilateral projection within different vertebrate species, as it is absent in species lacking binocular vision, such as chick and zebrafish and it is upregulated during metamorphosis in Xenopus when they transition from a non-binocular tadpole to an binocular adult frog (Petros et al., 2008).

b) Guidance molecules: EphB1 and ephrinB2

EphB1 and ephrinB2 are key players in retinal axon divergence at the optic chiasm. EphB1 is member of the Eph tyrosine kinase receptors expressed in DC retina at E13.5 and E14.5 and in the ventrotemporal crescent from E14.5 to E15.5. EphrinB2 is a member of the ephrin tyrosine kinase receptors and it is expressed in the optic chiasm radial glia from E12.5 at low levels to a peak phase at E15.5 and decreases thereafter. The interaction between EphB1 in the ipsilateral axons and ephrinB2 at the optic chiasm generates a repulsive axonal cue that leads to the divergence of the ipsilateral RGC axons from the midline, contributing to formation of the ipsilateral optic tract (Garcia-Frigola et al., 2008; Lee et al., 2008; Petros et al., 2010; Williams et al., 2003). While the Zic2 hypomorph mice seem to lack EphB1 (Herrera et al., 2003), as do the Foxd1 KO mice (Carreres et al., 2011), EphB1 is not a direct target of Zic2 (Garcia-Frigola et al., 2008; Lee et al., 2008). Nonetheless, the work of Lee et al indicates that Zic2 and FoxD1 are upstream of EphB1 and activate the transcriptional pathway for EphB1 expression (Garcia-Frigola et al., 2008; Lee et al., 2008).

c) Other ipsilateral RGC factors: Sert, Boc

The serotonin transporter (Sert) is an integral membrane protein responsible for the efficient uptake of serotonin from the extracellular space expressed in the ipsilateral RGCs in the VT retina. Sert expression is under the direct control of the transcription factor Zic2 in the ipsilateral RGCs in the ventrotemporal retina. *Sert* plays a role in the modulation of activity-dependent mechanisms during the refinement of the ipsilateral RGCs extension at the targets (Garcia-Frigola and Herrera, 2010).

Boc is a cell adhesion molecule that acts as a high-affinity receptor for sonic hedgehog (Shh). Boc is expressed in the ipsilateral RGC axons in the ventrotemporal retina and it as two known effects on the ipsilateral RGCs: (1) it sustains the expression of Zic2 in ipsilateral VT RGCs in a mechanism that includes the requirement of Boc to restrain the expression of Shh and Islet2 in the ventrotemporal retina (Sanchez-Arrones et al., 2013), and (2) in the ipsilateral RGC axons it interacts with Shh at the optic chiasm resulting in a repulsive cue that causes ipsilateral RGC axons to avoid the chiasm midline (Fabre et al., 2010).

EphB1 is expressed in the DC retina and in the permanent ipsilateral RGCs that originate in VT retina, while BOC and Sert are only associated with the permanent ipsilateral RGCs from the VT retina.

From the optic chiasm to targets

After crossing the chiasm, RGC axons travel along the lateral surface of the neuroepithelium in the diencephalon, forming the optic tract (OT). At the dorsal and ventral lateral geniculate nucleus (LGN) and Superior Colliculus (SC), RGC axons are organized in a topographic map that is fundamental for the coherent representation of the outside world and an eye-specific segregated map that is crucial to binocular vision.

In mice, an important study by Godement et al. (1984) traced RGC axons from the retina to the brain, using the tracer HRP. Unfortunately, most of these descriptions of prenatal development came from observations of single embryos at E14.5, E16.5 and E18.5, a small sample size that can be explained by the low yield of the *in utero* injections of HRP with the surgical techniques used then (Godement et al., 1984). Still, most of the observations described by Godement were confirmed later by other authors (Jaubert-Miazza et al., 2005).

Organization of ipsilateral and contralateral retinal ganglion cells axons at the optic tract

Godement at al. described the ipsilateral and contralateral RGC axons segregation in the optic tract, after the optic chiasm and before the projection to the LGN, after E16.5 (Godement et al., 1984). The RGC axons projecting ipsilaterally occupy the more lateral optic tract while the RGC

axons projecting contralaterally occupy a more medial position in the optic tract. The same segregation of ipsilateral and contralateral RGC axons in found in the rhesus monkey optic tract (Meissirel and Chalupa, 1994). The mechanism of organization of the ipsilateral and contralateral RGC axons in the optic tract and whether their organization and/fasciculation plays a role in axonal targeting is currently under investigation (Austen Sitko and Carol Mason, unpublished).

Retinal ganglion cell axon targets in the brain

RGC axons target multiple targets in the brain. Dorsal to the optic chiasm, RGCs axons target the suprachiasmatic nucleus that regulates circadian rhythms. Caudally and dorsally to the optic chiasm the RGC axons target the thalamus, the LGN, which is subdivided into dorsal (dLGN) and ventral (vLGN) nuclei separated by the intergeniculate leaflet (IGL). The dLGN is a major recipient of visual information, whereas the vLGN processes non-visual information (Huberman et al., 2008). A major target of RGC axons is the superior colliculus (SC), a dorsocaudal brain structure in mice. The SC in mammals corresponds to the optic tectum in lower vertebrates, such as fish and frog. In these lower vertebrates the tectum is the major target of the retinal projections. While in mice, most of the RGC axons project to the superior colliculus (Hofbauer and Drager, 1985), in primates the dLGN is the main target of these neurons and less than 10% of fibers project to the SC (Rodieck and Watanabe, 1993). Other non-visual areas in the brain are targeted by RGCs: the olivary pretectal nucleus (OPT) in the dorsal midbrain, the nucleus of the optic tract (NOT), the medial (MPT) and the posterior (PPT) pre-tectal nuclei, the caudal end of the olivary pretectal nucleus, and the medial terminal nucleus (MTN) (Badea et al., 2009b).

Topographic organization at the target – organization of the ipsilateral and contralateral axons at the target

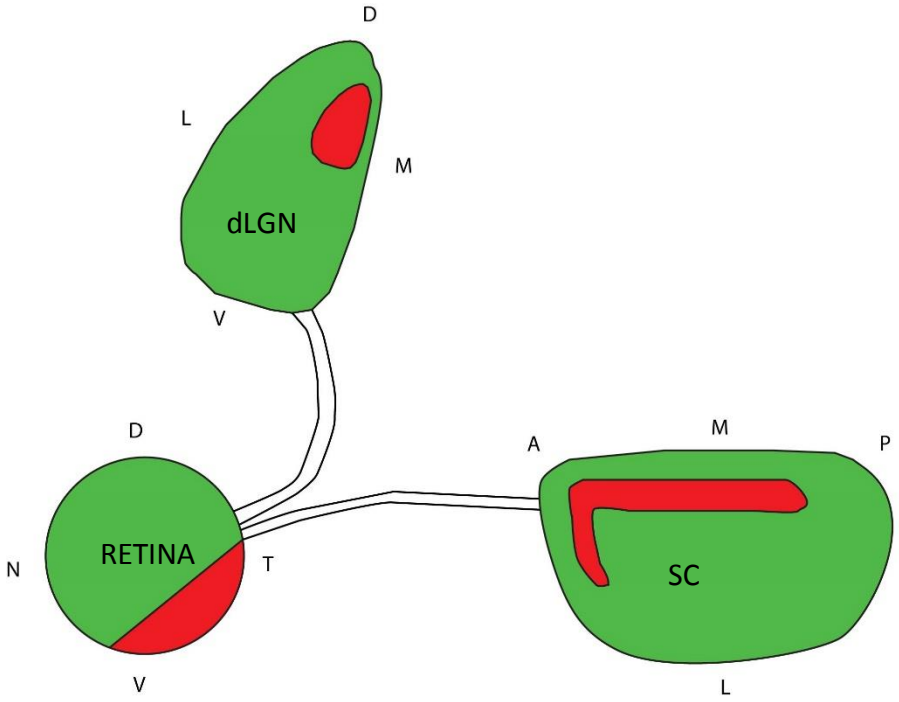


Figure I-6 Representation of the topographical organization of ipsilaterally and contralaterally projecting RGC axons in mice.
At the peak phase of axonal projection from the retina to the brain, RGCs from the ventrotemporal retina project ipsilaterally (red) while RGCs outside this area project contralaterally (green). Later, RGC axons in the ventrotemporal retina also project contralaterally. In adult mice, ipsilateral RGCs target a patch area in the dorsal Lateral Geniculate Nucleus (dLGN) while in the Superior Colliculus (SC) the ipsilateral RGCs occupy the more medial area and a rostral area expanding from the medial to the lateral SC. dLGN: Dorsal Lateral Geniculate Nucleus. SC: Superior Colliculus. Based in Cang and Feldheim, 2013.

The first RGC axons reach the OT after E14.5 and some axons reach the SC by E16.5.

dLGN: At E16.5, the primary RGC axons in the optic tract maintain their lateral position to the LGN, sending very few branches to the LGN. At E18.5 there is a progressive increase in the number of RGC axons in the optic tract and more RGC axons sending branches to the dLGN, but still not in a significant number (Godement et al., 1984). During the first postnatal week, crossed and uncrossed axons show substantial overlap throughout most of the LGN. Between the first and second week RGC axonal arbors show significant pruning. At P1, there is a

discordance between the ipsilateral and contralateral pattern of innervation of the dLGN: while the contralateral axons are distributed in the entire dLGN, the ipsilateral axons just start to invade the dLGN. At P1, the ipsilateral axons have simple morphology with few branches. Two days later, at P3, the ipsilateral and contralateral RGC projections overlap in a large area of the dLGN, while at P7 the contralateral axons retract from the ipsilateral area, leaving a patch innervated by ipsilateral RGC axons. The axonal ramifications at this stage of development are more complex than at P1, and with a preference to form complex branches at the future terminal zone. By the time of natural eye opening (P12–14) segregation is complete and retinal projections are organized into distinct eye-specific areas with extremely complex arborizations and no ectopic projections. (Jaubert-Miazza et al., 2005)

SC: Ipsilaterally and contralaterally projecting RGC axons are also segregated at the SC. These two populations target different layers and areas in the anteroposterior and mediolateral axis. Contralaterally projecting RGC axons target in the most superficial layer of the SC, the *stratum griseum superficiale*, while the ipsilateral RGC axons target a deeper layer, the *stratum opticum*. Furthermore, ipsilateral axons are distributed in an L-shaped patch, in the rostral SC, with ipsilateral axons extending along the mediolateral axis in the rostral SC where they form segregated patches and in the caudal SC, they project to a single medial area that extends caudally. RGC axons reach SC by E18 with simple RGC axons with no ramifications or very few until P0 (Godement et al., 1984; Sachs et al., 1986). As was described in the dLGN, in the SC there is an initial period of overshooting the appropriated targets in the SC (P1-3) , followed by a refinement of the axonal projection with elimination of “mistargeted” projections (P4-9) and arborization in the appropriate target area, until P14-21 (Sachs et al., 1986; Wu et al., 2000). Only at P3 do the ipsilateral RGC axons overshoot their projection to the *stratum griseum superficiale* and those mistargeted projections are eliminated by P8 (Godement et al., 1984; Wu et al., 2000).

Establishment of topographic maps at the target - EphA gradients in the topographical organization of the mouse visual system

As mentioned above, Ephs are receptor tyrosine kinases that bind to the ligand, ephrins. Since both Ephs and ephrins are membrane-bound proteins, the activation of the Eph/ephrin intracellular signaling is dependent on cell contact. Eph/ephrin signaling is bidirectional, with the ephrin interaction with Ephs classified as 'forward signalling' and the Eph induction of ephrin 'reverse signalling'.

Both Ephs and ephrins can be divided into two classes. Ephs can be subdivided into EphAs and EphBs and ephrins can be subdivided in ephrin-As and ephrin-Bs (1997; Klein and Kania, 2014). EphAs and EphBs are distinguished by sequence homology and pattern of binding to ephrins, while ephrins A and B are distinguished based on structure and function. Ephrin-As are glycosylphosphatidylinositol-linked to the membrane, whereas ephrin-Bs are transmembrane proteins with cytoplasmic domains with C-terminal PDZ binding motifs. The interactions between Eph and ephrins sub-families are promiscuous *in vitro*: each EphA can bind multiple ephrin-As, and each EphB can bind multiple ephrin-Bs (Gale et al., 1996), with some examples of crosstalk between EphAs and ephrinBs and EphBs and ephrinAs (Himanen et al., 2004).

In the rodent embryonic retina, EphA receptors are displayed in a temporal-nasal gradient, with the peak of EphA levels in the temporal retina. EphrinA is expressed in a complementary gradient to the EphA gradient, with the higher levels of ephrinA being expressed in the nasal retina. In the dLGN, SC and V1 area of the visual cortex, ephrin-A/EphAs are displayed along complementary retinotopic gradients. In the dLGN EphAs are distributed in a gradient of high-dorsal to low-ventral while the ephrinAs are distributed in a high-ventral to low-dorsal. In the SC, EphAs are distributed in a gradient of high-anterior to low-posterior, while the ephrinAs are distributed in a high-posterior to low-anterior. In the visual cortex, the EphAs are distributed in a peak of high at the center to lower at medial and lateral, while the ephrinAs distribute in of high-medial and lateral and low-center (Cang and Feldheim, 2013). The establishment of these membrane bound proteins together with the evidence that axons expressing ephrinAs are repelled by EphAs (Rashid et al., 2005) supports the hypothesis that these molecular gradients contribute to the formation of matching topographical maps in the retina and targets. As an example, RGCs from the temporal retina, with high levels of EphAs, project to the dorsal dLGN,

anterior SC and central V1 area of the visual cortex, being repelled from the high-ephrin areas in the target. Thus, axons project to an area at the target where their sensitivity to the repellent cues is minimized (Cang and Feldheim, 2013) helping to match the organization of the topographical between the retina and its targets.

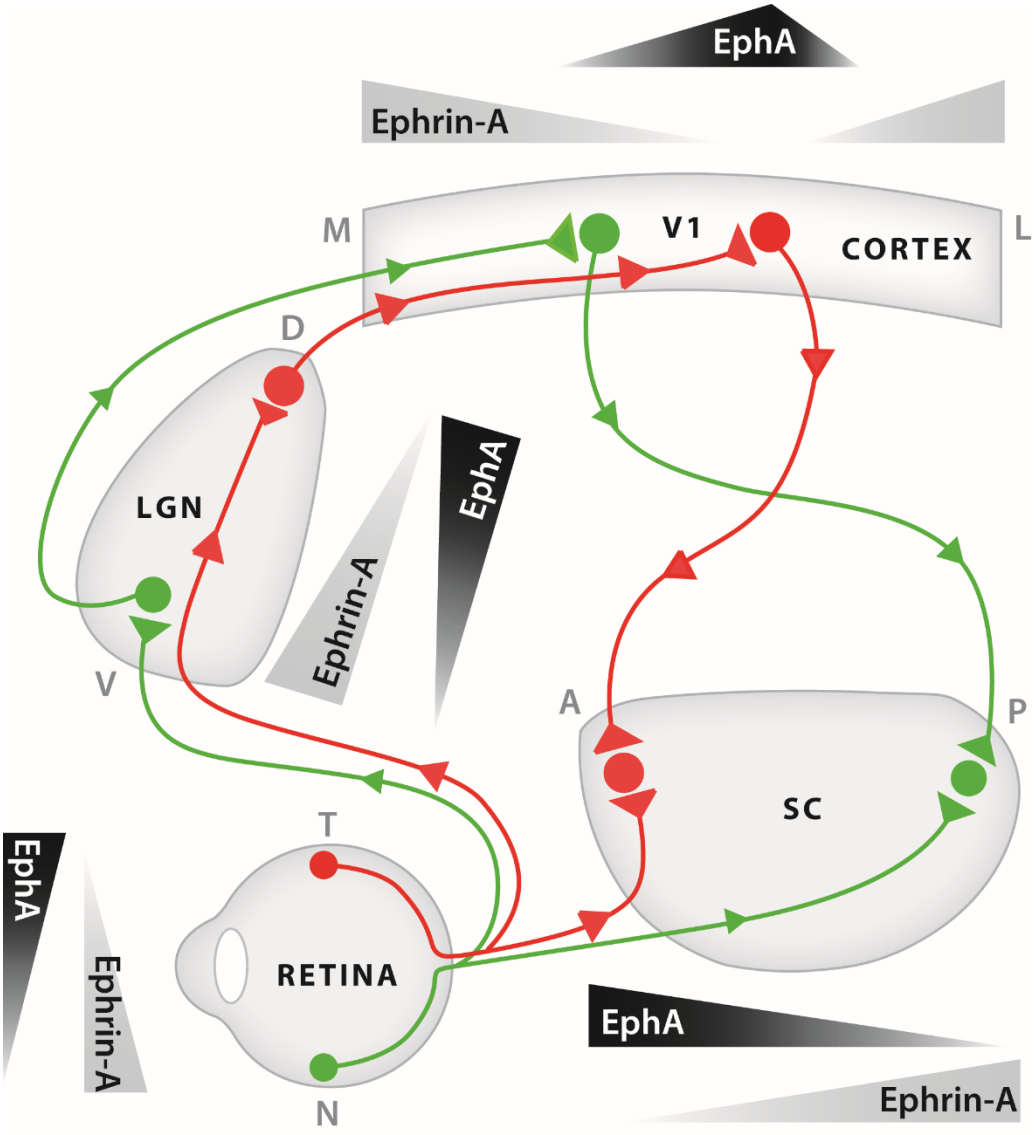


Figure I-7 EphA-ephrinA gradients in the establishment of the topographical organization of the visual targets.
Complementary gradients of EphA and ephrinA organize the matched topographical map of the retina in its targets. dLGN: Dorsal Lateral Geniculate Nucleus. SC: Superior Colliculus. Taken from Cang and Feldheim, 2013.

In the human embryonic retina the distribution of the EphA receptors is different from rodents. EphA is displayed along two gradients, with EphA levels decreasing from at the center of the retina to its periphery (Lambot et al., 2005).

Dynamics of innervation of retinal ganglion cell targets

The invasion of targets by the axons during development is a complex process still being unraveled. The invasion of visual targets by RGC axons is a useful model to study the mechanisms of target innervation. Different species have different strategies of innervation of their primary visual targets during development. Frogs and fish RGCs project directly to their final topographic position in their primary visual target, the optic tectum, forming branches restricted to their final terminal zone (Harris et al., 1987; Kaethner and Stuermer, 1992; O'Rourke et al., 1994). Harris et al., using time-lapse video studies also showed that Xenopus retinal axons slow down precisely when they reach their terminal zone at the target and form axonal branches in the terminal zone (Harris et al., 1987). It was also suggested that in these species the EphA/ephrinA gradients define where the RGC axons will target. Nevertheless, in chick and mouse the establishment of axonal projections to the appropriate topographical terminal zone in the target (in most studies the SC or LGN) requires multiple steps, listed here as described by (McLaughlin and O'Leary, 2005):

- Axon extension and overshooting: When RGC axons first innervate the SC, the primary projecting RGC axons enter the SC and extend posteriorly past the location of their final terminal zone, overshooting it. Ephrin-A5 forms a barrier that prevents the axons of overshooting further caudally to other targets, such as the inferior colliculus (Frisen et al., 1998).
- Topographic branching: After overshooting their terminal zone within the caudal regions, axons form interstitial branches along their extension in the target, with most of the branches being formed at the location of the final terminal zone. The targeting in the final terminal zone is defined by the EphA/ephrinA gradient.

- Branch guidance and arborization: After the initial formation of interstitial axonal branches along the axonal extension, the branches at near the terminal zone are selected to survive while the axons in other locations retract. It is suggested that the EphA/ephrinA gradients at the target also play a role in the selective survival of the axonal branches near the terminal zone (Yates et al., 2001), but the exact mechanism is not known. The process of branching might also be partially controlled by TrkB/BDNF interactions (Alsina et al., 2001; Kaneko et al., 2008).
- Map refinement: Refinement is the process of elimination of axonal projections projecting to inappropriate targets. This axonal pruning process is a dynamic process which requires multiple molecules, neurotransmitters, immune system molecules, glia, microglia and neural activity (Bjartmar et al., 2006; Chung et al., 2013; Huh et al., 2000; Penn et al., 1998; Schafer et al., 2012; Stevens et al., 2007; Upton et al., 1999). The refinement of RGC axons projection to the appropriate area in the dLGN is driven by random spontaneous retinal activity (Penn et al., 1998) that propagates in the retina called "retinal waves" (Meister et al., 1991), mediated by cholinergic synaptic transmission in the first postnatal week and followed then by glutamatergic transmission in the second postnatal week (Blankenship and Feller, 2010; Huberman et al., 2008; Katz and Shatz, 1996). New work has defined additional aspects of neural activity that are important such as the coordination and assynchronicity between the retinal waves in each eye postnatally, (Ackman et al., 2012; Zhang et al., 2012), and the compensation of the glutamatergic retinal waves if they are genetically inhibited, by the persistent cholinergic retinal wave during the development and refinement of eye-specific segregation at the target (Xu et al., 2016).

Experiments by Sachs et al., 1986 using anterograde labeling with HRP and by Dhande et al. 2011, in mutant mice that express GFP after the electroporation of a Cre plasmid at early postnatal ages, showed the dynamics of RGC axons projections to the targets with single cell resolution (Dhande et al., 2011; Sachs et al., 1986). These authors demonstrated that in early stages the same axon projects to multiple targets in the brain, and later the projections to inappropriate targets disappear.

Neurogenesis of retinal ganglion cells sub-types and timing of projection to the targets

RGCs are comprised of different sub-types with specific functional properties, patterns of stratification in the retina, targeting, and are beginning to be distinguished by molecular markers (Sanes and Masland, 2015). As mentioned above, in mice, image-forming RGCs project to image forming targets like the dLGN, while non-image forming RGCs project to other nuclei, such as the vLGN and SC. Osterhout et al. (2014) showed that there is a different strategy of target innervation between the early born Cadherin 3 (Cdh3)-expressing RGCs and the late-born Hoxd10- or dopamine receptor D4 (DRD4)-positive RGCs. The Cdh3 RGCs first project to multiple targets before eventually restricting their target regions, and the elimination of projections coincides with a significant decline in the number of Cdh3- expressing RGCs. In contrast, late-born DRD4 RGCs project specifically to the appropriate target from the beginning of their development (Osterhout et al., 2014). This experiments suggest that the strategy of RGC innervation of targets might be related to the time of birth of different subclasses of RGCs. Whether the disappearance of the mistargeted Cdh-3 RGC projections occurs through elimination of these RGCs or retraction of the inappropriate projections is still not clear.

Factors important for targeting

Mutations in the transmembrane protein, Ten_m3 expressed in the ventral area of the retina in the zone where ipsilateral RGCs originate alters the targeting and refinement of the ipsilateral RGCs in the dLGN and in the cortex (Leamey et al., 2007).

Mutations in the multi-domain protein Phr1, a regulator of synapse formation and axon guidance, also lead to defects in the mapping of RGC axons at the targets independently of the effects of activity or Eph/ephrin topographic maps (Culican et al., 2009).

At what level these molecules act and during which specific phase of development (axon guidance, refinement or synapse elimination) remains unclear.

Neurotrophic factors during development of the visual projections to the brain

The classic experiments conducted by Hamburger and Levi-Montalcini showed that the elimination of a target induces the cell death of the neurons that would project to that target. These authors then proposed that the target provided a trophic signal that assured projecting neuron survival (Hamburger and Levi-Montalcini, 1949). Later this signal was identified as Nerve Growth Factor, one of the neurotrophic factors (Cohen et al., 1954). Neurotrophic factors are a family of proteins that control aspects of survival, development and function of neurons such as synapse formation and synaptic plasticity, in both the peripheral and the central nervous systems (Reichardt, 2006). In mammals, there are four classical neurotrophins: neurotrophic growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Nevertheless, other proteins of different protein families also play a role in neuronal survival, such as the glial cell-derived neurotrophic factor (GDNF) family (Harvey et al., 2012; Reichardt, 2006). The neurotrophins interact with two distinct classes of receptors: the p75 neurotrophin receptor and the tropomyosin-related kinases (Trk), TrkA, TrkB and TrkC, triggering intracellular pathways that result in a cell survival or elimination (Harvey et al., 2012). The elimination of axonal projections in inappropriate targets has been attributed to the absence of appropriate neurotrophic factors in the target (Lom and Cohen-Cory, 1999), competition for neurotrophic factors at the target (Ma et al., 1998; O'Leary et al., 1986; Voyatzis et al., 2012), or the absence of appropriate receptors or trophic factors in the growing neurons themselves (Cohen-Cory et al., 2010; Spalding et al., 2004). Another hypothesis is that the growing axons while projecting to their target rely on intrinsic neurotrophic factor. When these axons reach these targets, they become dependent of target-derived neurotrophic factors (Marshak et al., 2007; Spalding et al., 2004).

I. Hypothesis on the mechanisms of formation of the central retina ipsilateral retinal ganglion cells

In the earliest studies of the DC ipsilateral RGC in the 1970's, three possible mechanisms have been postulated for the formation of transient ipsilateral projections from the central retina (Clarke and Cowan, 1976; Lund, 1975):

(1) The production of transient ipsilateral projections is a consequence of the formation of axonal collaterals at the chiasm that grow ipsilaterally, in RGCs in species in which the main projection is contralateral.
(2) The misrouting of axons to the ipsilateral side is due to mechanical factors that operate at the chiasm, such as the mechanical force of the contralateral optic nerve itself.
(3) The misdirection of fibers is due to non-mechanical or molecular factors that produce misrouting at the chiasm.

At the time that these varied hypotheses were postulated the molecular mechanisms of RGC laterality and targeting were not known.

The time-lapse studies performed by Sretavan and Reichardt (1993) in mouse embryos showed that when one eye was removed at E11.5 there were no alterations in the laterality of the crossed and uncrossed RGC axons when they reach the optic chiasm at E13-E14, making the second hypothesis of the misrouting of RGC axons due to mechanical interactions with the axons from the opposite eye less likely (Sretavan and Reichardt, 1993). These authors concluded that '*the initial pathfinding at the chiasm does not depend on binocular axon interactions, but on local cues that trigger differential growth cone responses'*. In these time-lapse experiments the authors did not observe axons ramifying or branching at the optic chiasm with one projection going to one OT and the other to the opposite OT, thus eliminating the first hypothesis. In the third hypothesis, the ipsilateral projection from the central retina is the result of the expression of molecules that guide the RGC axons to the ipsilateral OT. While the molecules for the permanent ipsilateral and contralateral projection have been identified (see further section on this Introduction) for the transient ipsilateral projection from the central retina, no specific molecular mechanism has been identified. In conclusion, the origin and

mechanism of formation of the ipsilateral projection from the central retina, either as a misdirected projection or a molecularly determined projection, remain unclear.

More recently, Raper and Mason (2010) suggested that the transient ipsilaterally projecting RGCs pioneer the mouse optic tract since they are the first to project to the optic tract. This topic will be expanded in a later section of this Introduction and Discussion. An alternative hypothesis is that the transient RGC projections are reminiscent of evolutionary traits that generate developmental neural plasticity and diversity, which would be beneficial to the ancestors since it would increase their capacity to adapt to new paradigms (Buss et al., 2006). Another hypothesis is that the transient ipsilaterally projecting RGCs do not have a role or guarantee an evolutionary benefit, but rather represent the by-product of an imperfect process of development that requires further mechanisms to refine visual circuit formation.

J. Hypotheses on the mechanisms of disappearance of the central retina ipsilateral retinal ganglion cells

A few experiments have addressed the mechanism of elimination of the central retina ipsilaterally projecting RGC axons/entire neurons. These experiments also constitute the basis of the paradigm that the selection of neurons that survive occurs via competition at the target. Land and Lund showed in rats that there is an early postnatal ipsilateral projection that occupies a wide area of the SC. This widespread ipsilateral projection disappears in the two first weeks after birth and the ipsilateral retinal projection occupies only a restricted area in the SC. If one eye is removed in early postnatal days (P0), the previously transient ipsilateral widespread projection to the SC persists into adulthood (Land and Lund, 1979). Other authors replicated these findings when mice were monoenucleated prenatally by a surgical procedure (Chan et al., 1999). Further studies showed that monoenucleation in rodents led to an increase in the number of ipsilaterally projecting RGCs from the non-VT retina (Chan and Guillery, 1993; Cowan et al., 1984). In this set of experiments the RGCs projection to the brain was labelled with WGA- HRP in postnatal albino rat and hamsters (Cowan et al., 1984; Insausti et al., 1984; Land and Lund, 1979) or with DiI in non-albino mice during prenatal days (Chan et al., 1999).

The projection from the SC to the retina was labelled with Fast Blue (Fawcett et al., 1984). Similar results were found when cats where monoenucleated during early post-natal days with non-VT ipsilateral RGCs surviving into adulthood (Lund et al., 1980). These experiments suggest that the competition between ipsilateral and contralateral RGCs axons projecting to the SC is a mediator of the disappearance of the ipsilaterally projecting RGCs from the central retina, since when the contralateral axons are eliminated by monoenucleation the ipsilateral RGC axons from the central retina remain. Further studies in albino rats also suggested that this competition at the target is mediated in some way by electrical activity, since reducing activity in the contralateral eye with the sodium channel blocking agent tetrodotoxin (TTX) also preserved the widespread ipsilateral projection in the SC and the survival of the central retina ipsilateral RGCs (Fawcett et al., 1984).

*C*ultures matching the retina and the SC (representing a visual target) of mice in the same dish were able to replicate these principles of circuit formation *in vitro* and to expand the molecular mechanism of elimination of inappropriate RGC projections when they implicated that ephrin-A-mediated elimination of exuberant projections does not involve developmental cell death (Voyatzis et al., 2012).

K. Molecular landscape of the <u>early</u> developing retina – E10.5- E13.5

FoxD/FoxG

The spatial distribution of FoxD/FoxG in the developing retina when the transient ipsilateral projection arises is important for defining the territories that would give rise to ipsilaterally and contralaterally projecting RGCs. Around the time that the first RGCs are born Foxd1 is expressed in the temporal retina and FoxG1 in the nasal retina (Herrera et al., 2004; Marcus et al., 1999; Pratt et al., 2004). It is not clear whether there is a factor associated with the Fox genes that predominates in the DC retina where the first RGCs are born.

Zic2

The transcription factor Zic2 is expressed in the optic stalk before E10.5, before the first RGCs are born. Nevertheless, it is not clear whether Zic2 is expressed at very low levels throughout the retina in the retinal progenitor layer, before E14.5 as detected by *in situ* hybridization

(Nagai et al., 1997). Only after E14.5, there is a clear Zic2 expression in the RGCs in the VT retina with immunohistochemistry and *in situ* hybridization; these RGCs become the permanent ipsilateral projection. (Herrera et al., 2003).

EphB1 and ephrinB2

Williams et al. (2003) performed in situ hybridization for EphB1 and ephrinB2. As described above, EphB1 is the guidance molecule expressed in RGCs that mediates a repulsive reaction when growing axons interact with ephrinB2 at the optic chiasm (Williams et al., 2003). In their experiments, EphB1 is expressed in the central retina at E13.5 and at slightly higher levels in the DC and temporal retina at E14.5. By E15.5 the EphB1 expression in the retina is more ventrotemporal. EphrinB2 is expressed at very low levels at the more medial area of the optic chiasm at E12.5-E13.5, in an area that seems not to be in contact with the more lateral area where the first DC ipsilateral RGC axons course while projecting from the optic nerve to the optic chiasm (Williams et al., 2003). However, it is possible that at E12.5 the developing optic chiasm is still a broader immature structure than it is at later ages.

Despite the fact that we know that EphB1 is expressed in the central retina at E12.5 it is not clear whether the ipsilateral RGCs within the central retina express EphB1, the contralateral RGCs or both. EphB1 expression seems more restricted to the temporal retina while ipsilateral central retina RGCs seems to project from a broader area in the retina, not only from the temporal aspect (Chan et al., 1999)

L. An evolutionary perspective - The transient ipsilateral retinal ganglion cells in different species

The presence of transient RGCs projecting ipsilaterally is not exclusive of mice or rats, the most common models used to study this projection. Other species present this population even if there is not a clear phylogenetic association for the etiology of these transient ipsilateral RGCs.

1. Chick

In adult chick, RGC axons project from the retina to the contralateral tectum (Fig. I-8). Nevertheless, the normal development of the chick visual projection from the eye to the

tectum also includes a transient ipsilateral retinotectal projection (McLoon and Lund, 1982)(Fig. I-8 B-C). At incubation day 3.5 and 4 the first RGC axons reach the ventral diencephalon with the majority of the fibers crossing the midline at the optic chiasm to the contralateral side, whereas about 10-15% do not cross and turn at a 90° angle at the optic chiasm and project ipsilaterally (Fig. I-8B) and by incubation day 15 the number of ipsilateral RGC axons in the tectum is near zero. The transient ipsilateral RGCs project from the central retina in a centrifugal gradient of intensity of this population of cells, with the higher density of ipsilateral RGCs in the central retina while the peripheral retina is free of ipsilateral RGCs.

Thanos and Bonhoeffer, 1984, using rhodamine to label the ipsilateral RGCs in chick and described that on their course to the tectum the ipsilateral axons do not branch in the optic nerve, chiasm or tract, and that when double retrograde labeling from both tecta was performed with two different tracers, no RGCs were stained both retrograde tracers at day 9 (Thanos and Bonhoeffer, 1984). These two experiments support the finding that these transient ipsilateral projections arise from ipsilateral RGCs and do not represent collateral branches of contralateral RGCs. Nevertheless, after day 9-10 the number of ipsilateral RGC axons decreased to an estimated 1% of the axons in the tectum at day 9 and near zero by day 15. In the same study, the authors described that the ipsilateral RGC axons arrive to the ventroanterior tectum at day 7, one day later than the contralateral axons. Ipsilateral and contralateral axons arrived at the midline at the same time, at incubation day 3.5-4, suggesting that this delay is not a consequence of a different time of exit of the retina between ipsilateral and contralateral RGC axons but likely the result of a slower progression of the ipsilateral RGC axons growth or a stalling/"waiting period" at some point of their projection from the chiasm to the tectum. Furthermore, the ipsilateral RGC axons defasciculated from optic nerve axonal bundles that also projected to the contralateral side of the brain, proving that ipsilateral and contralateral are not in separate axonal bundles along their projecting path in the optic nerve.

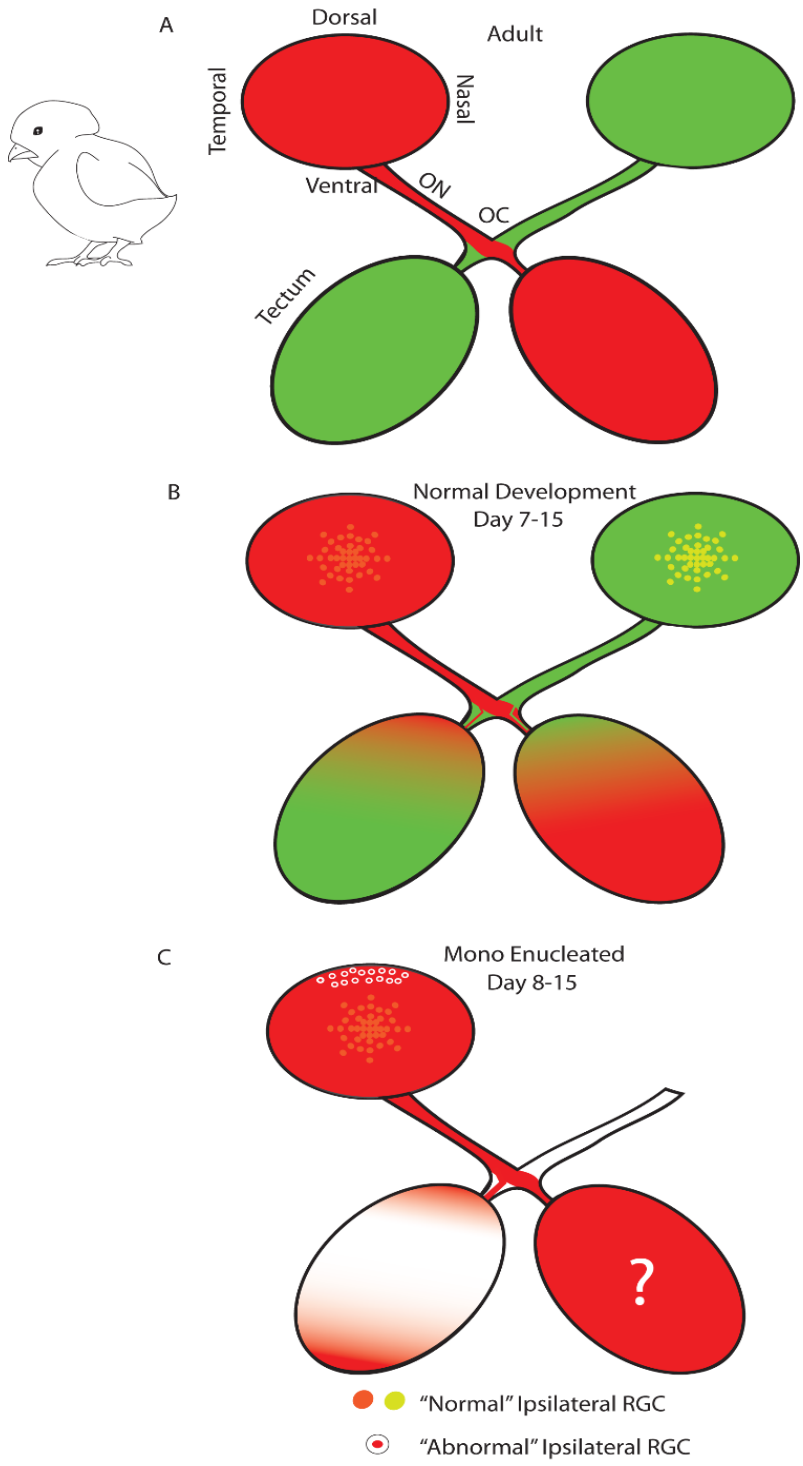


Figure I-8 The transient ipsilateral retinal projections to the brain in chick.

In chick the retinotectal projections directly target their final topographic position in the tectum (while in early mammals' development the RGC axons branch in an extensive area of a given target and later refine their projections to the appropriate topographical area). The transient ipsilateral RGCs follow a retinotopic organization similar to that of the contralateral RGC retinotopy in the tectum, with respect to the dorsoventral axis. All fibers originating in the dorsal retina lie ventrally (laterally) in the optic tract and on the tectum, whereas all fibers originating ventrally in the retina lie dorsally (medially) in the optic tract and on the tectum. Mono-enucleation performed before incubation day 3 in chick embryos leads to a substantial increase in the ipsilateral retinal projection (Ferreira-Berrutti, 1951; Mathers and Ostrach, 1979; O'Leary et al., 1983; Raffin and Reperant, 1975) consisting of two transient ipsilateral population in the monoenucleated chicks: one that is similar to the central retina transient ipsilateral retinal projection, and a second "abnormal" ipsilateral population that projects exclusively from the dorsal retina and targets the dorsoposterior tectum (Fig. I-8C) (Thanos et al., 1984). Interestingly, despite the absence of contralateral RGC axons in the tectum of mono-enucleated chicks the ipsilateral RGC axons disappear after incubation day 15, as in normal chick development (Thanos et al., 1984). If the disappearance of the ipsilateral RGC axons was purely dependent on a competition at the target with the contralateral RGC axons, it would be expected that the ipsilateral RGC axons would persist after incubation day 15 in the mono-enucleated chicks.

Interestingly, the loss of the ipsilateral projections in chick have a close temporal correlation with the wave of cell death in the ganglion cell layer in the retina, with 40% of RGCs being lost during development, with the peak period for this cell degeneration occurring from 12 to 16 days of incubation (McLoon and Lund, 1982).

2. Primates

The primate retina has a 50/50-40/60 ratio of ipsilaterally and contralaterally projecting RGCs, with the RGCs in the temporal retina projecting ipsilaterally and the RGCs in the nasal retina projecting contralaterally (Meissirel and Chalupa, 1994; Petros et al., 2008). The same is reflected in the OT with a proportional distribution of ipsilateral and contralateral RGCs in the

tract. These two populations of RGCs axons occupy different positions in the adult OT, with the contralateral RGC being more medial and the ipsilateral RGCs more lateral. Nevertheless, during development of the visual projections of the primate rhesus monkey (Macaca mulatta), Chalupa and Meisserel described a transient population of RGC axons projecting ipsilaterally and occupying the medial most position in the OT (Meissirel and Chalupa, 1994). From these experiments it not clear whether this transient ipsilateral RGC projection in the medial OT corresponds to the ipsilaterally projecting RGC from the central retina in mouse.

M. Transient projections as pioneers - Do the earliest retinal ganglion cell projections pioneer the retinal projections to the brain?

The early RGCs were proposed to be the pioneers of the optic tract as described previously in the section about hypothesis on the origin of the transient ipsilateral RGC axons from the central retina (Guillery, 1995; Raper and Mason, 2010). To our knowledge, evidence to support the existence of pioneer axon-axon mechanisms in mammals is lacking. In lower vertebrates and invertebrates, previous experiments suggest that some groups of neurons have special characteristics that make them “pioneers”, axons that are the first to project to a pathway and that facilitate the progression of “follower” axons, as represented in
Figure I-9 (Raper and Mason, 2010). It is hypothesized that the pioneer axons, the first axons to enter a new pathway, have broad growth cones, with an expanded area to explore/interact with the environment, while follower axons present an arrow shaped growth cone and grow faster (Bak and Fraser, 2003).

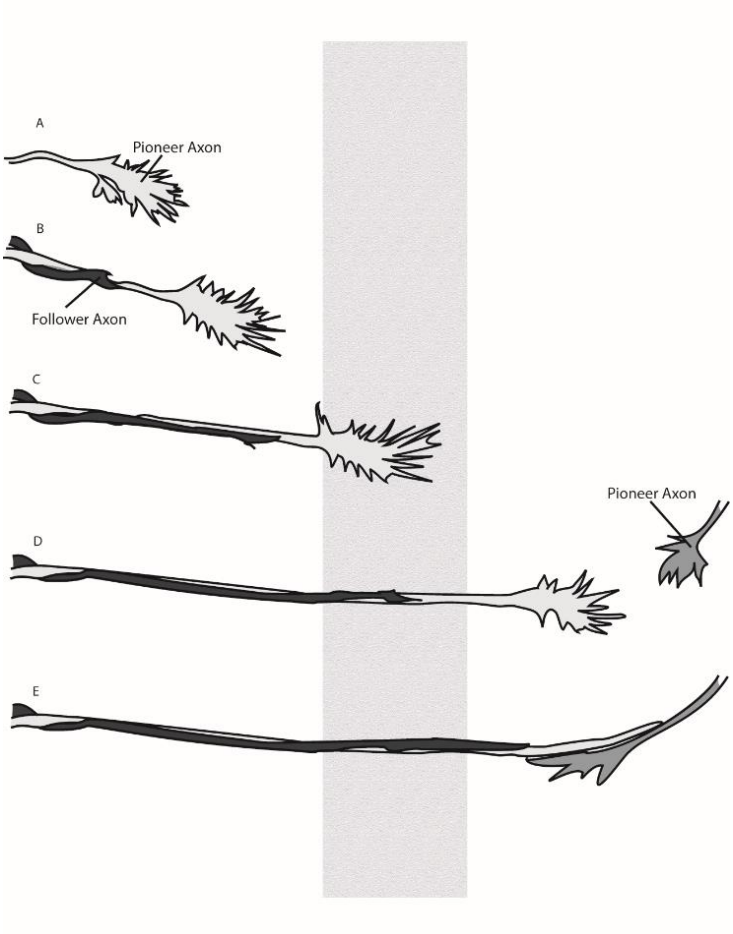


Figure I-9 Schematic representation of pioneer axons and followers axons. Taken on Bak and Fraser 2003.

The mechanism for pioneering could happen through isotypic or heterotypic axon-axon interactions or the pioneer projections could change the pathway extracellular environment facilitating the progression of further axonal extensions, as in the preformed guidance pathways hypothesis described below (Carney and Silver, 1983; Pittman et al., 2008). On the first scenario, in which the pioneering is exclusively dependent on axon-axon interactions, the elimination of pioneer axons would derail the projection of the follower axons, as was showed in the grasshopper's thoracic ganglion neurons in experiments performed by Jonathan Raper and described below (Bastiani et al., 1984; Raper et al., 1983a, d; Raper et al., 1984).

It is not clear whether pioneer axons are genetically different from the follower axons. An interesting example is the olfactory pathway in zebrafish. In this species, a group of transient pioneer neurons that prefigure the primary olfactory pathway before outgrowth of olfactory

sensory axons. These pioneer neurons are distinct from the permanent olfactory sensory neurons in terms of antigen expression, morphology and embryological origin, and when deleted there is an increase in the misrouting of the "follower axons" of the olfactory sensory neurons (Whitlock and Westerfield, 1998).

1. Preformed guidance pathways

Axon-axon interactions are not the only model of pioneering in development. Studies on cell migration and axon guidance in the developing distal auditory system of the mouse have proposed that both neuronal cells and the extracellular matrix environment play a role in the progression of axonal projections. Carney and Silver used transmission electron microscopy imaging to study the early phase of inner ear development. They described that migratory neurons delaminate from the otic epithelium and condense to form a funnel-shaped configuration that begins in the dorso-rostro-lateral wall of the otocyst and broadens to reach the auditory ganglion. There is a widening of extracellular spaces around the wall of the otocyst, temporally and spatially correlated with this migration and an increase in necrosis. Later, the first axons to project from the auditory ganglion into the otocyst extend moving along the cells of the preformed funnel-shaped neural tissue and their growth cones enter the otocyst at sites devoid of basement membrane and invade the wall of the otocyst moving tangentially along radially arranged cells that bridge the otocyst and the preformed funnel-shaped tissue. Considering these data, Carney & Silver (1983) proposed that the pathfinding of ganglion neuron projection from the auditory ganglion to the otocyst is dependent on preformed guidance pathways. The authors did not perform loss of function experiments, such as selective interference of the formation of the funnel-shaped tissue; studied whether there was an increase of necrosis in the preformed pathway; did not search for the expression of adhesion molecules on the axons or funnel-shaped tissue; or study the inhibition of the cell-matrix interaction through the molecular deletion of the adhesion molecules that could be responsible by axon-matrix interactions. As such, most of the hypotheses proposed in this study on preformed guidance pathways for the projection of the first axons from the auditory ganglion to the otocyst are not supported by any evidence. If the formation of these preformed

guidance pathways plays a role in the development of the otic projections, it is not clear whether they facilitate the progression of the first projecting axons or are essential for these axons to project and more axons to progress further.

2. Pioneer axons in the grasshopper

One the first controlled experiments that studied the subject of pioneering neurons and projections was the work of Raper et al, (1983-1984) using the development of the grasshopper's second thoracic ganglion as a model. In grasshoppers, the G neuron projects to the contralateral side of the thorax and ramifies in two perpendicular longitudinal branches, fasciculating with the longitudinal axons of the A and P neurons. When Raper et al, eliminated the A and P neurons with a laser, the contralateral G neuron's axon was unable to extend in the longitudinal axis. This result was replicated by the elimination of the P neurons alone, but not with the elimination of only the A neurons. These experiments suggest that the guidance and extension of the G neuron's axons in the longitudinal axis is dependent of the pioneering effect of the P neurons that facilitates the extension of the G neuron's axon. Since the G and P neurons' axons fasciculate together it was proposed that fasciculation was part of a mechanism in the pioneering function of the first axons (Bastiani et al., 1984; Raper et al., 1983a, d; Raper et al., 1984).

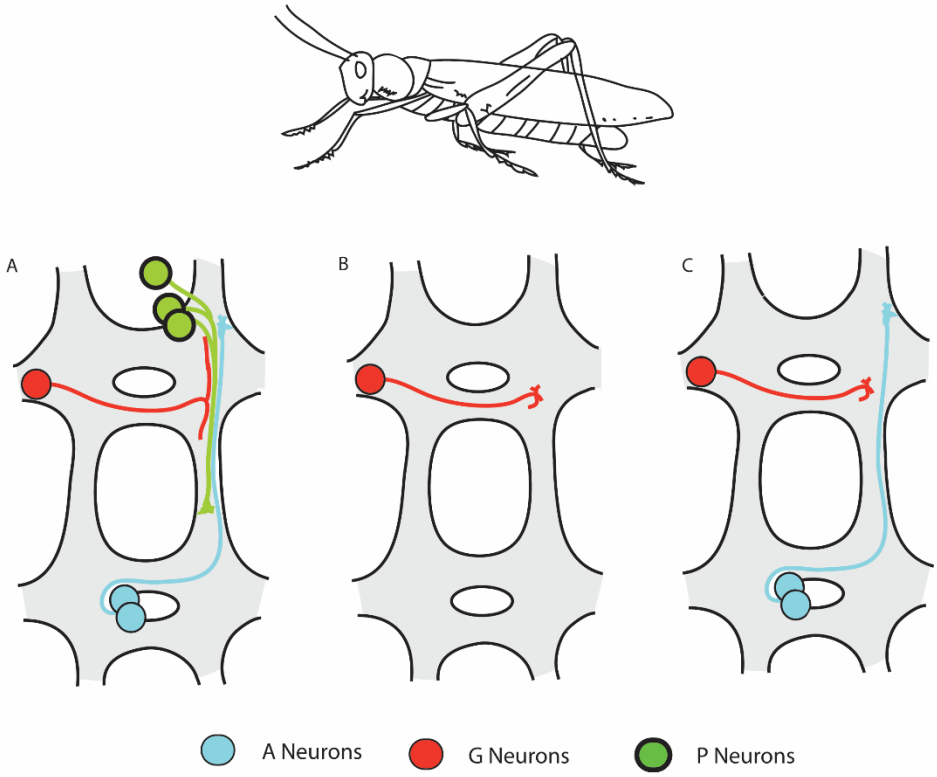


Figure I-10 Pioneer axons in grasshopper's second thoracic ganglion.
A-Normal development of the P neurons in the second thoracic ganglion. The G neuron axon fasciculate with fascicle formed by the three P neurons and two A neurons in the longitudinal axis. **B**- After the elimination of the A and P neurons the G neuron growth cone is not able to extend in the longitudinal axis, even if other axonal fascicules remain in area where the A and P neuron axons should be. **C-** The lack of extension of the G neuron axon longitudinally seen when A and P neurons are eliminated is similar to elimination the P neurons alone. Taken from on Raper et al. 1984.

3. Pioneer retinal ganglion cells in zebrafish

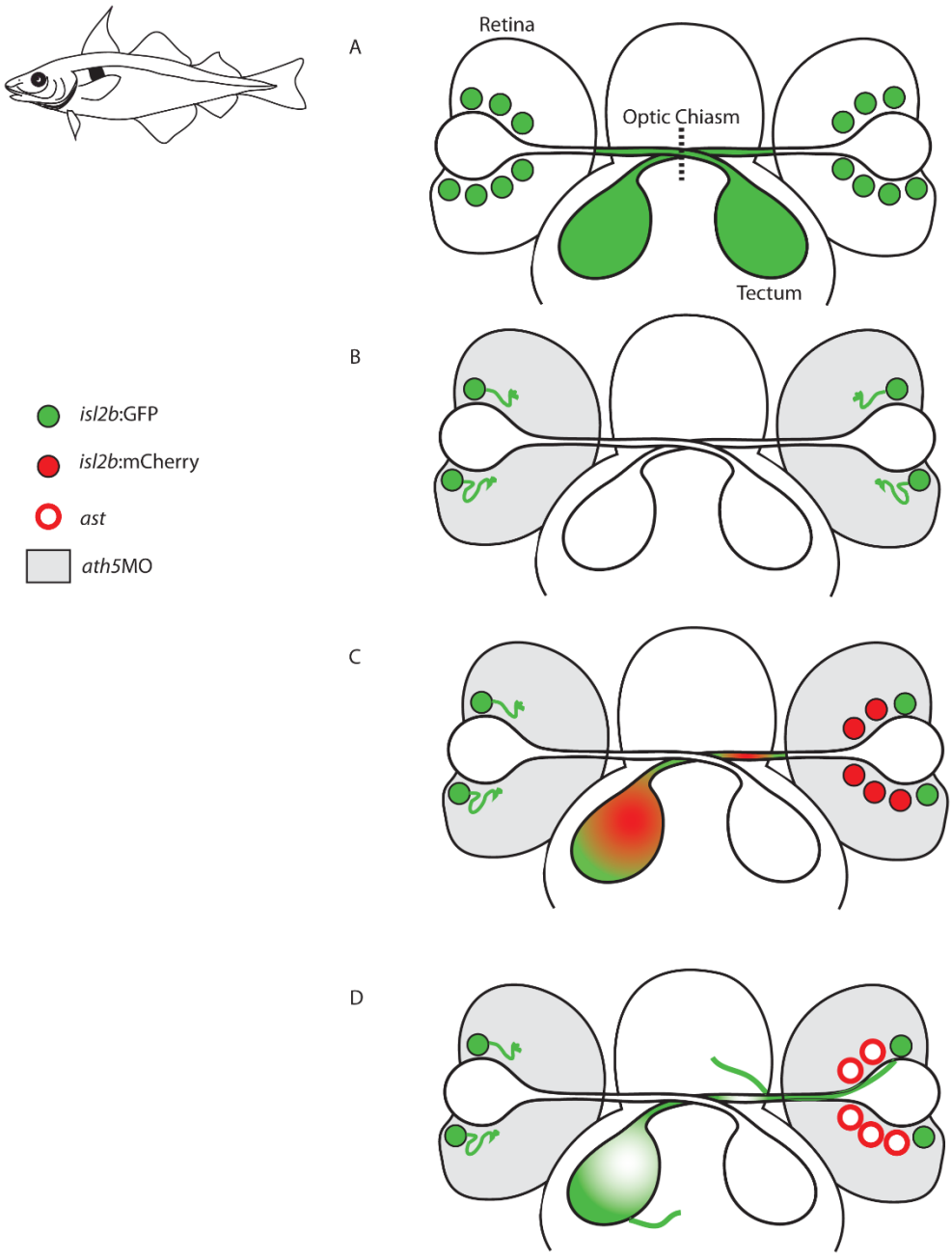


Figure I-11 Guidance of retinal axons by pioneer axons in fish.
Based in Pittman et al. 2008.

In a genetic model, Pittman and Chien knocked-down genes critical for RGCs differentiation at early ages in zebrafish, and as a result, no RGCs where born at those ages (Pittman et al., 2008). The RGCs born subsequently were unable to project from the eyecup to the brain. This experiment supports the hypothesis that the early RGCs have a role in the guidance of the later born RGCs.

To remove early RGCs, Pittman et al used a translation-blocking antisense morpholino oligonucleotide, ath5MO, to knock down function of ath5 in isl2b:GFP fish. Ath5 is a bHLH transcription factor expressed specifically in the zebrafish eye and required in a cell autonomous fashion for RGC differentiation. Other groups showed that the mutants of atonal homolog genes, ath5 in zebrafish or math5 in mice, have a change of fate of the retinal progenitors from RGCs to bipolar cells, resulting in mutant with almost no RGCs (Brown et al., 2001; Kay et al., 2001). In the Pittman et al. study, the authors used a morpholino instead of a complete knock out. The morpholino effect wanes with the dilution of its concentration with embryological development instead of a complete knock-out. In these experiment the use of an ath5MO resulted in the elimination of the early RGCs in the central retina and the persistence of the later born more peripheral RGCs. As a consequence, when these central RGCs were removed by injecting the ath5MO in the isl2b:GFP zebrafish, axons of late-born RGCs failed to exit from the eye into the brain (Figure I-11). Instead, the axons from peripheral RGCs grew in the RGC layer of the eye without invading the intraretinal region of the optic nerve. There were no evident major anatomical or molecular anomalies of the optic nerve head, with the area of the presumptive optic nerve head expressing the glial marker Pax2 and the guidance molecule netrin2. The replacement of the missing early born, central retina RGCs in the ath5MO isl2b:GFP zebrafish with transplanted progenitor cells from isl2:mCherry-CAAX rescued the exit of the RGC axons from the retina, with both host and donor RGC axons projecting to the topographically appropriate target in the tectum. Furthermore, imaging within chimeric eyes showed that late-born, peripheral RGCs host axons appeared to fasciculate with donor axons at least in the retina. These observations support the hypothesis that early RGCs are necessary and sufficient to guide later axons out of the eye.

To imply a cell autonomous role of adhesion molecules in the guidance of follower axons in Pittman et al, 2008, the authors performed a series of experiments transplanting cells from the astray (ast) mutants to wt and vice-versa, from the ast donor cells into ast host and wt donor cells into wt host. The astray mutant fish lack the Slit receptor Robo2 and present drastic eye-dependent, pathfinding errors that are not dependent on the expression of molecules in the brain (Fricke et al., 2001). In these series of studies on the expression of genes the wildtype was considered to be the not mutated or the brn3c:GFP fish (Xiao et al., 2005). Brn3c is, like Isl2b, a specific marker of postmitotic RGCs in zebrafish. Also, the host expressed a fluorescent protein, GFP, while the donor cells were not labeled by a fluorescent protein. In these experiments, ast cells were transplanted to wt zebrafish exposed to the ath5MO at a defined time that deleted the early central retina RGCs and not the later born peripheral RGCs, as described above. As in the previous experiments, when the early central retina population is replaced in the ath5MO zebrafish with cells from a wt donor, the most peripheral RGCs were able to project from the eye to the brain/tectum without frequent misrouting of axons. Nevertheless, when the donor cells were provided by an ast mutant into a wt;ath5MO host, some RGCs were misguided along the visual pathway and there was some axon defasciculation in the optic chiasm not present in the previous experiments. The number of misguided RGCs axons increased dramatically, when an ast;ath5MO host received cells from a ast donor. This phenotype was partially rescued when an ast;ath5MO host received cells from a wt donor, but the number of misguided axons was still greater than when a wt;ath5MO host received ast donor cells. These experiments showed that an ast host can misroute wt axons. And overall, these data support the hypothesis that adhesion molecules play a role in the axon-axon interactions, namely, fasciculation, that allow pioneer axons to guide follower axons. Nevertheless, these experiments identified one receptor, Robo2, but not its ligand. Also, from these experiment it is clear that central retina RGC pioneers axons have a role in the guidance of peripheral retina RGC axons outside the eye, but it is not clear whether the misrouting errors found in the some experiments with the ast mutant are a consequence of the lack of adhesion of the follower axons to pioneer central retina axons or another type of interaction not dependent on Robo2 axon-axon interactions, or of interactions of the Robo2 receptor with a ligand in the visual pathway. The localization of the

Robo2 ligand in other axons or in the visual pathway would contribute to elucidating some of the questions regarding the role of pioneer axons after the axons exit from the eye.
In the discussion of this publication the authors made an important remark: “However, we cannot formally exclude other possibilities. For example, early RGC cell bodies might secrete an attractant that draws later axons to the optic nerve head.” This is an important statement that is contrary to the interpretation of the ability of central retina RGC axons acting as pioneers of the later born, peripheral RGC axons, generally in disagreement with the notion of the existence of pioneer axon-axon interactions in the zebrafish eye.

4. Corpus Callosum “Pioneer” Axons

The Corpus Callosum is a band of nerve fibers connecting both cortical hemispheres.
Axons from the medial cingulate cortex are the first to cross the midline at E17.5, through the pathway of the corpus callosum. There neurons were called by Koester et al. (1994) “pioneer neurons” taking into account the definition: ‘Pioneer neurons have been defined as those that extend the first axons through a pathway’ (Koester and O'Leary, 1994). This definition does not take into consideration any functional evidence for pioneer neurons such as facilitating the progression of the follower fibers. In a more strict classification of pioneer axons that includes a functional role to the classification of pioneer neurons, these axons would not be classified as pioneers, since there is still no evidence of the functional role of these axons acting as pioneers.

N. Difficulties in studying transient neurons/projections

The development and destruction of transient projections is a dynamic process in development better studied with live imaging or time-lapse techniques (Campbell and Okamoto, 2013; Harris et al., 1987; Sretavan and Reichardt, 1993). Most of these live imaging studies were performed in *Xenopus* or fish, more suitable animal models in which to study the live dynamics of neural projections. Sretavan and Reichardt were able to study the dynamics of RGC axons projections in live mice *ex vivo*. Nevertheless, their approach, and that used by Godement et al. (1994) were not tested for the sufficiently longer time periods necessary to study the central retina

RGC axons *ex vivo*, from E11.5 to E17.5. In addition, the growing RGC axons take a very complex path from the ventral and anterior diencephalon to the posterior and caudal diencephalon in a difficult angle to perform live imaging and in a tissue undergoing rapid growth and transformation. A major hardship in the field of study of transient neurons in general but especially in the mouse retina is the lack of molecular markers for these neurons. In the case of transient neurons that have been hypothesized to be pioneer neurons, it is still not known whether they have the functional role in mammals. In lower vertebrates and invertebrates, the neurons proposed to be pioneering neural tracts were ablated with lasers (Bastiani et al., 1984; Raper et al., 1983a, d; Raper et al., 1984), or through genetic ablation of a population at a specific time, even if that population did not have a characteristic marker (Pittman et al., 2008).

II. Prospectus of the Thesis

This thesis aims to elucidate the extension and fate of the transient central retina ipsilaterally projecting RGCs using multiple methodologies to trace this population of RGCs and the contralateral RGCs projecting from the same area. While previous authors suggest that the central retinal ipsilaterally-projecting RGCs disappear after E16.5, it is not clear how far they project.

In Section 1 of the Results the successful targeting of the central retina ipsilateral RGCs will be described using *in utero* electroporation of GFP at E12, allowing the subsequent study of axonal projections from the central retina at different times from E15 until early post-natal days.

Three hypotheses on their fate, and given the results presented, each hypothesis will be discussed (Figure II-1).

In Section 2 and 4, alternative technical approaches were tested to target the central retina RGCs in early development, using viruses and transgenic mice with xx RGCs labeled, respectively, but these approaches presented suboptimal results when compared with approach tested in Section 1. In Section 3 a method was developed to combine the use of the lipophilic tracer DiI with immunohistochemistry, in order to mark the transient population selectively; this method required the use of detergents which were deleterious to the DiI, and a modification was made to overcome this failing. The experiments in this Section are important for further studies on the expression of the ipsilateral and contralateral central retina RGC axons and to validate the use of Brn3b conditional knock-out mice in Section 4.
Section 5 constitutes a tentative uncovering of the mechanism of disappearance of the ipsilateral central retina RGC axons described in Section 1, based on Caspases.

Model A: The central retinal ipsilaterally-projecting RGCs project along the OT but do not project to the SC or dLGN and then disappear.

Model B: The central retinal ipsilaterally-projecting RGCs project to the SC or dLGN, but do not arborize at the target and then disappear.

Model C: The central retina ipsilaterally-projecting RGCs project to the SC or dLGN, arborize at the target and then disappear.

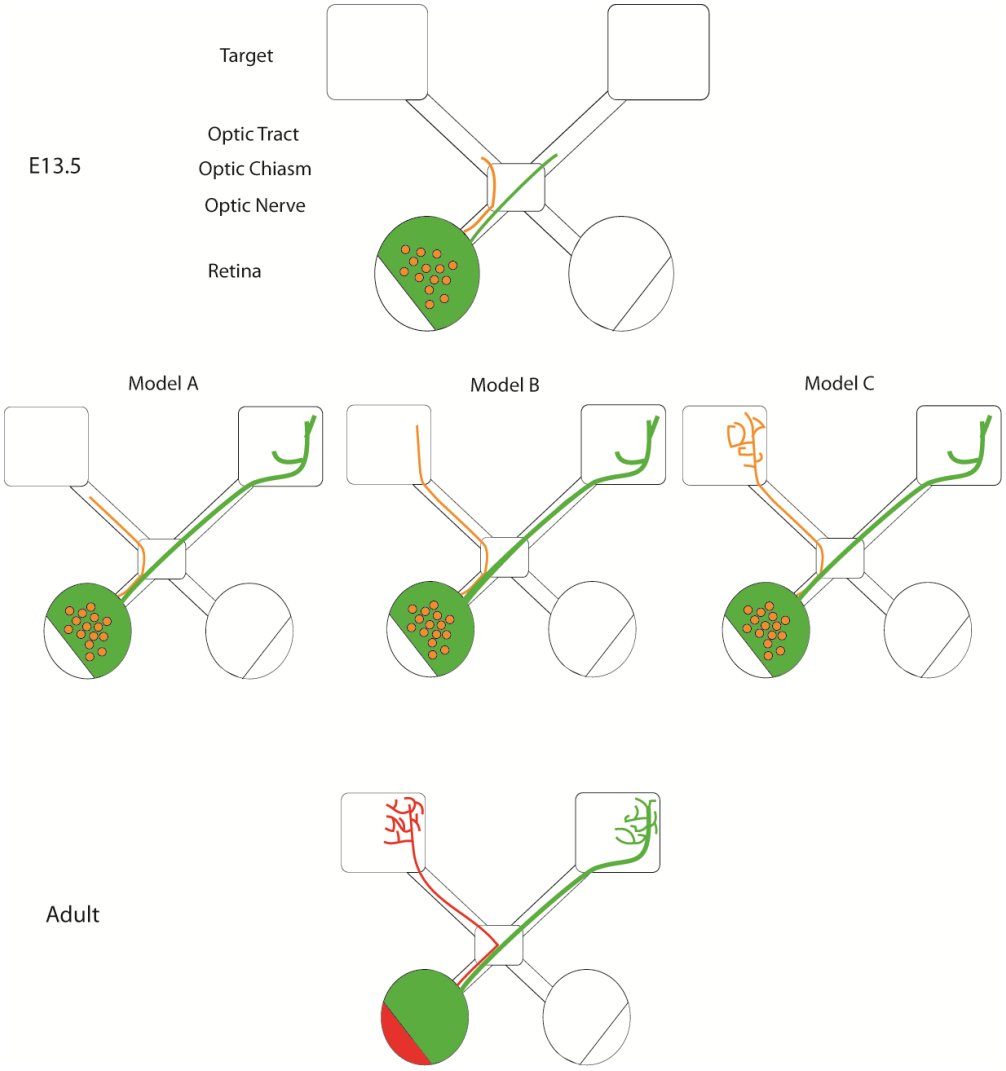


Figure II-1 Models on the disappearance of the central retina ipsilateral RGC axons during normal development.

The three different models represent hypotheses on the fate of the central retina RGC axons after E13.5. The central retina ipsilateral RGCs are represented in orange, while the permanent ipsilateral RGC from the ventrotemporal crescent are represented in red and the contralateral RGC in green.

III. Results

Section 1 –Transient ipsilateral retinal ganglion cell projections to the brain: Extent, targeting and disappearance.

Paper published as: *Célia A Soares and Carol A Mason. (2015). Transient ipsilateral retinal ganglion cell projections to the brain: Extent, targeting and disappearance. Developmental Neurobiology. DOI:10.1002/dneu.22291*

Abstract

During development of the mammalian eye, the first retinal ganglion cells (RGCs) that extend to the brain are located in the dorsocentral retina. These RGCs extend to either ipsilateral or contralateral targets, but the ipsilateral projections do not survive into postnatal periods. The function and means of disappearance of the transient ipsilateral projection are not known. We have followed the course of this transient early ipsilateral cohort of RGCs, paying attention to how far they extend, whether they enter targets and if so, which ones, and the time course of their disappearance. The dorsocentral ipsilateral RGC axons were traced using DiI labeling at E13.5 and 15.5 to compare the proportion of ipsi- versus contralateral projections during the first period of growth. *In utero* electroporation of E12.5 retina with GFP constructs was used to label axons that could be visualized at succeeding time points into postnatal ages. Our results show that the earliest ipsilateral axons grow along the cellular border of the brain, and are segregated from the laterally-positioned contralateral axons from the same retinal origin. In agreement with previous reports, although many early RGCs extend ipsilaterally, after E16 their number rapidly declines. Nonetheless, some ipsilateral axons from the dorsocentral retina enter the superior colliculus (SC) and arborize minimally, but very few enter the dorsal lateral geniculate nucleus (dLGN) and those that do extend only short branches. While the mechanism of selective axonal disappearance remains elusive, these data give further insight into establishment of the visual pathways.

Introduction

The development of neuronal circuits is a dynamic process involving the formation of projections that do not persist in mature circuits (Luo and O'Leary, 2005). The elimination of transient projections ranges from small-scale events such as local pruning of axonal branches and synaptic boutons, as in the vertebrate neuromuscular junction (Tapia et al., 2012), to large-scale elimination of major axon projections or their collaterals (Luo and O'Leary, 2005; Stanfield and O'Leary, 1985) and wholesale elimination of neurons themselves, as in programmed cell death (Francisco-Morcillo et al., 2014).

An example of long axon elimination is the transient projection of retinal ganglion cells (RGCs) from the dorsocentral (DC) retina of rodents to the ipsilateral side of the brain (Colello and Guillery, 1990; Cowan et al., 1984; Petros et al., 2008). During the development of the mouse visual circuit, RGCs in the ventrotemporal (VT) crescent of the retina project to the ipsilateral side of the brain from embryonic (E) day E14.5 to E16.5, while RGCs outside this crescent project contralaterally (Petros et al., 2008). RGC axons diverge ipsi- or contralaterally at the ventral midline of the brain and form the optic chiasm (OC) (Petros et al., 2008). After E16.5, RGCs from the VT crescent also project contralaterally. This plan comprises the permanent binocular circuit. However, during the first phase of retinal development, from E10.5-13.5, the first-born RGCs clustered in the DC retina project either contra- or ipsilaterally (Colello and Guillery, 1990; Drager, 1985; Guillery et al., 1995). The ipsilateral DC RGCs cannot be retrogradely labeled postnatally and thus their projections and the cell bodies themselves have been thought to disappear (Colello and Guillery, 1990; Petros et al., 2008). In mice, as assessed by DiI labeling, the ipsilateral RGC axons from the DC retina decrease to a negligible level by E16.5 (Chan et al., 1999; Colello and Guillery, 1990). In the rat, this central retinal ipsilateral RGC population persists after birth, and the few axons that project to the brain decrease over time (Cowan et al., 1984). However, the details of the transient ipsilateral RGC projections such as how far they extend, whether they enter targets and their behavior within target regions, the time course of their disappearance, and their function are not well understood.

Anterograde labeling with DiI has been widely used to label axonal projections in fixed developing nervous tissue (Colello and Guillery, 1990; Marcus and Mason, 1995), and can be

used to chart the time course of axonal projections. However, this approach provides snapshots of the status of developing cells and is not prospective, whereby cells could be labeled at early stages then the time course of projections followed. In order to chart the early DC ipsilateral RGC projection at later developmental stages, we used *in utero* electroporation of a GFP plasmid at E12.5. This strategy allows prospective analysis of the number, projection, and disappearance of this cohort of RGC axons.

In this study, we used both DiI labeling and GFP *in utero* electroporation to track the earliest ipsilateral fibers from retina to brain. We observed that while the DC ipsilateral RGC axons enter the optic tract first, they do not progress as far as the contralateral axons from E13.5 to E15.5. The number of ipsilateral RGC axons increases until E16.5 and sharply decreases thereafter, but a few remaining axons project to the SC. Moreover, while the a few early-growing ipsilateral RGC axons enter the SC and elaborate arbors at postnatal ages, these RGC axons do not make substantial projections to the more proximal dorsal lateral geniculate nucleus (dLGN). In addition, at the time of the early ipsilateral RGC axon decrease, most have not yet reached their target, suggesting that their disappearance may not be related to target-derived factors (Luo and O'Leary, 2005).

Methods

Animals

C57BL/6J mice were kept in a timed pregnancy breeding colony at Columbia University. Procedures for the care and breeding of mice follow regulatory guidelines of the Columbia University Institutional Animal Care and Use Committee. Noon of the day on which a plug was found was considered E0.5.

Tissue fixation

Embryos were removed from mothers anesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively, in 0.9% saline), and before E16.5, fixed by immersion in 4% paraformaldehyde (PFA) in phosphate buffer (PB) (pH 7.4) overnight, or at E16 and thereafter, embryos were injected with additional anesthetic, and perfused intracardially with 4% PFA/PB, and post-fixed overnight at 4°C.

DiI Labeling and Quantification

Anterograde labeling was performed on fixed tissue using 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) or 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA) (Molecular Probes) as previously described (Plump et al., 2002) (Pak et al., 2004). Briefly, the lens was removed from the eye of a fixed embryonic head and a small crystal of DiI or DiA was placed on the optic nerve, at E13.5 and E14.5 when the first-born RGCs from the DC retina have extended their axons ipsilaterally (the transient population of ipsilateral RGCs) and contralaterally, or on the DC retina at E15.5 to label the same populations. The position of the DiI labeling at E15.5 was confirmed in whole mounts or frontal sections through the eye, and only the cases with DiI labeling in the DC retina were used for analyses. Heads were incubated in a solution of 1% PFA in phosphate buffer saline (PBS) for 4 days (E13.5 or younger embryos) or 7 days (E15.5 embryos) at room temperature. Whole heads were vibratome sectioned frontally at 100µm. The samples with evidence of leakage of DiI into radial glia in the ventral diencephalon were discarded.

The extent of RGC axon projections from the ventral midline along the optic tract (OT) to the most dorsal RGC axon tip was measured with ImageJ software (version 1.48, NIH). To determine the proportion of ipsilateral fibers relative to the total number of labeled fibers, the full retina was labeled with DiI at E13.5 and E15.5, and the pixel intensity was quantified in the OT as in previous studies (Erskine et al., 2011; Escalante et al., 2013; Petros et al., 2009b). At E13.5 the first section after the optic chiasm was selected, and at E15.5 the first and second section after the optic chiasm were analyzed. To measure the pixel intensity in the OT with ImageJ software, twelve micron square regions of interest (ROI) were defined at three points along the OT, 500, 750, and 1000 µm dorsal to the ventral midline. Pixel intensity was calculated in the contralateral (cROI) and ipsilateral (iROI) OT in each of the sections described above, and an additional ROI was selected outside the tissue area in order to calculate the background pixel intensity. The background pixel intensity value was subtracted from the individual pixel intensity values. The ipsilateral ratio was calculated as (iROI / (iROI + cROI)) x 100, as previously described (Erskine et al., 2011; Escalante et al., 2013; Petros et al., 2009b) and expressed as a

percentage. The ipsilateral ratio was calculated at each distance at E13.5 and E15.5, and the values of the 3 segments averaged for each case.

In utero electroporation

In utero electroporation was performed as previously described (Garcia-Frigola et al., 2007; Matsuda and Cepko, 2007; Petros et al., 2009b). Pregnant female mice carrying E12.5 embryos were anesthetized with an intraperitoneal injection of ketamine-xylazine (100 and 10 mg/kg, respectively, in 0.9% saline). A solution of 5µg/µL membrane-bound GFP plasmid (Addgene plasmid 14757) (Matsuda and Cepko, 2007) + 0.03% Fast Green dye in distilled water was loaded into a graduated glass micropipette and approximately 0.3 µl was injected into the sub-retinal space. Tweezer-type electrodes (CUY650-P7, Nepa Gene) were then placed around the embryo's head, with the '+' electrode on the side on which the retina is injected, and five 50 ms square current pulses were delivered (25V) at 950 ms intervals using an electroporator (CUY21EDIT Square Wave, Nepa Gene). After repeating this procedure for other embryos, the peritoneum was sutured and the skin was stapled closed. For pain management, the mother was injected with buprenorphine (0.1mg/kg, SC), immediately before surgery and every 8-12h, up to 72h after surgery. The embryos were allowed to develop normally for 2-12 days. Previous studies have shown that electroporation of GFP plasmids into the subretinal space at E13.5 labels retinal progenitors that become postmitotic two days later (Garcia-Frigola et al., 2007). Here we electroporated retinal progenitors at E12.5 and by the time GFP is expressed, differentiated RGCs extend axons that cross the chiasm midline, as viewed at E14.5. GFP-labeled cells were consistently seen in the central retina from which both transient ipsilateral RGCs and permanent contralateral RGCs arise.

Tissue processing of electroporated embryos and pups

Embryos and pups were sacrificed as described above. The left retina of each embryo or pup was dissected, immunostained for GFP (rabbit polyclonal anti-GFP, 1:1000, Invitrogen), flattened as a whole mount, and confirmed for successful electroporation. Retinal whole mounts were imaged and ImageJ was used to calculate the GFP^+ retinal area and pixel intensity of GFP^+ cells. Only the cases in which the GFP^+ area comprised more than 5% of the total retinal

area were used for further analysis. Whole heads or brains were vibratome sectioned frontally at 100µm and immunostained for GFP.

Immunohistochemistry

Electroporated retinas and brain sections were blocked in 10% donkey serum (DS) + 1% Triton20 in PBS and then incubated with rabbit GFP antibody (1:1000, Life Technologies) + 1% DS + 1% Triton20 in PBS, overnight at 4°C. After 3 PBS washes for a total of 1 hour, tissue was incubated in Alexa488 anti-rabbit GFP antibody (1:500, Life Technologies) for 3h at room temperature.

Image processing and quantification

Whole mounts and sections of GFP electroporated and DiI labeled brains were imaged on a Zeiss AxioImager M2 microscope with an AxioCam MRm camera, and Neurolucida software (v 11.01, MicroBrightField Systems), using 5x, 10x, or 20x objectives. Images were analyzed with ImageJ software (version 1.48, NIH).

Quantification of electroporated RGC axons in the optic tract

Frontal sections containing RGC axons in the first 500 microns caudal to the optic chiasm (OC) were selected to quantify the number of ipsilateral axons. The number of ipsilateral RGC axons was quantified in multiple sections, but care was taken not to quantify axons twice. Since the GFP+ area varies through samples, the number of ipsilateral axons in the OT was normalized to the GFP^+ area in the retina as previously (Petros et al., 2009b) by calculating the number of ipsilateral GFP^+ axons in the OT/ GFP^+ area in the retina (mm^2).

Axon reconstruction in the superior colliculus

All frontal sections containing the superior colliculus (SC) were selected for analysis. Using ImageJ software, a 100x100µm grid was aligned on these images with the midline considered sector 0. The area occupied by the contralateral axons or the coordinates of the ipsilateral axons was manually identified and recorded. The coordinates of each individual ipsilateral axon and the area occupied by the contralateral axons in the SC were represented in a gridwork schematized with Adobe Illustrator CS3 software.

Statistical analyses

Data were plotted in Excel software (Microsoft) and analyzed with GraphPad Prism 5. Means and standard error of means (SEM) were calculated for each group. Data were statistically analyzed using Mann-Whitney U test, ANOVA, or Kruskal–Wallis one-way analysis of variance, where appropriate. p values smaller than 0.05 were considered significant.

Results

In the mouse, the first RGCs are born in the DC retina. Some of these RGCs projecting ipsilaterally and others contralaterally, and the two subpopulations are intermixed within the DC retina before E15.5. The axons of each population reach the ventral diencephalon, where the optic chiasm forms, at E12.75, and by E13.5 they project to the optic tract (OT) (Marcus et al., 1995). The transient nature of the ipsilateral projection from the DC retina has been described (Colello and Guillery, 1990; Petros et al., 2008), but the extent of axon growth past the chiasm into the OT has not been documented. Here we used DiI labeling and electroporation of GFP to chronicle how far distally the early ipsilateral RGCs extend, the proportion of ipsilateral versus contralateral projections over time, and whether these RGCs innervate dorsal Lateral Geniculate Nucleus (dLGN) and Superior Colliculus (SC) targets.

DiI labeling of the transient ipsilateral RGC projection from central retina

To determine how far the contralateral and ipsilateral axons of the DC RGCs project in the early period of RGC axon growth, we labeled RGC axons by placing DiI on the optic nerve head of one retina in E13.5 and E14.5 embryos and measured the extent of the ipsilateral and contralateral projection from the DC retina in brain sections. After E15.5, however, since the retinal projection includes axons from the central retina (and not only from the DC retina as in previous ages) as well as the permanent ipsilateral RGCs from VT retina (Colello and Guillery, 1990), we labeled only the DC retina at E15.5 to selectively visualize the early ipsilateral component in Fig. 1A-D, G. As previously reported (Marcus and Mason, 1995), we found that the ipsilateral axons are the first to reach the OT by E12.75 (data not shown). The extent of the retinal axons was measured along the length of the OT from the ventral midline to the distal most axonal tips. Since the DC ipsilateral axons are the first retinal cohort to reach the OT, and

assuming that ipsilateral and contralateral axons grow at the same rate, we predicted that the DC retinal ipsilateral axons would extend farther than the contralateral axons at early ages. However, surprisingly, we found that the central retinal ipsilateral axons extend to a similar distance as the contralateral axons in the OT at E13.5, but by E14.5 and E15.5, the contralateral cohort projects past the ipsilateral cohort (Fig. 1C, G). There was no statistically significant difference in extent of axon growth between these two populations at E13.5 (ipsilateral: 1616 µm ± 149, contralateral: 1513 µm ± 153, Mann Whitney p= 0.54, n= 7 embryos) (Fig. 1D). At E14.5 and E15.5 there is a trend for the contralateral axons to project farther than the DC ipsilateral axons (E14.5, ipsilateral: 1783µm ±124, contralateral: 2325µm ±85, n=4; E15.5, ipsilateral: 1443µm ±170, contralateral: 3076µm ±108, n=5. At E14.5 and E15.5, the difference between the extent of growth of ipsilateral and the contralateral axons from DC retina was statistically significant (Mann Whitney E14.5, p= 0.029; E15.5, p= 0.0079). It is important to note that whereas the contralateral axons continue to extend more caudally, the distal extent of the DC ipsilateral axon projection remains relatively constant, with the majority reaching only below the future dLGN, and remaining at this relative distance from E13.5 to E15.5 (p= 0.47). The extent of contralateral axon growth, on the other hand, increases over this period, (p= 0.0014).

In addition to revealing the limits of projection of DC ipsi- and contralateral RGC axons at these two stages, DiI labeling along with DiA labeling of the other eye revealed the relationship between the ipsilateral and contralateral fibers from each eye (Fig. 1E-G). We found that the DC ipsi- and contralateral axons were segregated in the OT (Fig. 1F-G). At E13.5, the ipsilateral DC RGC axons occupy a more medial position in the OT compared with the contralateral RGCs (Fig. 1F). Labeling of the DC retina at E15.5 indicated that most of the ipsilateral axons from this retinal region continue to occupy the medial-most position in the OT, but a few are positioned in the contralateral RGC territory (Godement et al., 1984) (Fig. 1G). In these preparations, a reduction in number of DC ipsilateral axons in the OT from E13.5 to E15.5 is also apparent (Fig. 1F-G).

Quantification of DiI fluorescence intensity enables inference of the relative abundance of axons in a tract, but cannot provide information on the actual number of axons. The

percentage of ipsilateral axons relative to contralateral at E14.5 in the OT was previously estimated to be 8% by DiI anterograde labeling (Erskine et al., 2011), but this value was not known for ages E13.5 and 15.5. Therefore, we next estimated the percentage of ipsilateral axons in the proximal OT at these ages, in each case labeling the entire RGC projection by placing a crystal on the optic nerve head and inferring values from pixel intensity of DiI labeling (Erskine et al., 2011) (Fig. H-L). Measures were taken at three areas along the OT: 500, 750, and 1000 µm from the ventral midline in frontal sections. First, we determined whether there was a statistically significant difference in the ipsilateral ratio (iROI/ (iROI + cROI)) for the brains examined at E13.5 within these three areas (n = 6 embryos; E15.5, n=7 embryos). As there was no difference in the ipsilateral ratio in the three OT segments (E13.5, p= 0.12; E15.5, p=0.13), the ipsilateral ratios from the three areas were averaged, and one mean value per case was considered. In whole eye-labeled preparations at E13.5, the first age at which RGC axons are seen within the OT, the ipsilateral RGC axons were estimated to comprise 20.07% of the total RGC labeled axon population in the OT (± 1.09, n=6) (Fig. 1H-J). By E15.5, there was a dramatic decrease in the overall proportion of ipsilateral to contralateral axons in the OT to 5.32% (± 0.88, n=7), even with the addition to the ipsilateral projection in the OT of the permanent ipsilateral axons from VT retina (Fig. 1H, K-L).

The ipsilateral RGC axons in the OT comprise a heterogenous population of DC and VT RGCs. Nevertheless, most of these ipsilateral axons occupy the lateral-most territory of the OT where the ventrotemporal ipsilateral RGC axons are located (Godement et al. 1984, Sitko and Mason, unpublished). A few fibers continue to occupy a medial position in the OT; these are presumably the DC ipsilateral axons, as they are seen only after DiI labeling of the DC retina (Fig. 1L).

Thus, ipsilateral axons from the DC retina are the first to enter the OT at E12.5, and the contralateral RGC axons reach the OT at E13.5, confirming previous reports (Marcus and Mason, 1995). Even though the ipsilateral axons are the first to arrive in the OT, after E13.5 they are overtaken in their extent by the contralateral axons. These two populations of DC retina RGC axons are well segregated at E13.5 in their position in the OT, with the ipsilateral axons medially and the contralateral laterally. At early stages of development (E13.5-14.5) the

DC ipsilateral RGC cohort comprises a greater proportion of the RGC axons within the OT relative to contralateral axons (Fig. 1H-J) and by E15.5 the total ipsilateral projection (including the DC transient ipsilateral projection (Fig. 1G) as well as the first axons to extend from the permanent VT ipsilateral cohort (Fig. 1L)) decreases relative to the contralateral RGC axons (Fig. 1H).

Prospective labeling of RGCs by early electroporation of GFP

Prospective labeling, i.e., "fate mapping", of axon projections is difficult to perform with DiI, especially at postnatal ages when DiI is less effective as an axonal marker. To trace the projection of RGC axons from the DC retina prospectively, we electroporated membrane-bound green fluorescent protein (GFP) into embryonic retinas *in utero* at E12.5, allowing RGC labeling *in vivo* and visualization of RGC axons at selected later stages of development, into the postnatal period. E12.5 is the earliest age at which it is technically feasible to label the retina without labeling the brain, since before that age, the subretinal space is connected to the brain ventricles. The injection and electroporation of a GFP plasmid into the subretinal space at E12.5 predominantly targets RGC precursors in the central retina (Garcia-Frigola et al., 2007; Petros et al., 2009a) (Fig. 2A-B). In our experiments, the central retina including both the dorsal and ventral central regions was consistently labeled with GFP. Thus, *in utero* electroporation of GFP at E12.5 labels a cohort of RGCs different from those labeled with DiI as described above. While DiI labels most of the RGCs projecting to the brain at the time of DiI application, electroporation of GFP in the subretinal space at E12.5 labels progenitor cells that will differentiate into RGCs a day or two later, and allowing prospective examination of RGC axon projection at later ages. Embryos were collected at E14.5, 15.5, 16.5, P0, and P4. To determine whether the GFP signal from a standard injected concentration of GFP plasmid, deteriorated through time after electroporation, pixel intensity in the GFP^+ area in the retina was measured in retinal whole mounts at the time points listed above. The pixel intensity was similar across the ages examined, with no statistical difference (Min: 0, Max: 255, Mean: 40.3 ± 2.26, p= 0.078, n = 30). This argues against the possibility that the observed decrease in ipsilateral RGC axons over time is a consequence of loss of GFP expression. Moreover, the contralateral projection is strongly labeled with the membrane-bound GFP in the most distal point of their extent, implicating that

the GFP signal remains robust from E12.5 until P4, and that this prospective labeling technique is valid and useful for developmental studies.

After electroporation of GFP at E12.5 into the central retina, axons of GFP^+ RGCs were seen to reach the diencephalon at E14.5 (Fig. 2C), and these axons were usually tipped with growth cones (Fig. 2C'). One day later, at E15.5, ipsilateral RGC axons (Fig. 2D) make a turn away from the ventral midline into the OT both in the medial (Fig. 2D, arrows) and lateral OC (Fig. 2D, arrow heads), and many axons were positioned in the ipsilateral and contralateral OT.

Even though only a subset of RGCs are labeled with this technique, after E14.5 we were able to quantify the relative number of ipsilateral RGC axons in the proximal portion of the OT (Fig. 3A, CA), as in Figure 3C-C'. The number of ipsilateral axons varies from E15.5 to P4 ($p= 0.0092$) and at E15.5 and E16.5 is rather similar (E15.5: 8.02 ± 2.60, n=6; E16.5: 8.81 ± 2.91, n=7, $p= 1$) but after E16.5, this number declines (E17.5: 2.64 ± 1.11, n=6; P0: 1.38 ± 0.75, n=6; P4: 0.36 ± 0.23, n=5). The decline is more accentuated when comparing the number of ipsilateral axons in the OT between E16.5 and P0 and P4 (E15.5 vs E17.5: $p=0.12$; E15.5 vs P0: $p=0.0505$; E15.5 vs P4: $p= 0.040$; E16.5 vs E17.5: $p= 0.10$; E16.5 vs P0: $p=0.014$; E16.5 vs P4: $p=0.0025$) (Fig. 3B).

Next we determined how far the ipsilateral RGC axons electroporated at E12.5 project from E14.5-P4, and whether they invade the SC and/or dLGN. We quantified the number of axons in 500µm sectors along the OT from the ventral midline to their most dorsal extent at 4000 µm in frontal sections (Fig. 4A, B). A greater number of central retinal ipsilateral axons is in the proximal optic tract at E15.5 and E16.5 than at later ages (Fig. 4C, G, H), but their number is still markedly lower than the contralateral RGC axons from the same cohort of electroporated RGCs (Fig. 4E), and few of these axons extend beyond 3000µm distal to the midline. In Fig. 4D, at E16.5, a central retinal ipsilateral axon with a growth cone, and therefore presumably still extending, is seen in the OT (Fig. 4D, arrow). At E17.5 and thereafter the number of axons decreases but the remaining axons extend farther than at earlier ages (Fig. 4G, H). There was no evidence of axonal degeneration, i.e., large axonal swellings disconnected from neurites.

Thus, the DC retinal RGCs, electroporated at E12.5 with GFP reach the OT after E14.5. The central retinal ipsilateral RGC axons from this group of cells project along the OT primarily between E15.5 and E16.5. Subsequently, their number decreases abruptly. At the time of

decline of central retinal ipsilateral RGC axon number, E16.5 to E17.5, and prior to this time, most axons do not seem to have projected to the SC.

Target entry of RGC axons electroporated at E12.5.

Previous studies in rat indicated that the central retinal RGC axons project to the SC at postnatal ages (Cowan et al., 1984) but whether the central retinal ipsilateral RGC axons project to this and other targets is not clear (Godement et al., 1980; Godement et al., 1984). In the brains electroporated at E12.5 and analyzed up to P4, we determined the extent of the contralateral and ipsilateral RGC axonal projection from the central retina to targets such as the dLGN and SC. At postnatal ages the RGC axons reach the LGN and SC roughly at the same time but enter and arborize later in the dLGN than in the SC (Dhande et al., 2011). We observed that very few electroporated RGC axons enter the LGN area at E16.5 and E17.5, and bifurcate with short branches along the dorso-ventral axis of the dLGN, both the central retinal contralateral (Fig. 5A, C) and ipsilateral RGC axons (Fig. 5B, D). Nevertheless, while contralateral axons begin to show further complexity within the dLGN by P0 (Fig. 5E), ipsilateral axons continue to display simple morphology (Fig. 5F), similar to that seen during prenatal ages, and never increase in number. At P4 the contralateral axons have very complex arbors that are focused in the appropriate topographic retino-recipient region in the dLGN for the DC retina (Pfeiffenberger et al., 2005) (Fig. 5G, arrow). Again at P4, the ipsilateral RGC axons display morphologies similar to that seen at previous stages, i.e., simple relatively unbranched terminations (Fig. 5H).

The SC is the first target in the brain to receive retinal projections, with the earliest axons entering at E15.5 (Godement et al., 1984). In mouse, from E16.5 to P0 RGC axons extend in the SC, overshooting their topographically appropriate target (McLaughlin and O'Leary, 2005). Only at P2 do they start to form branches in the topographically appropriate area in the SC. At P4, RGCs begin to prune branches projecting outside the final target area. This refinement process is established by P10 (Feldheim and O'Leary, 2010). In the cohort of RGCs electroporated at E12.5, the first axons reach the rostral SC at E16.5 and invade the SC at E17.5 (Fig. 6). At E17.5 the RGC axons project to the most superficial dorsal area of the SC but do not invade the inner-most area of the SC, in agreement with previous reports (Godement et al., 1984). The central retinal ipsilateral RGC axons electroporated at E12.5 project to the rostral-most area of the SC

(Fig. 6A, A'), while the contralateral axons project more caudally (Fig. 6A-C). By P0, the contralateral RGC axons occupy most of the SC, and invade deeper layers of the SC than at E17.5 (Fig. 7A).

In order to better visualize the rostral-caudal distribution of the RGC axons in the SC, a schematic reconstruction of the SC was created, showing the area occupied by the central retinal contralateral axons in green, and individual central retinal ipsilateral RGC axons as red tracings (Fig. 7E-J). At P0, the few remaining ipsilateral RGC axons from the central retina project more caudally in the SC compared with E17.5, but do not project as far caudally as the contralateral axons in the same cohort of electroporated RGCs (Fig. 7B, E-G). Only by P4 do the central retinal ipsilateral RGC axons reach the caudal SC. At the same age, it is possible to notice a slight decrease in the contralateral RGC axon territory, likely reflecting pruning of the axons outside of the appropriate topographic area (Fig. 7H-J). Both central retinal contralateral and ipsilateral axons do not show complex branches at P0, when compared with axons at P4. At P4, the contralateral RGC axons form complex arbors in a specific area in the SC (Fig. 7C). A few ipsilateral axons formed a branched arbor but others were much more simple (Fig. 7D, H-J). Thus, the central retinal ipsilateral and contralateral RGC axons electroporated in the retina at E12.5 show quite different behaviors projecting to and within their targets. Although the contralateral and ipsilateral axons extend past the dLGN by E17.5, neither population enters the dLGN until after E17.5. The few ipsilateral axons that do enter have only simple branches through P4, while the contralateral axons form full arbors by that time. In the SC, the ipsilateral RGC axons project into this target later (P0) than the contralateral RGC axons, and although they branch, they do not reach the same complexity at P4 as the contralateral axons.

Discussion

Using DiI labeling and *in utero* electroporation of GFP, we have followed the progression and waning of the transient ipsilateral retinal ganglion cell (RGC) axons projecting from the DC and central retina in the early period of RGC axon growth. As seen by DiI labeling, the ipsilateral RGC axons projecting from the DC retina at E13.5 comprise a greater proportion of axons

extending in the OT compared with the contralateral axons at that time and compared with the RGC axons forming the permanent ipsilateral projection from the VT retina. Subsequently there is a precipitous drop in the proportion of ipsilateral to contralateral RGC axons that originate in the DC retina. RGC precursors that are electroporated in the central retina at E12.5, enter the OT at E14.5 and that can be followed until P4, display similar timing of elimination. The ipsilateral RGC axons observed after both labeling paradigms the DC/central retina seem to disappear through a process independent of interactions with their targets since so few of the ipsilateral RGC axons observed in this study ever reach targets in the brain beyond the dorsal OT just rostroventral to the LGN. While the mechanism of the disappearance of the early ipsilateral RGC axons is not understood, these data provide a more detailed picture of the ephemeral ipsilateral RGC projection than in previous studies.

<u>After ipsilateral RGC axons from the DC retina reach the proximal optic tract, their number falls abruptly</u>

The disappearance of ipsilateral RGC axons from the DC retina has been acknowledged, but previous studies analyzed this projection in mice with whole eye anterograde labeling with HRP (Godement et al., 1987), retrograde labeling from the OT with DiI and examined only the retina (Colello and Guillery, 1990), or by retrograde labeling from the SC with Fast Blue dye in rats (Cowan et al., 1984). None of these studies measured the extent and targeting of the transient DC retinal ipsilateral projection during the early stages of development. In our measures of the extent of projection of the early RGC projection from E13.5 to E15.5 using anterograde DiI labeling, we found that the distance to which the majority of the early DC ipsilateral RGC axons project is stable over the first few days of growth, indicative of stalling, while the contralateral RGC axons labeled at the same ages progress more distally in the OT. Since the development of the retinal projection is a dynamic process and we analyzed DiI labeling in fixed tissue, we could not determine with certainty whether any of the early DC ipsilateral RGC axons target the SC and retract. Nevertheless, in our analysis, the ipsilateral RGC axons from both the DC and the central retina from labeling by electroporation extended only up to the ventral aspect of the future dLGN. Live imaging in semi-intact preparations or *in utero* would resolve whether RGC axons project any farther or retract after reaching the SC.

At E13.5, the proportion of ipsilateral RGC axons within the OT compared to contralateral axons is 20% of the total projection as estimated from DiI labeling, whereas by E15.5 the relative proportion of ipsilateral RGC axons is only 5%. This early higher percentage of ipsilateral axons, when RGCs project only from the DC retina, can be explained by two hypotheses that are not mutually exclusive. First, the DC ipsilateral RGC axons take a shorter path from the optic nerve to the OT (Fig, 2D) and populate the OT before the DC contralateral RGC axons that are still crossing the midline at E12.75. Thus, the proportion of DC ipsilateral RGC axons is relatively high at E13.5 when compared with the DC contralateral RGC axons. Second, the ipsilateral-to-contralateral ratio of 20% could be due to an inaccurate early midline crossing, as a consequence of the immaturity of the OC and its factors that selectively attract/repel selected populations of axons from the midline at E13.5. The DC ipsilateral RGC axons enter the chiasm region at E12.75 when the chiasm expresses the repellant EphrinB2 at low levels (Williams et al., 2003). However, the early DC ipsilateral RGC axons do not grow close to the midline and thus should not interact with this cue, even though the early DC RGCs express EphB1 at E13.5 (Marcus and Mason, 1995; Williams et al., 2003).

In the optic tract, the first DC ipsilateral RGC axons are segregated from the DC contralateral RGCs

At E13.5 the DC ipsilateral RGCs are the first to grow into the OT, and occupy the most medial position in the OT. As the DC contralateral RGC axons enter the tract, they course lateral to the ipsilateral RGCS. At this early age, it is striking that the two populations are segregated from one another. This lateral-medial organization might simply reflect a chronotopic mode of growth, as found in the ferret visual system (Walsh and Guillery, 1985), with each successive cohort layering on top of the previously extending cohort. In support of chronotopic organization of different RGC axon cohorts, the permanent ipsilateral RGCs from VT retina project later and occupy an even more lateral position in the OT compared with contralateral RGC axons (Godement et al., 1984); Sitko and Mason, unpublished).

The ipsilateral-contralateral segregation of axons in the OT is relevant to the suggested role of the earliest RGC axons as pioneers of the OT, readily experimentally analyzed in zebrafish

(Pittman et al., 2008) and insect (Raper and Mason, 2010). Axon-axon interactions have been proposed to be a mechanism underlying axon order in tracts that then mediate segregated targeting (Imai and Sakano, 2011). However, our data argues against the hypothesis that the first ipsilateral RGC axons that project to the OT at E13.5 pioneer the OT by axon-axon interactions. The segregation of the early ipsi- and contralateral RGC axon cohorts in the OT may reflect homotypic interactions among fibers of each subpopulation rather than axon-axon interactions across these two populations as would be expected if the early ipsilateral fibers played a pioneering role.

Difference in innervation of targets by early ipsilateral versus contralateral RGCs from the central retina

Electroporation *in utero* of a GFP plasmid at E12.5 targeted the central retina and allowed visualization of the retinal projections from the central retina to both sides of the brain. We described two differences in target innervation between the projections of the early ipsilateral and contralateral central retinal RGCs. First, while central retinal contralateral RGC axons project to the dLGN after P0 and make complex arbors by P4, the ipsilateral RGC axons project only short branches to the dLGN that branch minimally, if at all. A previous study that labeled RGCs in the peripheral retina by electroporation at postnatal ages found no difference in the features of dLGN innervation by the contralateral and ipsilateral axons, especially their morphology, although they noted that arborization of RGC axons in the dLGN at P4 lagged behind the arborization in the SC by almost one week (Dhande et al., 2011). However, in the Dhande study the permanent ipsilateral RGCs from the VT retina were visualized, whereas in our study the transient ipsilateral RGCs in DC/central retina were labeled. Second, there is a delay in the progression of the central retinal ipsilateral RGC axons in the OT from E15.5 to E17.5 and in the rostral-caudal axis of the SC at P0, compared with the extension of the contralateral axons electroporated in the same cohort. Nonetheless, the timing of branching in the SC seems similar in both contra- and ipsilateral populations.

The strategy of innervation of RGC axons to their targets seems to change throughout development (Osterhout et al., 2014). Early-born RGC axons that project as early as E15.5 innervate multiple targets and subsequently retract the projections from inappropriate targets.

However, later-born RGC axons accurately project to their appropriate and final targets. Various RGC subtypes have different birth dates, molecular markers, projections and functional roles (Hong et al., 2011; McNeill et al., 2011; Osterhout et al., 2014; Osterhout et al., 2011; Triplett et al., 2014). To date, molecular markers for the transient ipsilateral RGCs from DC and central retina are lacking and thus it is not clear whether the transient RGCs observed in the present study have a distinct molecular profile or belong to a RGC subtype. Judging from the time of their projection, the transient ipsilateral RGCs labeled from the DC retina with DiI and the central RGCs electroporated with GFP at E12.5 could belong to the first group of early-born, early-projecting, non-imaging forming RGCs expressing cadherin 3 and cadherin 4 that project from the DC retina at E14.5 (Osterhout et al., 2014).

Mechanisms of elimination of transient axonal projections

Several mechanisms for the disappearance of axonal projection can be invoked. Caspases have been newly implied in non-apoptotic roles such as pruning of axonal branches (Campbell and Okamoto, 2013; Simon et al., 2012). We attempted to determine whether caspase 3 and 6 are expressed in the contralateral and ipsilateral RGC axons when they are in the optic tract and approach the dLGN, with and without GFP labeling. Although a few retinal cells expressed caspase 3, we were not successful in detecting these proteins in RGC axons, and thus cannot implicate this mechanism for RGC axon transience. One explanation for the inability to retrogradely label the transient ipsilateral RGC projection is that RGC cell bodies migrate away from the DC region (Guillery et al., 1995) . This hypothesis is unlikely since when the central retina was electroporated at E12.5 and observed later, no labeled RGCs were observed in the peripheral retina.

The elimination of axonal projections in inappropriate targets has been attributed to the absence of appropriate trophic factors in the target (Lom and Cohen-Cory, 1999; Yamaguchi and Miura, 2015), or the absence of appropriate receptors or trophic factors in the growing neurons themselves (Cohen-Cory et al., 2010; Harvey et al., 2012). In support of these hypotheses, growing RGC axons have an intrinsic supply of neurotrophic factors supporting growth toward targets but when axons reach their target they become dependent on target-derived neurotrophic factors (Marshak et al., 2007; Spalding et al., 2004). The ipsilateral RGC

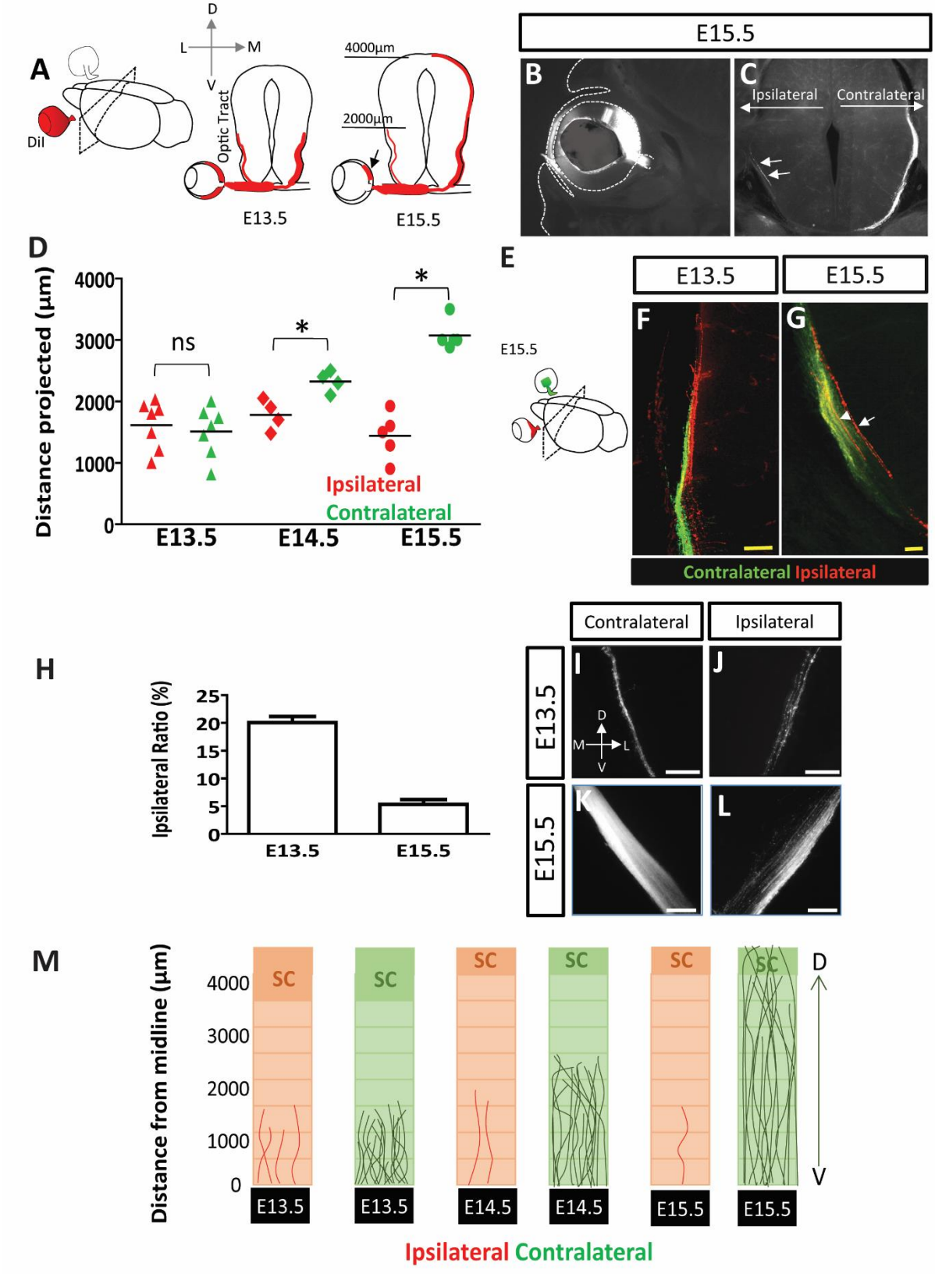
axons that we electroporated at E12.5 in the central retina grow more slowly than the contralateral RGC axons, and the slower progression could reflect a reduced intrinsic supply of neurotrophic factors before reaching the target. In addition, we did not observe the majority of early-projecting ipsilateral RGC axons reaching their first retinal target, the SC, or entering the dLGN, implying that the DC and central retinal ipsilateral axons do not perceive target-derived neurotrophic factors that would ensure their progression toward and entry to the target and/or survival.

Other mechanisms underlying the disappearance of this projection include interactions with glial cells in the OT or between the ipsi- and contralateral cohorts within the OT. The central retinal contralateral RGC axons might express factors at their surface important for support from OT astroglia, or microglia (Pont-Lezica et al., 2014), that precludes the ipsilateral cohort from fasciculating in the optic tract with the contralateral cohort. However, to date, we have not been able to distinguish early DC ipsilateral from contralateral RGCs by transcription factor expression or surface molecules (our unpublished data).

Summary and conclusions

Anterograde Dii labeling and in utero electroporation of GFP have provided new details on the transient ipsilateral projection from the retina distal to the optic chiasm and visual targets in the brain. DiI provides a snapshot of the early stages of RGC axon growth from the DC retina and GFP electroporation at E12, a prospective chronicle of the extent, targeting and disappearance of the transient ipsilateral RGC projection from the central retina. Both approaches have shown for the first time that the majority of the transient ipsilateral RGC axons do not innervate targets, and provides precise spatiotemporal information on their disappearance. This study will provide a basis for further analysis of this transient projection by fate mapping, and investigation of the mechanisms underlying its elimination.

Figures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Ipsilateral Ratio (%)
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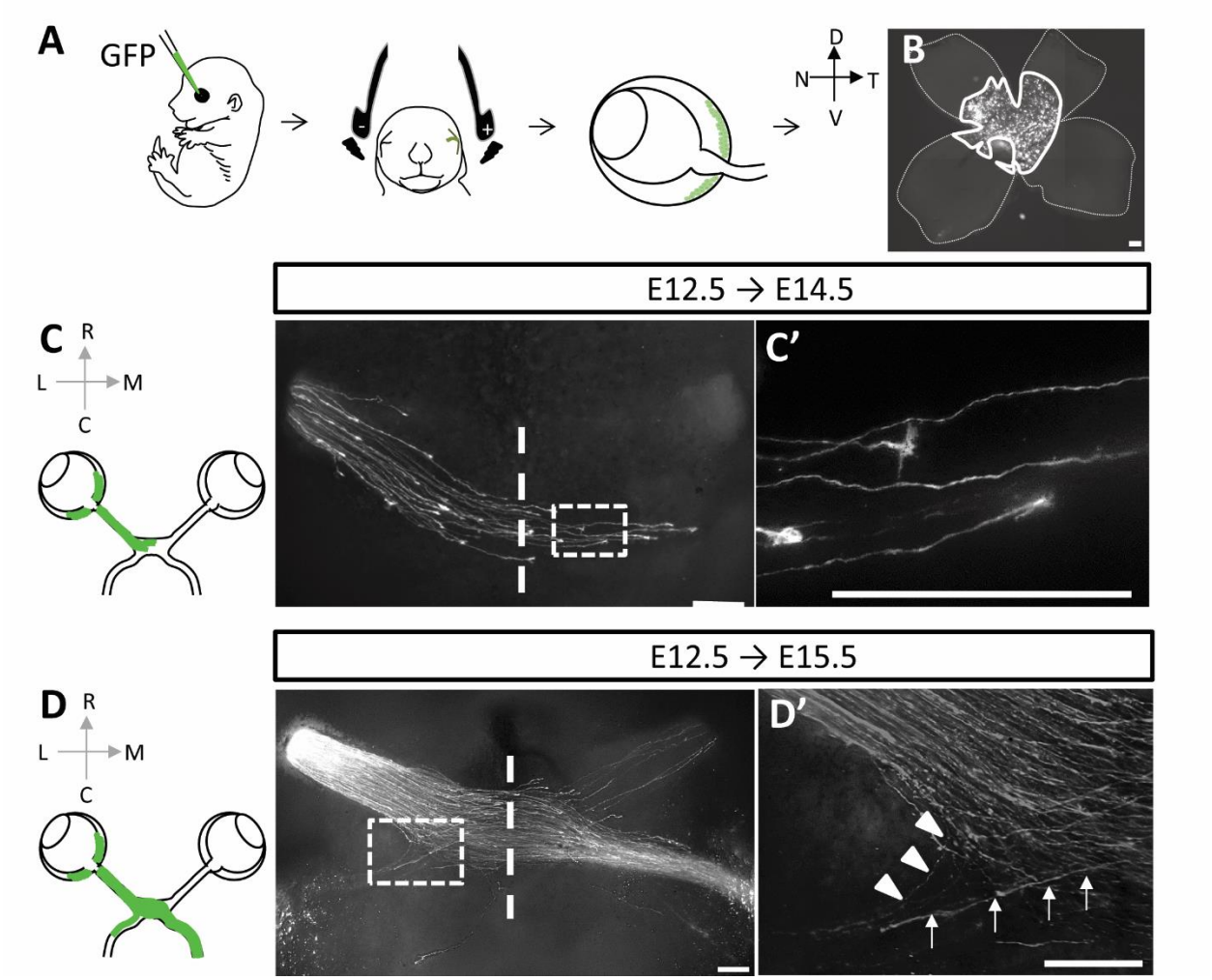
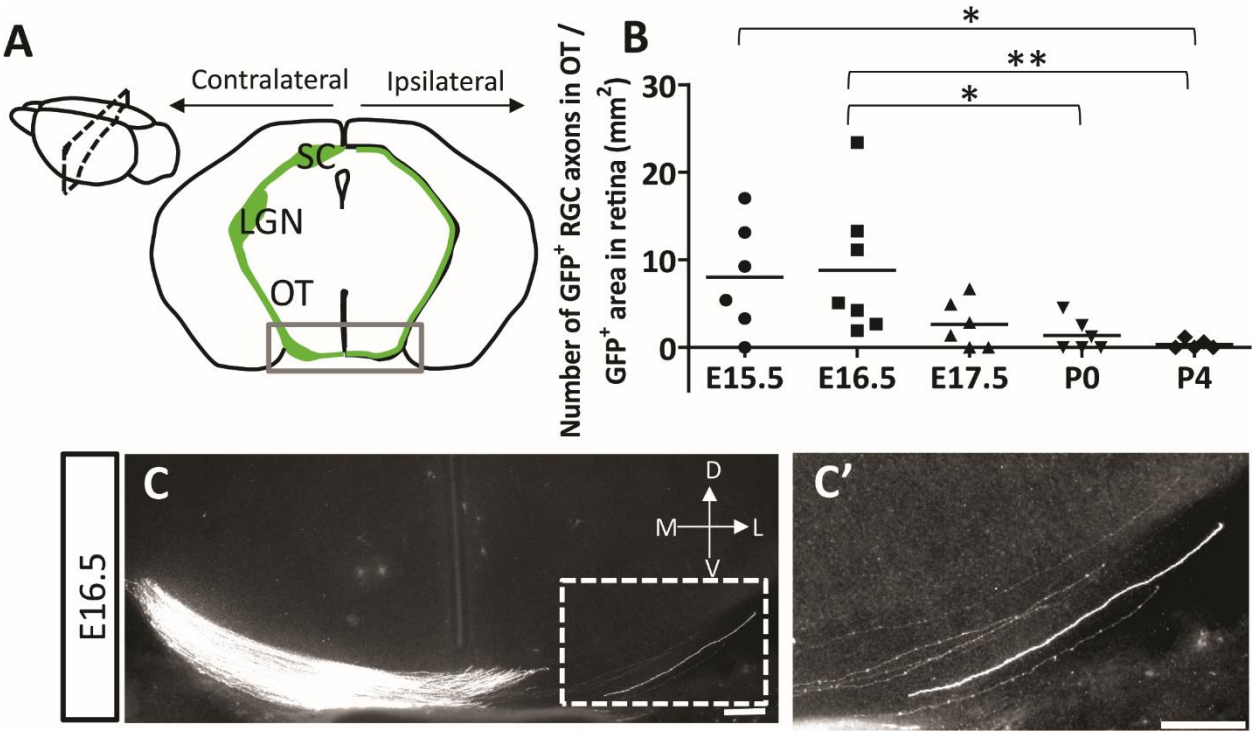
Section1 - Figure 1 Retinal ganglion cell axon projections in the first stage of extension – DiI labeling.

(**A, B**) DiI was applied to the retina of fixed mouse embryos to visualize retinal ganglion cell (RGC) projections to the brain, either to the whole retina at E13.5 and E14.5 (left) or only to the dorsocentral (DC) retina at E15.5 (right, and B). (**C**) DiI labeling in the ipsilateral and contralateral optic tracts at E15.5 when only the DC is labeled. (**D**) The distance that RGC axons extend from the ventral midline to the dorsal thalamus over time. While the extent to which the contralateral RGC axons project varies from E13.5 to E15.5 ($p= 0.0014$), the extent of the ipsilateral RGC projection does not ($p= 0.47$). Each mark = a single brain, horizontal bar = mean, $p< 0.05$. (**E-G**) DiI or DiA was applied in fixed embryos, to the entire retina at E13.5, when only RGCs from DC retina extend axons, and to the DC retina at E15.5, and the brain sectioned frontally. (**F**) At E13.5, DC ipsilateral axons occupy a more medial position in the OT compared with contralateral RGCs. (**G**) At 15.5, most DC ipsilateral RGC axons continue to occupy the medial-most position in the OT (arrow) but a few ipsilateral axons are positioned in the lateral OT mingled with contralateral axons (arrowhead). (**H**) DiI crystals were applied to the whole retina at E13.5 and E15.5, and the embryos were sectioned frontally. The proportion of ipsilateral to contralateral RGC axons was estimated from pixel intensity (PI) of DiI in the Region of Interest (ROI) within the OT: PI ipsilateral ROI / (PI ipsilateral ROI + PI contralateral ROI) x 100, expressed as a percentage. The ipsilateral RGCs within the OT at E13.5 represent 20.07% (±1.09), n=6, of the total projection in both OTs. At E15.5 the percentage of ipsilateral RGCs decreases to 5.32% (±0.88), n=7. $p= 0.0012$. Data represents mean ± SEM. (**I-L**) Representative frontal sections through the contra and ipsilateral OT after DiI labeling of the optic nerve head in fixed brains at E13.5 and E15.5, performed as in A. Note that at E15.5 (K, L), the entire retinal projection was labeled, and thus includes the transient ipsilateral axons from central retina and the permanent ipsilateral RGCs from ventrotemporal retina. (**M**) Schematic representation of the extent and relative number of retinal projections from the midline to the optic tract (OT) and superior colliculus (SC). At E13.5 when the first axons project to the OT, the ipsilateral and contralateral projections project to the same distance. After E14.5 the contralateral projection projects farther than the ipsilateral axons. *$p< 0.05$, ** $p< 0.01$. SC: superior colliculus, OT: Optic tract, ROI: region of interest. D/L/M/V: dorsal, lateral, medial, and ventral. All scale bars= 100µm.



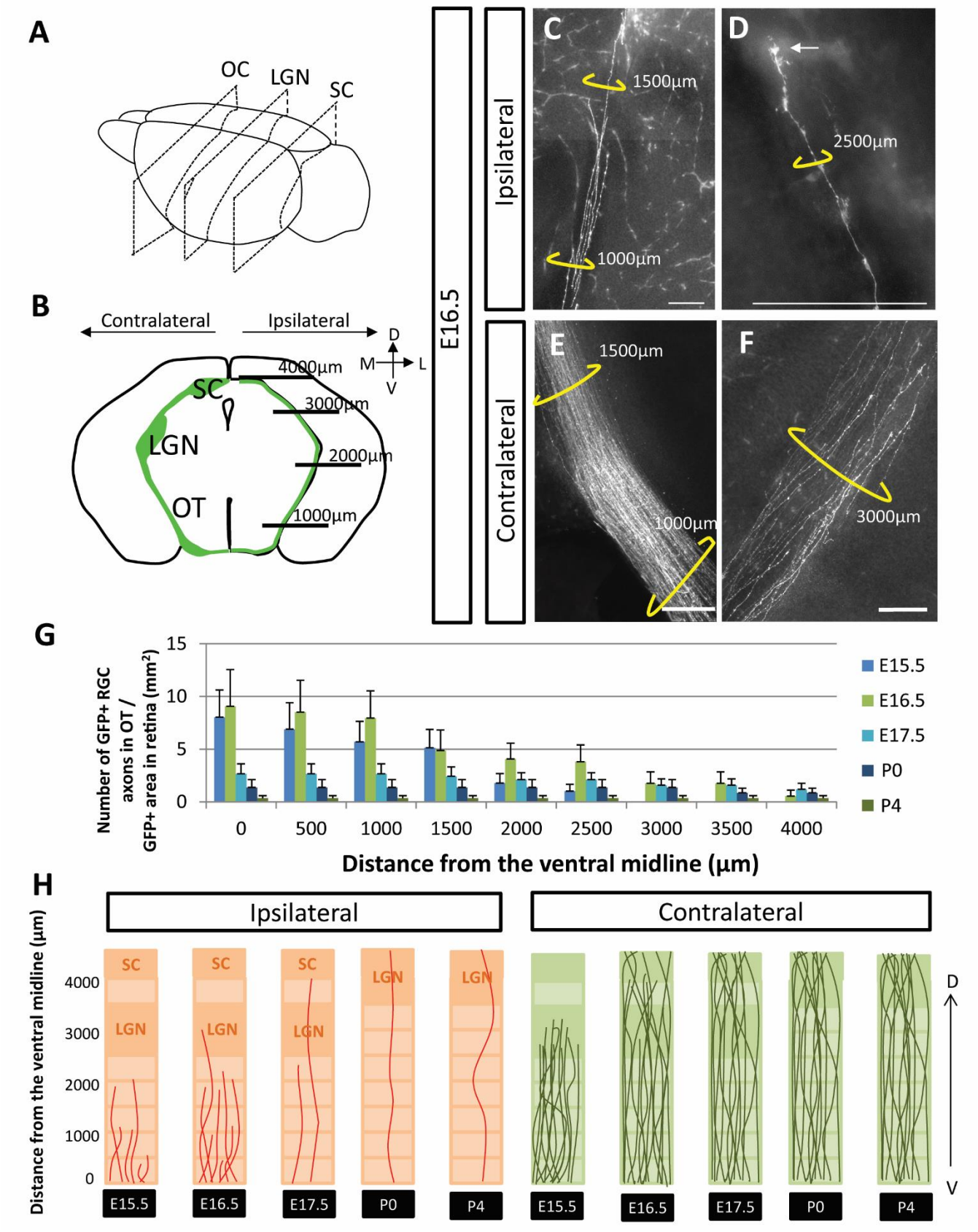
Section 1 - Figure 2 Electroporation of GFP into the central retina at E12.5 labels RGCs that cross the midline at E14.5.

(A) Mouse embryos are electroporated *in utero* with a GFP plasmid in the subretinal space at E12.5. Right scheme shows frontal section of eye through the optic nerve indicating the site of electroporation in the central retina. (**B**) Retinal whole mount at E16.5 confirming that only the central area of the retina is targeted with GFP (outlined area). **(C, C')** Left, scheme of E14.5 retina, optic nerves and chiasm. Center, in a whole mount of the ventral diencephalon, axons of RGCs electroporated at E12.5 reach the optic chiasm (OC) midline at E14.5 and have growth cones. This suggests that the cohort of RGCs targeted by electroporation at E12.5 have not yet extended axons at the time of electroporation and reach the OC two days later. (**D, D'**) Left, scheme of E15.5 retina, optic nerves and chiasm. Center, whole mount; many more RGCs from the central retina have crossed or turned away from the midline. Some ipsilateral axons turn more medially in the OC (arrows) while others exit the chiasm more laterally (arrowheads) (**D'**). White dashed vertical line: optic chiasm midline. D/N/T/V: dorsal, nasal, temporal, and ventral. C/L/M/N/R: caudal, lateral, medial, nasal and rostral. All scale bars= 100µm.



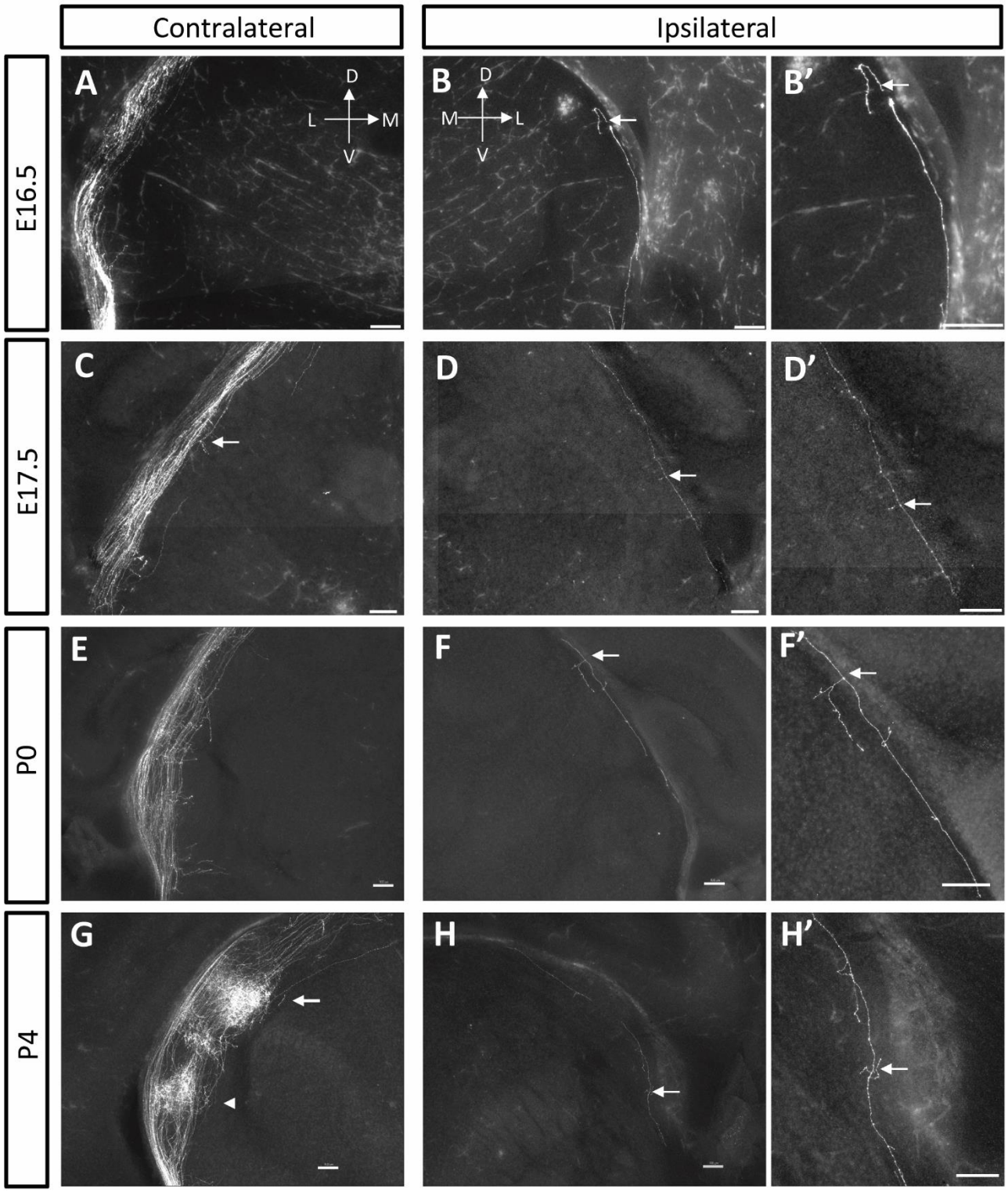
Section1 – Figure 3 In the cohort of RGCs electroporated with GFP at E12.5, the number of DC ipsilateral axons decreases after E16.5.

(**A-B**) After electroporation of GFP plasmids into E12.5 retina (see Fig. 2), the number of GFP-labeled ipsilateral RGC axons was quantified in 500 micron sections through and caudal to the optic chiasm (OC) at ages E15.5-P4. (**B**) The number of ipsilateral axons in the optic tract (OT) progressively decreases after E16.5 to nearly 0. Horizontal bars = mean. Mann Whitney test E15 vs P4 p=0.0398; E16 vs P0 p= 0.0140; E16 vs P4 p= 0.0025. (**C**) An example of the area selected for quantification at E16.5 with ipsilateral axons electroporated with GFP at E12.5 shown at higher magnification in Fig. 3C'. *p< 0.05, ** p< 0.01. D/L/M/V: dorsal, lateral, medial, and ventral. All scale bars = 100µm.


A
OC
LGN
SC
B
Contralateral
Ipsilateral
D
M
L
V
4000µm
3000µm
2000µm
1000µm
SC
LGN
OT
E16.5
Ipsilateral
Contralateral
C
1500µm
1000µm
D
2500µm
E
1500µm
1000µm
F
3000µm
G
Number of GFP+ RGC axons in OT / GFP+ area in retina (mm²)
15
10
5
0
0
500
1000
1500
2000
2500
3000
3500
4000
Distance from the ventral midline (µm)
E15.5
E16.5
E17.5
P0
P4
H
Distance from the ventral midline (µm)
Ipsilateral
Contralateral
4000
3000
2000
1000
0
SC
LGN
E15.5
E16.5
E17.5
P0
P4
D
V

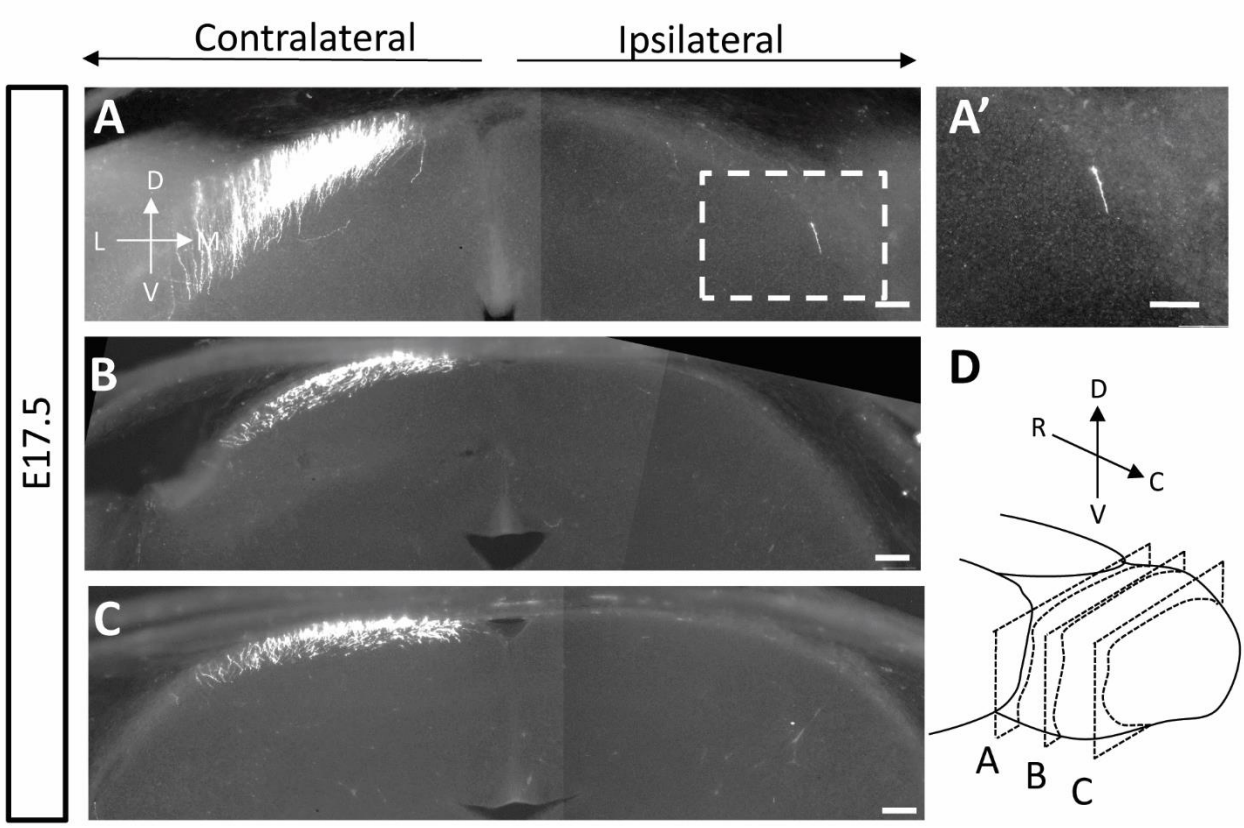
Section1 – Figure 4 In the cohort of RGCs electroporated with GFP at E12.5, only a few central ipsilateral axons project distal to the optic tract after E16.5.

(**A**) Schema of brain indicating sampling of frontal sections through the optic chiasm (OC), lateral geniculate nucleus (LGN) and a rostral section through the superior colliculus (SC). (**B**) Schema of the RGC axon projection in the brain in frontal view, indicating the distance measured from the OC midline through the optic tract (OT) to dLGN and SC targets. (**C-F**) The optic tract at different distances from the ventral midline, at E16.5. (**C**) Few GFP^+ axons from RGCs from the central retina are in the proximal ipsilateral OT at E16.5 compared with the contralateral projection (C, D vs E, F). (**D**) In the same cases as in C., the few ipsilateral axons that extend along the OT have growth cones. (**E**) At E16.5, many GFP^+ RGC axons project contralaterally. (**F**) The contralateral RGC axons project further along the OT than the ipsilateral RGC axons (**G**) The number of central retina ipsilateral RGC axons was quantified in contiguous 500µm sectors beginning from the ventral midline to the SC. A greater number of ipsilateral axons from the central retina are in the proximal optic tract at E15.5 and E16.5 than at later ages, but few axons extend beyond 3000 µm from the midline. After E16.5 only a few ipsilateral axons extend farther. (**H**) Scheme of the contralateral and ipsilateral axons labeled by electroporation at E12.5 in the central retina, by number and length, from E15.5 to P4. The darker shaded bars represent the LGN and the SC. The contralateral axons from the central retina extend toward and reach targets compared with ipsilateral axons from the central retina at the same developmental stage. dLGN: dorsal lateral geniculate nucleus, OT: optic tract, SC: superior colliculus. D/L/M/V: dorsal, lateral, medial, and ventral. All scale bars= 100µm.


Contralateral
Ipsilateral
E16.5
E17.5
P0
P4
A
B
B'
C
D
D'
E
F
F'
G
H
H'
D
L
M
V

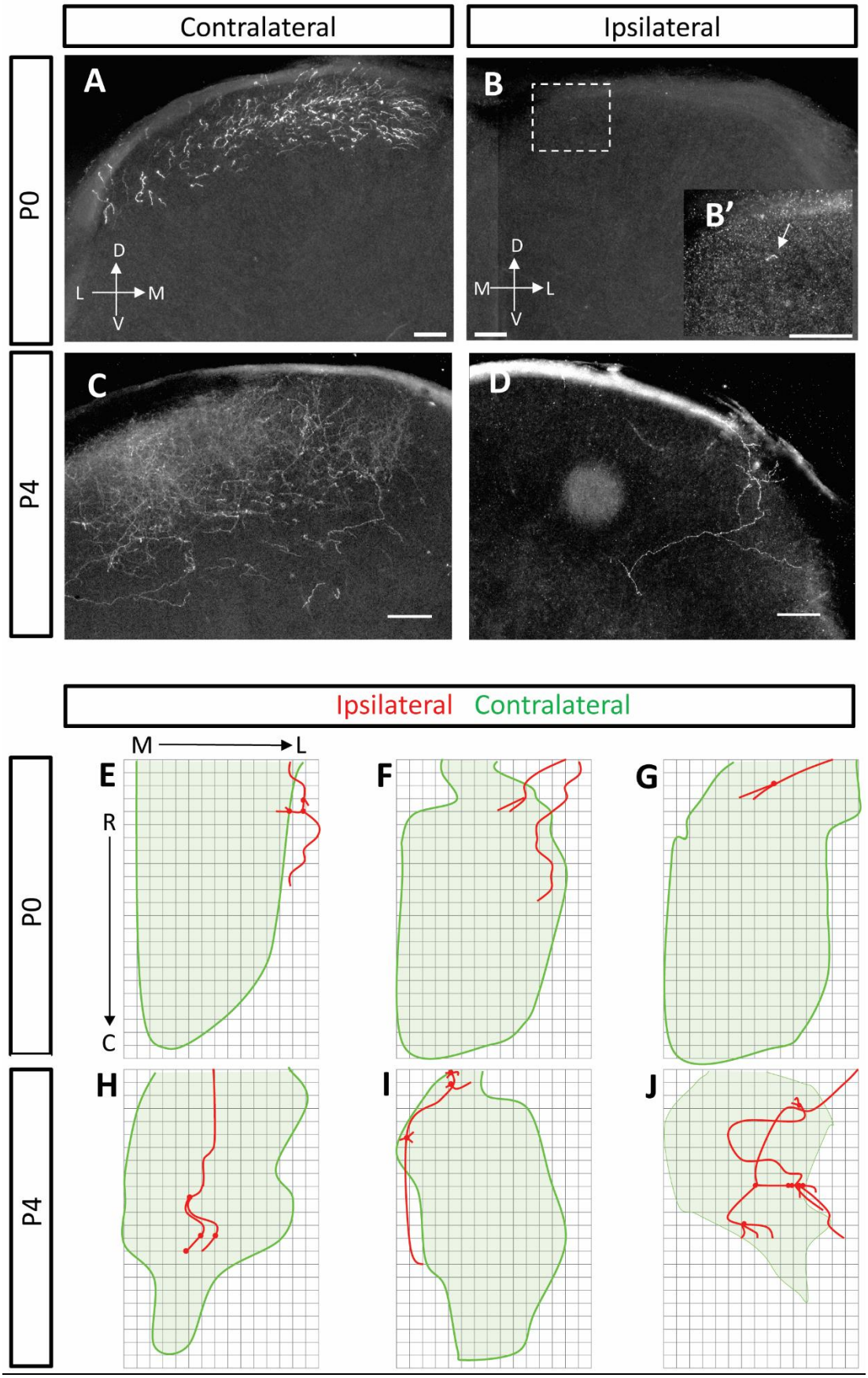
Section1 – Figure 5 Few ipsilateral RGCs electroporated at E12.5 in the central retina project to the dorsal lateral geniculate nucleus.

(**A-H'**) Frontal sections through the dorsal lateral geniculate nucleus (dLGN) at E16.5, E17.5, P0 and P4, after electroporation of GFP into the central retina at E12.5 (see Fig. 2). (**A-B'**) At E16.5, central retinal axons project contralaterally (A) or ipsilaterally (B) in the optic tract, adjacent to the future dLGN. Few contralateral axons project into the dLGN area. (B, B'). In B', one axon sends a short branch (arrow) to the dLGN. (**C-D'**) At E17.5 only a few contralateral and ipsilateral central retinal RGC axons project short branches into the dLGN (arrows). (**E-F'**) At P0, contralateral RGC axons enter the dLGN while the few ipsilateral axons from the central retina that remain have modest projections to the LGN (arrow). (**G-H'**) At P4 the contralateral projections from the central retina form complex branched arbors in a medial patch of the dLGN (G, arrow) and ventral LGN (G, arrowhead). (H, H') On the opposite side of the brain, the ipsilateral RGC axons have a morphology similar to ipsilateral axons at P0, i.e., simple arbors with only a few short branches. D/L/M/V: dorsal, lateral, medial, and ventral; LGN, lateral geniculate nucleus. All scale bars= 100µm.



Section1 – Figure 6 Few RGCs electroporated at E12.5 in the central retina project to the superior colliculus at E17.5.

(**A-D**) Frontal sections through the superior colliculus (SC) at E17.5, 200µm apart (D), displaying RGC axons electroporated with GFP at E12.5 in the central retina. The contralateral RGCs target along the rostral-caudal axis of the SC at E17.5 (A-C) while the ipsilateral counterparts project into the SC in a more rostral portion of the SC (A'). C/D/L/M/R/V: caudal, dorsal, lateral, medial, rostral, and ventral. All scale bars= 100µm.


Contralateral
Ipsilateral
P0
P4
A
B
B'
C
D
D
L
M
V
M
L
D
V
Ipsilateral Contralateral
M
L
R
C
E
F
G
H
I
J

Section1 – Figure 7 Some ipsilateral RGCs electroporated at E12.5 in the central retina project to and arborize in the superior colliculus at P0 and P4.

(**A-D**) Frontal section of the superior colliculus (SC) at P0 and P4 after electroporation of GFP into central retina at E12.5. (**A-B**) At P0, the contralateral RGCs electroporated in central retina project to most of the SC (pale green shaded area). At the same age, only a few central retina ipsilateral axons, with simple morphology, are seen (B and B'). (**C**-**D**) At P4, many more contralateral axons projecting into the SC are branched when compared with RGC axons P0. The few ipsilateral axons projecting into the SC are also more branched than at P0. (**E-J**) Reconstruction of projections to the SC of RGCs electroporated at E12.5 in the central retina. Each square represents a 100 x 100µm area in the SC. The green shading represents the area occupied by the electroporated contralateral RGCs. The red tracing represents individual ipsilateral RGC axons in the same cases in which the contralateral projections were estimated. At P4, ipsilateral axons project more caudally in the SC and have more branches when compared with P0. Note that the electroporated central ipsilateral RGC axons project to the lateral SC unlike the permanent ipsilateral RGCs from ventrotemporal retina, which project medially (not shown). C/D/L/M/R/V: caudal, dorsal, lateral, medial, rostral, and ventral. All scale bars= 100µm.

Section 2 - Alternative approach to label the central retina retinal ganglion cells – viral vector

Abstract

Some viral vectors have birthdating properties that allow them to birthdate the transfected cells and to fate-map the cells born at the time of the injection. Using a viral vector carrying the plasmid of a fluorescent protein would be a convenient approach to birthdate and fate-map the earliest projections from the retina including the transient ipsilateral retinal ganglion cells. Such an approach was tested, injecting a human Adeno type 5 CAG-mCherry viral vector in the retina at E12.5. The results were suboptimal because the virus produced uneven labeling in the cell body and axons, that would make further studies of fate-mapping and quantification of the number of axons less accurate than the alternative method tested in the previous section: the *in utero* electroporation of green fluorescent protein. In addition, the viral vector readily leaked to multiple areas in the brain.

Rationale

The dorsocentral (DC) RGC in the retina are the first retinal ganglion cells (RGC) to be born in the retina around E10.5. These DC RGCs project ipsilaterally or contralaterally. To understand more about the transient ipsilateral RGCs from this retinal region, I aimed to label these cells and follow their axonal projection through time. This can be achieved by timing their birthdating as one inroad, in combination with tracing their axons. Methods that would simultaneously enable birthdating and tracing of this population are expected to allow fate-mapping the ipsilateral and contralateral projection of these RGCs.

Viral vectors are a useful method to label cells during development to map their cell fate (Miyata et al., 2010; Turner and Cepko, 1987). Some viral vectors only transfect cells during specific phases of cell division, such as the M-phase of the cell cycle. If a virus transfects a cell at the time of the last M-phase of the cell cycle, that cell will have a higher concentration of the viral vector than the cells transfected and that underwent further cell division, diluting the concentration of the viral vector (Hashimoto and Mikoshiba, 2004).

Hashimoto et al. developed a human Adeno type 5 (Ad5) CAG-mCherry viral vector (Hashimoto et al., 2011). This Ad5 viral vector, carrying an epigenetic mCherry plasmid, transfects cells undergoing cell division, more specifically at the M-phase of the cell cycle, and they express their cargo briefly after transfection. This virus acts as a birthdating method since it transfects the cells only for a brief period of time (within 4h of the Ad5 injection) (Hashimoto and Mikoshiba, 2004).

I expected to use the birthdating properties of the Ad5 CAG-mCherry virus to label and trace the central retina RGC axons to study their projection trough time in mice.

Methods

Animals: C57BL/6J mice were kept in a timed pregnancy breeding colony at Columbia University. Procedures for the care and breeding of mice follow regulatory guidelines of the Columbia University Institutional Animal Care and Use Committee. Noon of the day on which a plug was found was considered E0.5.

Viral vector: Hashimoto et al. developed an Ad5 viral vector from a replication incompetent human Ad5 adenoviral vectors (Adex series) that lacked the E1A, E1B, and E3 region. An expression unit was inserted into the E1A–E1B deleted region to express mCherry.

The initial high-titter viral stock of 10^{11} plaque-forming units (pfu)/mL in 10% glycerol/PBS was diluted in aliquots with a concentration of 10^7 pfu/mL, and stored at -80°C.

In utero injection of the Ad5 viral vector: Pregnant mice with E12.5 embryos, were anesthetized with ketamine/xylazine (100 and 10 mg/kg, in 0.9% saline). 0.3-1µL of viral solution with a concentration of 10^7 pfu/mL was injected to the sub-retinal space. The viral vector titer was delivered with a pulled glass micropipette attached to Picospritzer II, from General Valve Corporation. The glass micropipette was filled with mineral oil first and then with the viral vector. After the injections, the abdomen was closed.

Animal sacrifice and tissue processing: The embryos were collected at E16.5 by caesarean section with the mother anesthetized with ketamine/xylazine. After decapitation the embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) (pH 7.4) for 8h and the eyes dissected to detect the mCherry fluorescence in the retinal wholemount. For cryosectioning,

whole heads or whole retina were embedded in 20% sucrose overnight, embedded in OCT (Fisher Scientific), frozen in dry ice, stored at -80°C for at least 24h, and cryosectioned frontally at 20µm.

Immunohistochemistry: The cryosections were blocked in 10% donkey serum (DS) + 0.5% Triton20 in PBS and then incubated with goat Brn3 antibody (1:200, Santa Cruz) + 1% DS + 0.5% Triton20 in PBS, overnight at 4°C. After 3 PBS washes for a total of 1 hour, tissue was incubated in Cy5 anti-goat antibody (1:1000, Life Technologies).

Image processing and quantification: Retinal sections were imaged on a Zeiss AxioImager M2 microscope with an AxioCam MRm camera, and Neurolucida software (v 11.01, MicroBrightField Systems), using 5x, 10x, or 20x objectives. Images were processed with ImageJ software (version 1.48, NIH).

Results

Five embryos from different mothers were successfully injected with the mCherry Ad5 viral vector, but one embryo showed microphthalmia in the injected eye and was not used for further analysis. The viral vector targeted a wide area in the retina and not only the central retina, with a diffuse and inconsistent pattern of expression instead of a small patch with an intense concentration of targeted cells (Fig. 1A). The retinas were dissected, sectioned and immunostained for the RGC marker, Brn3, to verify whether the RGCs were labelled by the Ad5-mCherry viral vector (Fig.1B).

The mCherry Ad5 viral vector apparently leaked to areas outside the sub-retinal space, targeting the trigeminal ganglion and cells near the third ventricle (Fig. 1C a,b). Unfortunately, while the mCherry fluorescence was strong in some non-retinal neurons, such as in the trigeminal ganglion, in the RGC axons the mCherry fluorescence was faint and segmented (Fig. 1C a-c).

Discussion

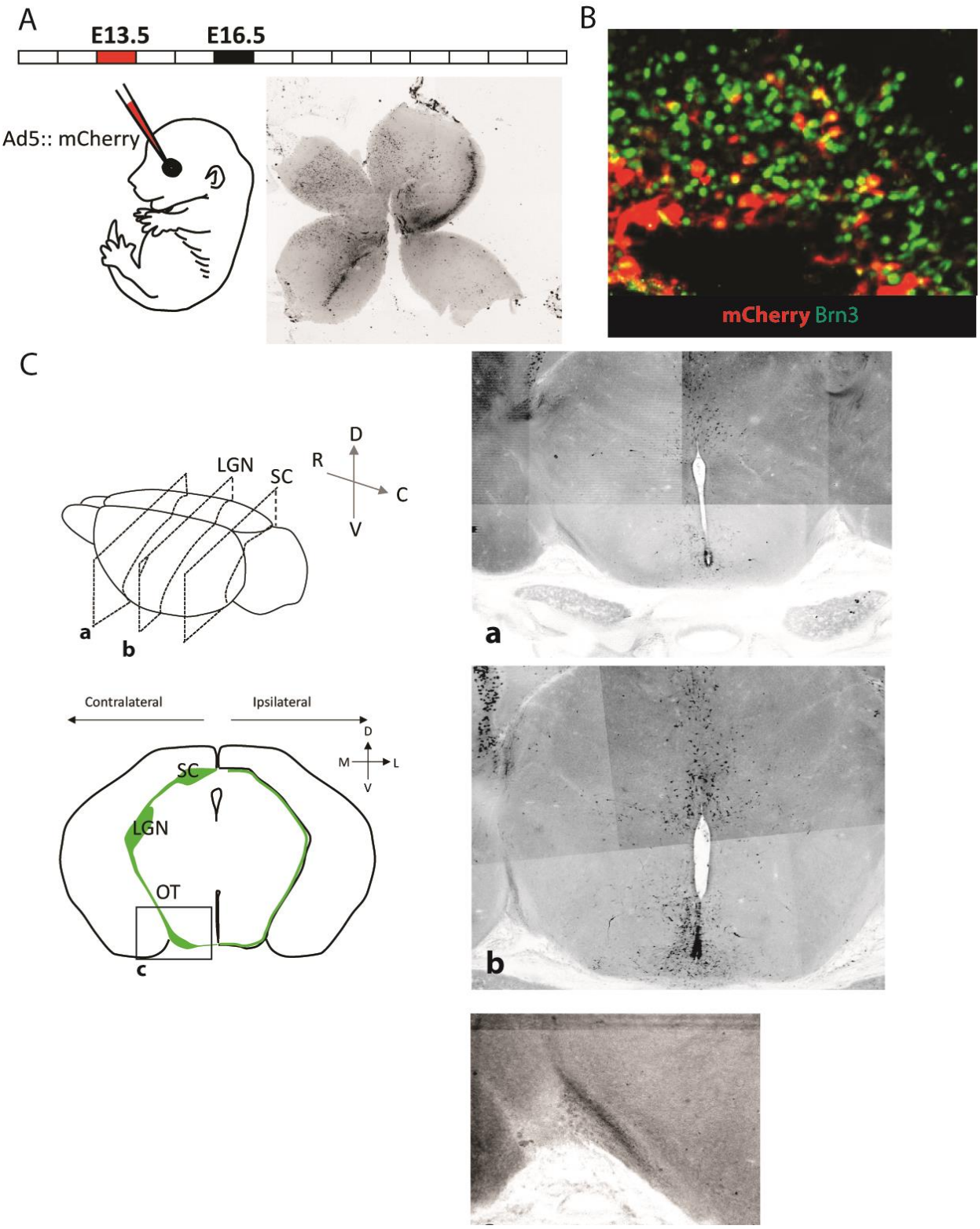
The early transient ipsilateral projection from the central retina to the brain has distinct characteristics such an early date of birth, after E10.5, and it is predominantly located is in the DC retina (Drager, 1985). To perform 'fate mapping" of this population it would be ideal to use a birthdating method that targets only cells born at early stages of development and that also labels the RGCs axons, so it would be possible to identify the ipsilateral and contralateral RGCs. Viral vectors are a good candidate to be the ideal fate mapping method. Nevertheless, in our experiments the results using an Ad5 viral vector were suboptimal. The Ad5-mCherry virus labelled the RGC cell bodies but the expression within their axons was inconsistent and uneven or segmented. Nevertheless, the accidental expression of mCherry in the trigeminal ganglion neurons and neurons/cells at the midline showed that this virus might consistently label other neurons. It can be suspected that even if neurons share the same tropism for the Ad5-mCherry, the expression of the cargo in the viral vectors could be different. Another hypothesis is that the RGC axons could take longer to fill by axonal transport of the vesicles transporting the mCherry protein from the nucleus to the axonal tip, while some neurons could take less time to fill with mCherry. Furthermore, the expression of the mCherry could have been intensified by immunostaining for mCherry, a strategy that was not attempted at the time these experiments were performed. The major caveat of the use of this Ad5-mCherry for the purpose of birthdating, tracing and statistical analysis is the inconsistent mCherry labeling of the retina. The alternative technique to label the earliest central retina RGC axons, the electroporation of green fluorescent protein (GFP), is more consistent in the labeling of the retina in a patch with a high and consistent expression of GFP.

If such experiments using this Ad5-mCherry vector to birthdate neurons are attempted in the future, it would be important to combine S-phase birthdating methods, such as EdU and inject this before the Ad5-mCherry M-phase transfection, to verify whether the use of Ad5-mCherry as a birthdating method is as effective in the retina as it is reported in the cerebral cortex and cerebellum (Hashimoto et al., 2011; Hashimoto and Mikoshiba, 2004).

Viral vectors could be useful for additional experiments on the tracing of the central retina projection. If a genetic marker was known, a Cre mouse could be generated in which the

candidate promotor would express a membrane-bound fluorescent protein after the deletion of a STOP codon by Cre. To label only the axonal projection from one eye, a viral vector carrying a Cre plasmid could be injected in one eye *in utero* at an early age. In optimal conditions only the RGCs expressing the genetic marker of interest would express the fluorescent and it would be possible to study the projection of the RGCs expression that gene. Nevertheless, if the genetic marker is expressed earlier the Cre recombination could occur at the progenitors labelling RGC born at different ages. If this was the case, it would be possible to study the projection of the RGC expressing the marker but it would be difficult to chronicle the timing of the progression since additional RGCs could be continuously generated.

Figures


A
E13.5
E16.5
Ad5:: mCherry
B
mCherry Brn3
C
LGN
SC
D
R
C
V
a
b
a
Contralateral
Ipsilateral
D
M
L
V
SC
LGN
OT
c
b

Section2 – Figure 1 Experiments on the use of an Ad5-mCherry viral vector to label the early born RGCs from the central retina.

(**A**) The viral vector Ad5-mCherry was injected in the embryo's sub-retinal space, *in utero*, at E13.5. The embryos were collected at E16.5 and their retina was dissected to verify the mCherry expression. In the E16.5 transfected retinas, the mCherry labeling was inconsistent, being sparser in some areas than in others. (**B**) Some of the cells labelled with mCherry are positive for the RGC marker Brn3 (green). (**C**) The mCherry Ad5 viral vector targets the retina but also cells near the ventricles and the trigeminal ganglion. In c) the faint labelling of the optic tract RGC axons by mCherry is shown at higher magnification. It is not possible to distinguish individual axons in these embryos.

Section 3 – Profile of the central retina retinal ganglion cells - Optimization of a method to combine retrograde labeling with DiI and immunohistochemistry, in mouse embryonic retina

Abstract

The molecular profile of a population of cells is an important feature that characterizes those cells and can elucidate the molecular mechanism of cell identity and function. It is unknown whether the central retina ipsilateral and contralateral retinal ganglion cells (RGC) present different molecular profiles. Ipsilateral and contralateral RGC can be identified by retrograde labeling from the optic tract after E13.5 with a lipophilic tracer. Nevertheless, the combination of lipophilic tracer with the immunohistochemistry for candidate genes requiring the use of detergents is troublesome since the detergents damage the lipophilic tracer. In this section, different detergents were tested for the combination of a lipophilic tracer with immunohistochemistry and digitonin proved to be the detergent that was less damaging to labeling with a lipophilic tracer. Subsequently, we tested two candidate genes, Brn3a and Brn3b, by immunohistochemistry but no difference was found in the expression of these two proteins in the ipsilateral and contralateral RGCs.

Rationale

There are no known molecular markers to distinguish ipsilaterally from contralaterally projecting RGCs in the central retina during early development RGCs as there are for molecular markers to distinguish the ipsilaterally projecting RGCs from the ventrotemporal crescent and contralateral RGCs (Petros et al., 2008). The technique used to identify the ipsilaterally and contralaterally projecting RGCs from the central retina is their axonal projection. Methods that label the axons from the brain to the retina, by retrograde labeling, are a good option to distinguish ipsilateral and contralateral RGCs in the retina. There are a few labeling methods to label retrogradely from the axon to the cell body, but most require live tissue or are only feasible when performed at later ages (E16.5), when RGC axons project to the optic tract or have projected to targets; these include rhodamine-dextran labeling (Drager, 1985; Williams et

al., 2003). One alternative labeling technique is the use of the lipophilic membrane tracer 1, 1'-dioctadecyl-3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI) and that can be applied in fixed brain. These lipophilic membrane tracers require the use of overnight fixed tissue when used in E13.5 and E14.5 embryos to label ipsilaterally and contralaterally projecting RGCs. E13.5 and E14.5 are the times in development in which the RGC axons have already crossed the optic chiasm midline or have projected ipsilaterally, and there is still a considerable number of central retina ipsilateral RGCs to study (Chan et al., 1999). One major inconvenience of the use of DiI is their partial incompatibility with the use of detergents necessary to permeabilize the cell membrane in combination with the identification of intracellular epitopes by the antibodies used in immunohistochemistry, since DiI is a lipophilic dye and detergents damage the rich lipophilic cell membranes. This characteristic makes the combination of DiI and the immunohistochemistry challenging, and also is a major disadvantage for the identification of intracellular candidate factors expressed by ipsilateral vs contralateral RGCs. Nevertheless, alternative detergents such as digitonin have been used and showed a better maintenance of the DiI labeling even after the membrane permeabilization with these detergents (Matsubayashi et al., 2008). These detergents constitute an alternative that can allows identification of ipsilaterally and contralaterally projecting RGCs in the retina with DiI labeling in the RGCs from the optic tract after they have crossed the optic chiasm, and to perform a candidate molecules screening using immunohistochemistry for intracellular epitopes.

The combination of retrograde labeling with DiI and immunohistochemistry is useful to explore candidate gene expression to determine the molecular identity of the central retina RGCs and whether there are genes differentially expressed in ipsilateral and contralateral RGCs in the central retina. We verified the expression of two transcription factors, Brn3b and Brn3a, in the early central retina RGCs. We choose to test these transcription factors for two reasons: one of the aims of these experiments is to optimize the technique of combining DiI labeling with immunohistochemistry for intracellular epitopes, so we choose to test this combination using antibodies that showed good signal in tissue fixed overnight (a necessary fixation procedure for the DiI labeling at early ages). We also choose to test these the expression of these two transcription factors first, because we would be using the Brn3b and potentially the Brn3a

conditional knock-out mice in further experiments (See Results Section 4 of this thesis). Therefore, it was necessary to verify whether Brn3b and Brn3a would label the central retina ipsi- and contralaterally projecting RGCs and whether these proteins would be differentially expressed in ipsi- and contralateral RGCs.

Methods

Animals: C57BL/6J mice were kept in a timed pregnancy breeding colony at Columbia University. Procedures for the care and breeding of mice follow regulatory guidelines of the Columbia University Institutional Animal Care and Use Committee. Noon of the day on which a plug was found was considered E0.5.

Animal sacrifice and tissue processing: The embryos were collected at E13.5 by caesarean section with the mother anesthetized with ketamine/xylazine. After decapitation the embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) (pH 7.4) overnight.

Dissection and DiI labeling: Wholemount retrograde labeling was performed on fixed tissue using 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes). E13.5 fixed embryos heads were sectioned frontally with a blade in a section caudal to the optic chiasm. A DiI crystal was inserted in the optic tract (OT). Heads were incubated in a solution of 1% PFA in phosphate buffer saline (PBS) for 4 days (E13.5 or younger embryos) or 7 days (E15.5 embryos) at room temperature. Whole heads were vibratome sectioned frontally at 50µm. After sectioning the embryos the contralateral OT was observed and only the cases with DiI labeling in only one optic tract were used for further analyses.

Immunohistochemistry: After the labeling with DiI and vibratome sectioning, the free floating vibratome sections were blocked in 10% donkey serum (DS) + 1% Triton20 in PBS or 1% Triton20 in PBS or digitonin (1:500 from a aliquot at 50 mg/ml) and then incubated overnight at 4°C with goat Brn3b-H18 antibody (1:200, Santa Cruz), Brn3b (antibody from Tudor Badea Badea, NIH-NEI) or rabbit Brn3a (1:1000, Millipore) + 1% DS + the same detergent used in the initial incubation, 1% Triton20 in PBS or 1% Triton20 in PBS or digitonin (1:500 from a aliquot at 50 mg/ml). After 3 PBS washes for a total of 1 hour, tissue was incubated in Cy3 anti-goat or anti-rabbit antibody (1:1000, Life Technologies).

Image processing and quantification: Retinal sections were imaged on a Zeiss AxioImager M2 microscope with an AxioCam MRm camera, and Neurolucida software (v 11.01, MicroBrightField Systems), using 5x, 10x, or 20x objectives. Images were processed with ImageJ software (version 1.48, NIH).

Results

Three detergents (Triton-X, Tween20, and digitonin) were tested for the permeabilization of the cellular membranes necessary for the immunohistochemistry of intracellular transcription factor. Digitonin was previously used by Matsubayashi, (2008) to combine DiI with other staining techniques that require the cellular membrane permeabilization (Matsubayashi et al., 2008). In the present study, this detergent proved to be superior to the others (Triton-X and Tween20) to maintain stable DiI staining, with better definition of axonal anatomy. Still, at the neuron's cell body, the staining becomes blurred, in some cases, for all the detergents, decreasing the number of samples useful for analysis.

After labeling the RGC axons with DiI applied to the optic tract and it labelled retrogradely the ipsilateral and contralateral RGCs, we performed immunohistochemistry for Brn3b and Brn3b. We verified that Brn3b is expressed in most of the central retina RGCs at E13.5 but not all (n=7 embryos). Both ipsilaterally and contralaterally RGC projecting RGCs express Brn3b, so their laterality of projection was not associated with a differential expression of Brn3b. Similar results were obtained for the expression of Brn3a in the central retina RGCs at E13.5. Both ipsilateral and contralateral RGCs express Brn3a (n=3 embryos). Thus, although the technique worked well, these genes were not differentially expressed in the central retinal in ipsi- versus contralateral RGCs, and therefore could not be used as markers of the early ipsilateral vs contralateral transient RGCs from central retina.

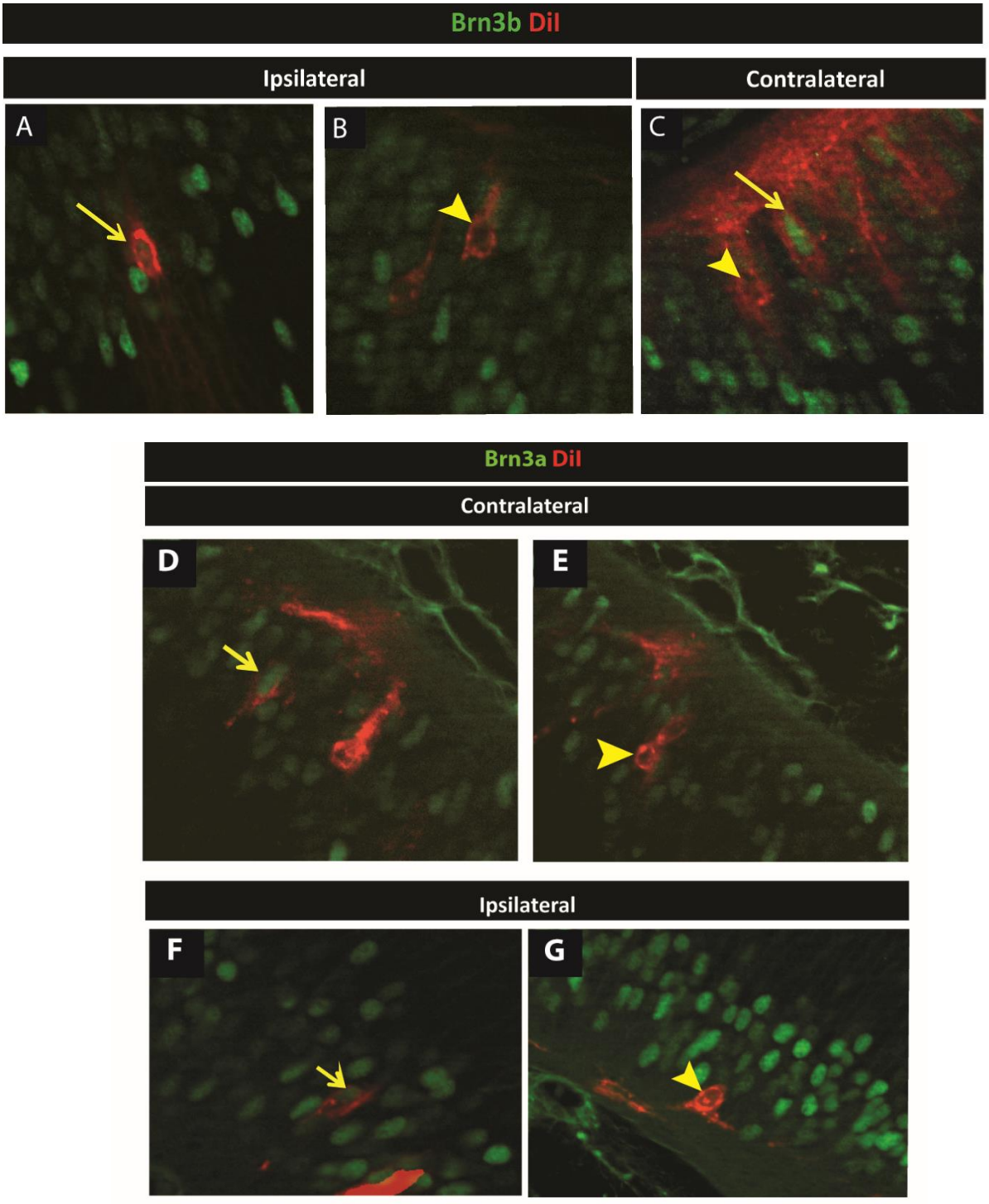
Discussion

The identification of the molecular profile of the early central retina RGCs projecting ipsilaterally remains elusive. Still, here we show that it is possible to combine the labeling method DiI, to distinguish ipsilateral and contralateral RGCs, with immunohistochemistry and RGC cell markers that require the use of detergents. If we were to perform additional

experiments in the future, we would test other known markers for ipsilateral and contralateral RGCs. Nevertheless, DiI labeling at E13.5 requires the overnight fixation of the embryonic tissue, and some antibodies such as the antibody for the contralateral RGC marker Islet1/2 (antibody from Jessell lab, Columbia) does not label tissue fixed overnight. But new antibodies might be developed to this and other markers and could be combined with the technique optimized in this thesis section. Additionally, an experiment that it is possible to perform, is the use of mutant mice that express a marker, such as alkaline phosphatase or a fluorescent protein, under the control of the promoter of a known gene of interest and combine the DiI labeling with immunostaining for a membrane bound marker with a extracellular epitope (that will not need the permeabilization with detergents) or even an intracellular marker that could go through the permeabilization process described in the experiments of this thesis section.
To analyze markers such as FoxD, FoxG or SERT, it would be feasible to label the ipsilateral and contralateral RGCs with DiI as previously described in the Methods section of this section; convert the DiI fluorescent signal with 3,3'-Diaminobenzidine (DAB) to a brown insoluble precipitate and then perform a *in situ* hybridization to the markers described.
Another inconvenience of the requirement of a strong tissue fixation for the DiI labeling in early development of retinal projections is the incompatibility with live tissue screening techniques. If it was possible to sort ipsilateral and contralateral RGCs at E13.5 in viable live tissue, other techniques of molecular profiling could have been used, such as RNA sequencing, to determine whether ipsilateral and contralateral RGCs from the central retina have a different genetic profile; to identify the molecules that distinguish these two populations; to identify potential markers for the ipsilateral and contralateral in the central retina, and to understand whether the molecules that determine the ipsilaterality of the VT RGCs are the same for the central retina ipsilaterally projecting RGCs.

In summary, even if the present experiments did not allow the identification of specific markers for the central retina ipsilateral RGCs, it showed that there is variability in the expression of the RGC markers Brn3b and Brn3a in the central retina. Furthermore these experiments validate the use of the Brn3b conditional knock-out mice for further experiments on the studies of the central retinal projection.

Figures


Brn3b DiI
Ipsilateral
Contralateral
A
B
C
Brn3a DiI
Contralateral
D
E
Ipsilateral
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Section3 – Figure 1 Brn3b and Brn3a expression in the central retina RGCs at E13.5. The retinal projection to the brain were retrogradely labelled with DiI from the optic tract at E13.5. (**A-C**) The RGCs projecting ipsilaterally can express Brn3b (Fig. 1 A) or not (Fig.1 B). A similar result was observed for the contralaterally projecting RGCs at E13.5 (Fig.1 C). (**D-G**) The expression of Brn3a could be detected both in contralateral (Fig.1 D) or ipsilateral (Fig.1 F) RGCs in the retina at E13.5. Nevertheless, not all contralateral (Fig.1 E) or ipsilateral (Fig.1 F) RGCs expressed Brn3a

Section 4 - Alternative approach to label the central retinal ganglion cells – Brn3b conditional knock out.

Abstract

The first retinal ganglion cells (RGCs) that extend to the brain of the mouse are located in the dorsocentral (DC) retina. These RGCs extend to either ipsilateral or contralateral targets, but the ipsilateral projections from the central retina are transient. To understand the context of the disappearance of the transient central retina ipsilateral projection, one possible approach is to fate-map this projection. A Brn3b conditional knock-out mouse was used to label the earliest central retina RGCs and their projection, taking advantage of the ability to temporally induce the conditional knock-out. However, irrespective of the time of induction, the number of RGCs labeled was too intense to distinguish individual axons, making any analysis challenging. In this study, nonetheless, transient expression of Brn3b at the ventral midline that was previously unknown, was observed.

Rationale

Brn3b is a transcription factor expressed in post-mitotic RGCs in early phases of differentiation (Pan et al., 2008). Dr. Tudor Badea, (National Eye Institute, National Institution of Health, Maryland) created a *Brn3b* conditional knock-out under a Rosa26 promoter that removes the *BRN3B* gene (Badea et al., 2009a; Badea and Nathans, 2011) . In this construct, a tetracycline transactivator (rtTA) is under the control of ubiquitously expressed promoter: Rosa26. rtTA is expressed in an inactive form that is activated by doxycycline (Dox). The activated protein links to the Tetracycline Response Element (TRE) and transcribes an inactive form of CreER(T). The Cre system is active only after the induction by a tamoxifen active metabolite, 4-hydroxy-tamoxifen (4-HT). This active form knocks-out Brn3b and also a STOP signal that inhibits the expression of the human placental alkaline phosphatase (AP). So, in the cells undergoing recombination BRN3B is deleted and AP expressed.

These mice present some characteristics that could be useful for labeling the earliest RGCs. First, using different doses of Dox, it is possible to obtain variation in the number of cells labeled in the Brn3b-positive neurons, from strong to sparse labeling. Second, by manipulating the timing of the 4-HT injection, it is possible to have temporal control of the induction of Brn3b expression (Badea and Nathans, 2011). We hypothesized that this technique could be used to label the early born RGCs from the central retina and label their projections, which would allow fate-mapping of the ipsilaterally and contralaterally projecting RGCs. Since the tamoxifen induction and Cre-recombination is not a birthdating method, to identify the early-born central retina RGCs it would be necessary to additionally inject a birthdating marker, such as EdU. Then it would be necessary to trace single neurons from the central retina, to identify them as early born RGCs, and by their axonal extension to identify them as ipsilateral or contralateral RGCs. The type of labeling necessary to identify single Brn3-positive expressing AP would be a very sparse labeling resembling the results of Golgi staining.

Methods

Conditional KO Induction: These experiments were performed in non-albino C57BL/6 mice. The time of the conception date was determined by examining the vaginal plug and the day the plug was found was dated as E0.5. All mouse handling procedures used in this study were approved by the National Eye Institute Animal Care and Use Committee (ACUC). The construct of the mutant mice is schematized in Fig. 1. In the Brn3b conditional knock-out (CKO) mice a tetracycline transactivator (rtTA) is under the control of the promoter Rosa26. rtTA is expressed in an inactive form that is activated by doxycicline (Dox). The activated protein links to the Tetracycline Response Element (TRE) and transcribes an inactive form of CreER(T). The Cre system is active only after the induction by a tamoxifen active metabolite, 4-hydroxy-tamoxifen (4-HT). This active form knocks-out Brn3b and also a STOP codon previously inhibiting the expression of the human placental alkaline phosphatase (AP). Bn3b CKAP/+; ROSA26-rtTACreER and Bn3b CKAP/-; ROSA26-rtTACreER pregnant females were feed with food pellets containing Dox (1.75 mg/g) at gestational days described in Annex A. In some experiments, pregnant mice were fed for half the day with food pellets containing Dox. We tried to administer the Dox in a

solution in the drinking water but the pregnant mice refused to drink this solution. At a gestational day described in Annex A, 4-HT in sunflower seed oil vehicle was delivered by intraperitoneal injection.

Genotyping: embryo genotyping was performed by our collaborators in the Badea Lab, NIH.

Tissue processing: Pregnant mice were anesthetized with ketamine and xylazine, and fixed with 4% paraformaldehyde in PBS by intracardiac perfusion. Embryo heads were sectioned on 100µm vibratome sagitally sections and histochemically stained for AP as described by Badea et al, 2003. For the AP histochemical reaction, the vibratome sections were washed twice in PBS with 2mM MgCl2, transferred to PBS, and heated in a water bath for 2h at 65°C to inactivate endogenous AP activity. AP staining was performed in 0.1M Tris, 0.1M NaCl, 50mM MgCl2, pH 9.5, 0.34 g/ml nitroblue tetrazolium (NBT), and 0.175g/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), overnight at room temperature with gentle agitation. After staining, tissues were washed three times for 20 min in PBS, 0.1% Tween 20, and postfixed in PBS with 4% paraformaldehyde overnight. Vibratome sections were dehydrated through an ethanol series and then cleared with 2:1 benzyl benzoate (BB)/benzyl alcohol (BA).

Imaging: sections were imaged in a glass dish filled with BB:BA using a Zeiss Axioplan 2 microscope, AxioCam camera and Axiovision software. Further imaging processing was performed with ImageJ software (NIH).

Results

The optimization of the protocol of induction was challenging: We tested several different protocols of induction and analyzed more than 80 embryos. We have been able to achieve sparse labeling with lower doses of 4-HT and Dox when the embryos were collected at earlier stages of RGC growth, at E12.5 (Fig.1 A-B and Fig. 2). At E12.5 the RGC axons have not yet crossed the midline, and it is not possible to identify them conclusively as ipsilateral or contralateral (Fig. 2E-F). However, when the embryos were collected at later ages, even with low doses of 4-HT and Dox, a high number of RGCs were labeled (Fig. 1C-D). With this extensive number of RGCs labelled in the retina it is impossible to identify single RGCs and trace them.

Brn3b is expressed at the ventral midline: An additional finding from these experiments is that Brn3b is expressed in a location not previously described, at the ventral midline of the diencephalon, where the optic chiasm is being organized at E12.5 and at E13.5 (Fig. 2E and 3). At E12.5 we detected strong AP staining in some cells at the midline. These cells have a have an elongated shape and a small nucleus (Fig.3 E12.5). At E13.5, the distribution of these Brn3b positive cells is more evident. As sections progress from rostral to caudal, the area that these cells occupy dramatically expands, from a narrow central cluster to a large sparser zone with the cells extending from the walls of the third ventricle outward. In the caudal sections, some of the radial cells have large cell bodies; these are likely tanycytes, specialized glia that extend from the third ventricle behind the optic chiasm into the hypothalamus. At later ages, the number of these cells at the midline and caudally, lateral walls of the third ventricle, is reduced. We performed immunohistochemistry for Brn3 and Brn3b with different antibodies, but these antibodies were not able to detect Brn3b expression in the ventral midline. Since other areas that express high levels of Brn3b were stained with these antibodies, we think that the Brn3b levels at the midline are lower than the antibody threshold of detection.

The differences in the phenotype between the Brn3b CKO/+ and Brn3b CKO/- were inconsistent: So far, the brain sections described in the previous results were from mice heterozygous for Brn3 CKO/+. When comparing the RGC axonal extension in Brn3b CKO/+ (Fig. 4B-C) with the Brn3b CKO/- (Fig. 4D-G), the results were inconsistent. The comparison between the Brn3b CKO/+ and Brn3b CKO/- was performed in embryos from the same litter. In some litters, the RGC axons of Brn3b CKO/+ and Brn3b CKO/- embryos projected to the same areas in the brain (Fig. 4 B-E), while in other litters the Brn3b CKO/- RGCs axons were delayed in their projection, extending their axonal tip to areas less caudally than the labelled RGC axons of Brn3b CKO/+ embryos from the same litter (Fig. 4F-G).

Discussion

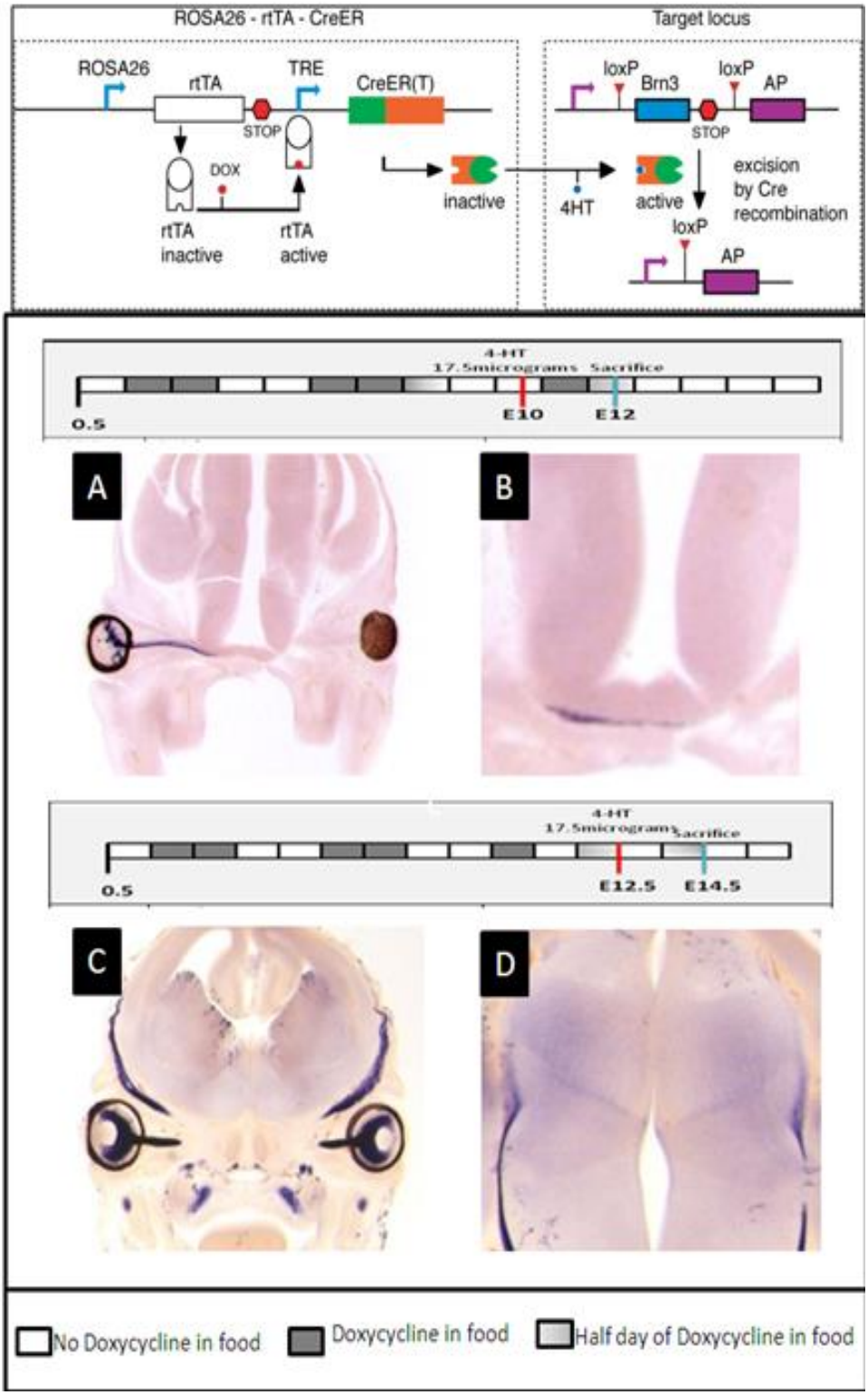
To explain why a greater number of RGCs is labeled when the embryos were collected later (E14.5, E15.5), we propose the hypothesis that Brn3b recombination is occurring in the

progenitors, such that all RGCs that are derived from progenitors undergoing recombination after 4-HT injection express AP. As a result, these mice have a high number of RGCs derived from the same progenitor that does not allow us to identify or follow single cells.

To overcome the problem of the greater number of cells that were recombined and that does not allow identification of single cells, we suggest alternative approaches, such as the injection of a viral vector containing a plasmid with Cre or the electroporation of Cre in one single eye. Still, this technical approach in Brn3b CKO mice is not superior to the approach of electroporating GFP in *wild-type* mice for our purpose of studying the projection of the early central retina RGCs (See first Section of the Results in this thesis). The approach of locally delivering Cre to the retina could be useful when studying specific sub-types of RGCs with a known genetic marker under the control of Cre, to describe the early projection of specific RGCs sub-types.

The expression of Brn3b at the ventral midline was not described previously, to our knowledge, but it has been hypothesized. Erkman et al, in 2000 described axon pathfinding defects of the Brn3b KO mice. They found defects of fasciculation and axon guidance in multiple sites of the RGC trajectory. Since no Brn3b expression had been described along the optic tract and the factors that shape the optic chiasm such as sonic hedgehog expression in the ventral diencephalon did not show major alterations, the pathfinding defects in the Brn3bKO were hypothesized to be cell-autonomous (Erkman et al., 2000). Nonetheless, Erkman et al. did not exclude the hypothesis that Brn3b might be expressed at the midline, but the methods available at the time did not allow the detection of this transcription factor (Erkman et al., 2000). Our data supports the second hypothesis, i.e., that Brn3b is expressed in cells along the pathway. Thus, the pathfinding defects in the Brn3b KO might be not purely cell-autonomous.

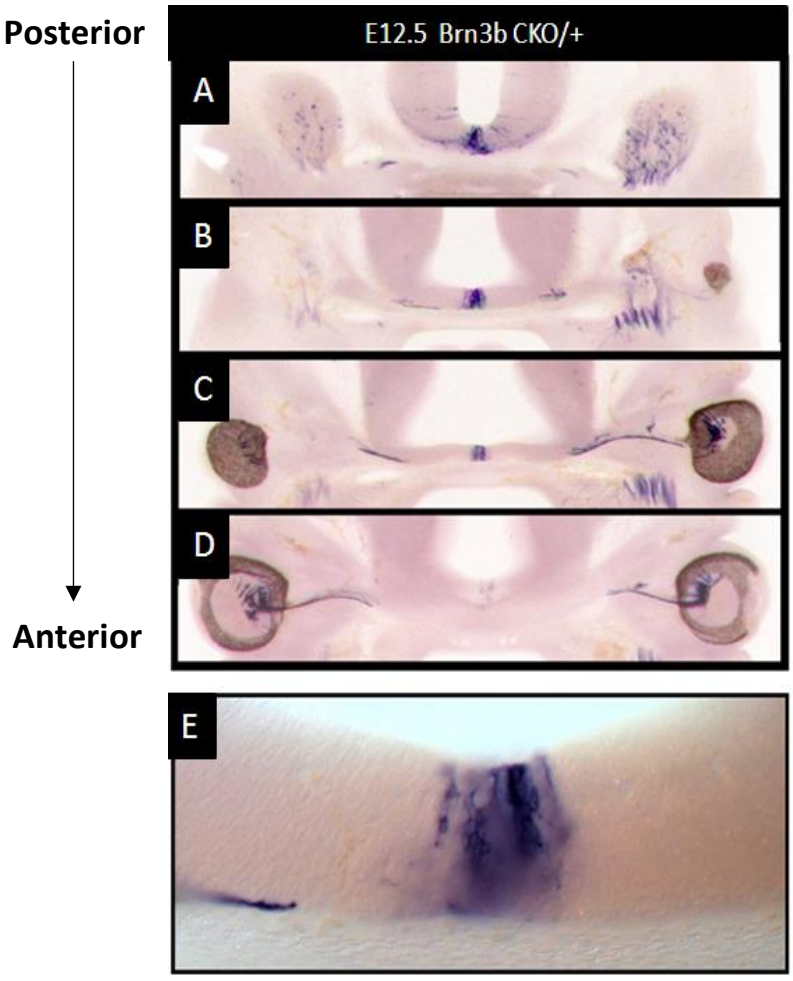
Figures

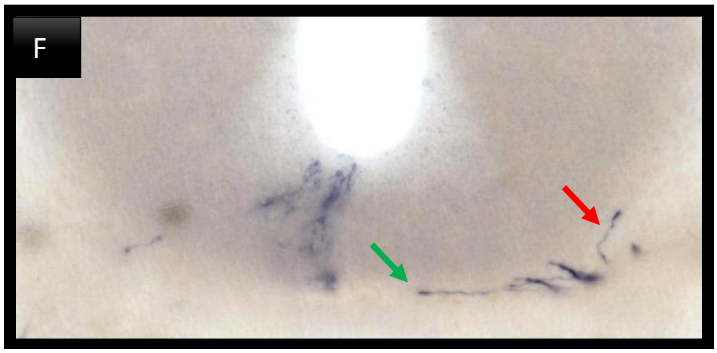

ROSA26 - rtTA - CreER
Target locus
ROSA26
rtTA
TRE
CreER(T)
STOP
DOX
rtTA inactive
rtTA active
inactive
4HT
loxP
Brn3
loxP
AP
STOP
active
excision by Cre recombination
loxP
AP
4-HT
17.5micrograms
Sacrifice
0.5
E10
E12
A
B
4-HT
17.5micrograms
Sacrifice
0.5
E12.5
E14.5
C
D
No Doxycycline in food
Doxycycline in food
Half day of Doxycycline in food

Section 4 - Figure 1 The construct for the Brn3b conditional knock-out and examples of induction protocols.

Upper Scheme: In the Brn3b conditional knock-out (CKO) mice a tetracycline transactivator (rtTA) is under the control of the promoter Rosa26. rtTA is expressed in an inactive form that is activated by doxycicline (Dox). The activated protein links to the Tetracycline Response Element (TRE) and transcribes an inactive form of CreER(T). The Cre system is active only after the induction by a tamoxifen active metabolite, 4-hydroxy-tamoxifen (4-HT). This active form knocks-out Brn3b and also a STOP codon previously inhibiting the expression of the human placental alkaline phosphatase (AP). Image from Tudor Badea's Lab.

(**A-D**) Examples of the possible protocols of induction on the Brn3b CKO. In the protocol of Fig.1 A-B the embryos were collected at E12.5 after the protocol represented in the scheme above the Figures. (**C-D**) Brn3b CKO mice collected at E16.5 with a high number of RGCs labelled with AP.


Posterior
E12.5 Brn3b CKO/+
A
B
C
D
Anterior
E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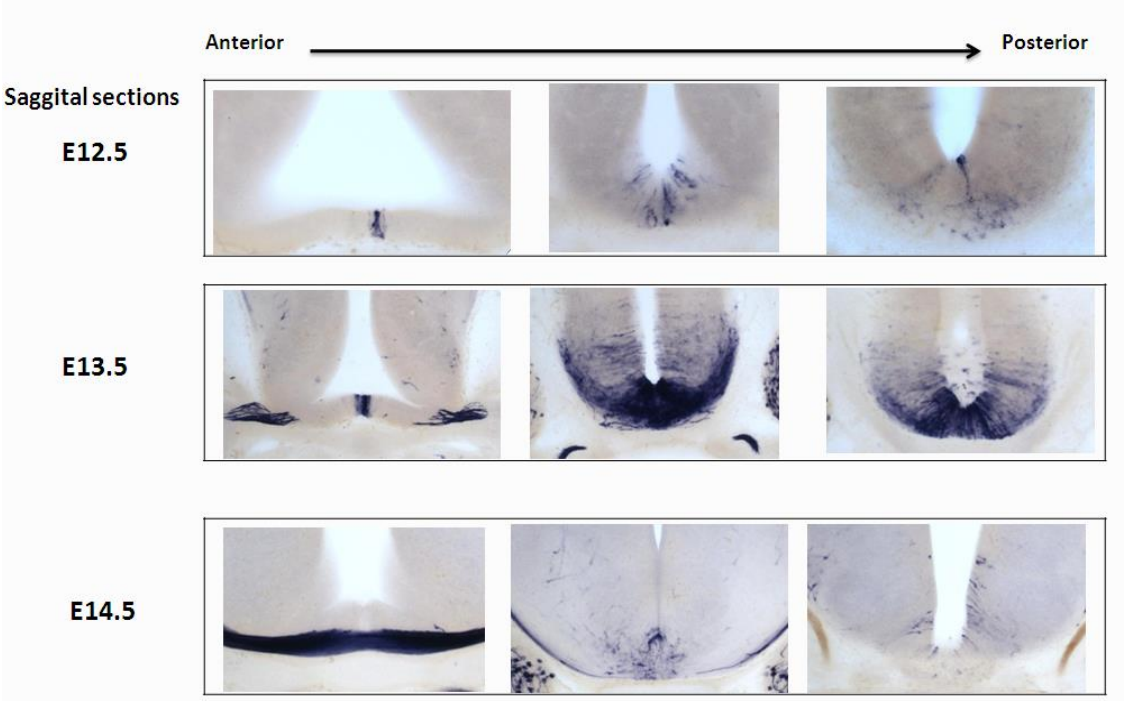
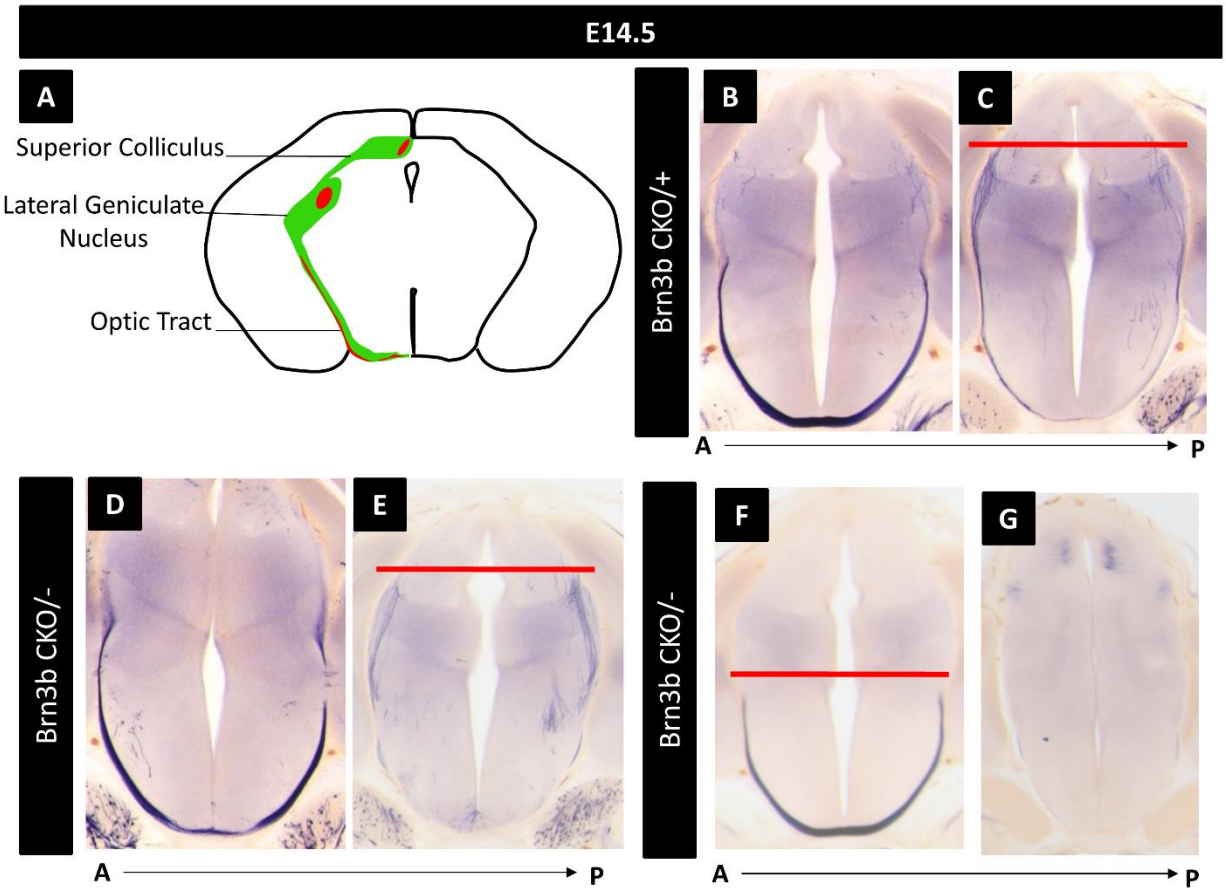
Section 4 - Figure 2 Progression of the retinal ganglion cells axons in the Brn3b CKO/+ at E12.5.

In the cases in which more sparse labeling was achieved it was possible to see the progression of the retinal ganglion cells (RGC) axons from the retina to the optic chiasm. This more sparse labeling was only achieved when the embryos were collected at early ages (E12.5). Nevertheless, at this age most of the labeled axons did not make the decision to cross or not the midline. Still, it is possible to see some axons that appear to be directing their growth cone to the midline (green arrow, Fig. 2F) or in the more lateral area of the chiasm, they seem to be projecting to the ipsilateral optic tract. Panel E is a higher magnification of B. In this Figure it is also possible to visualize the expression of Brn3b at the ventral midline, likely in the midline radial glia, and not described previously.



Section 4 - Figure 3 Brn3b expression at the ventral midline in sagittal sections.
In the Brn3b CKO transient expression Brn3b positive cells was detected in the ventral midline. The Brn3b positive cells occupy a V-shaped are with the vertex in the more rostral ventral midline, expanding laterally and caudally. In more posterior sections, radial glia extended from the lateral walls of the third ventricle. Some cells with large nuclei are likely tanycytes, extending into the hypothalamus. The number of Brn3b positive cells increases until E13.5, decreasing thereafter.



Section4 - Figure 4 The labeling and extent of growth of RGCs in Brn3b CKO/$^{+}$ vs Brn3b CKO/$^{-}$ is not consistently different.

(A) The rate of progression of the Brn3b positive RGC labelled with alkaline phosphatase (AP) (in blue in Fig. 4B-G) where accessed in E14.5 embryos using the future areas of targeting as an anatomical reference. (**B-C)** In this example of a Brn3b CKO/+ at E14.5 the RGC axons labelled with AP projected to an area near the Lateral Geniculate Nucleus (LGN) and the beginning of the Superior Colliculus (SC). (**D-E)** In this Brn3b CKO/- the progression of the RGC axons is similar to the progression seen in the Brn3b CKO/+ (Fig. 4B-C). (**F-G**) In this example of a Brn3b CKO/- no RGC axons were visualized to progress further than the optic tract area ventral to the LGN. The samples presented in this figure came from the same litter. A: Anterior. CKO: Conditional knock-out. P: Posterior.

Section 5 - Study on the axonal expression of caspases in the central retina retinal ganglion cell axons.

Abstract

The mechanism of selective elimination of extension during development remains elusive. Caspases have been suggested to play a role in the elimination of inappropriate projections independent of their apoptotic role, through their action in axons. The expression of activated caspase 3 and caspase 6 in RGC axons was investigated in E17.5 mice by immunohistochemistry in retinal ganglion cells (RGCs) electroporated with GFP in utero at E12.5. We were unable to detect the expression of these epitopes in axons even though they were detected in RGC cell bodies. Such results could be the consequence of these two caspases not being expressed in axons or their expression level being below the threshold of detection of the antibodies used in the immunohistochemistry.

Rationale

Caspases have been implicated in the process of cell elimination in programed cell death (PCD) during development. Recently, the caspase cascade has been also been implicated in the local process of axon degeneration, arborization and dendrite pruning (Campbell and Okamoto, 2013; Nikolaev et al., 2009; Simon et al., 2012), as described in the Introduction of this thesis. The initiator Caspase 3 and its downstream effector, Caspase 6, are thought to be involved in the process of axonal degeneration of inappropriate RGC axonal arborizations in the superior colliculus (Simon et al., 2012). We proposed the hypothesis that caspases could also be expressed locally at the axons of central retina ipsilateral RGCs, and play a role in the decrease in the number of ipsilateral RGC axons from the central retina observed after E16.5 (See first section of Results in this thesis) (Soares and Mason, 2015). To address this hypothesis we performed immunohistochemistry for activated caspase 3 and caspase 6 in embryos that were electroporated in the central retina with membrane-bound GFP at E12.5 and were collected at E17.5. After electroporation of the central retina at E12.5 in one eye, the RGC axons projecting

from eye were labelled with GFP and it was possible to distinguish the ipsilateral and contralateral RGC axons in the optic tract. We used this tissue to determine whether RGCs express activated Caspase 3 and Caspase 6.

Methods

Animals: C57BL/6J mice were kept in a timed pregnancy breeding colony at Columbia University. Procedures for the care and breeding of mice follow regulatory guidelines of the Columbia University Institutional Animal Care and Use Committee. Noon of the day on which a plug was found was considered E0.5.

Electroporation in utero of GFP: The protocol for electroporation was described in the first section of this thesis. The embryos used for this set of experiments were electroporated at E12.5 and sacrificed at E17.5 as described in the precedent section.

Animal sacrifice and tissue processing: The mother was anesthetized with ketamine/xylazine and embryos were collected at E17.5 by caesarean section. After decapitation, heads were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) (pH 7.4) for 2h, and washed three times in PB, 20 min for each wash.

Tissue processing: For cryosectioning, whole heads or whole retina were embedded in 20% sucrose overnight, embedded in OCT (Fisher Scientific), frozen in dry ice, stored at -80°C for at least 24h and cryosectioned frontally at 20µm.

Immunohistochemistry: The cryosections in a slide were blocked in 10% donkey serum (DS) + 0.5% Triton20 in PBS and then incubated with rabbit activated caspase 3 antibody (1:250, Cell Signaling) or rabbit caspase 6 antibody (1:50, 1:200, 1:1000, Cell Signaling) and chicken IgY GFP antibody (1:500, Aves Lab) or Brn3 (1:200, Santa Cruz) + 1% DS + 0.5% Triton20 in PBS, overnight at 4°C. After 3 PBS washes for a total of 1 hour, tissue was incubated in anti-chicken antibody (1:500, Life Technologies) for 4h, washed again in PBS and mounted in Aquamount with a coverslip.

Image processing and quantification: Retinal sections were imaged on a Zeiss AxioImager M2 microscope with an AxioCam MRm camera, and Neurolucida software (v 11.01, MicroBrightField

Systems), using 5x, 10x, or 20x objectives. Images were processed with ImageJ software (version 1.48, NIH).

Results

The Caspase 3 and Caspase 6 antibodies were able to detect epitope expression at the tested experimental conditions.

Antibodies for Caspase 3 and Caspase 6 both labelled the cell body of neurons in the brain during development. In the retina, very few cells showed positivity to the caspase antibodies but of those cells that did, both progenitor cells and post mitotic RGCs ($Brn3^+$ cells) were positive for Caspase 3 (Fig. 1A). The embryonic tissue in which Caspase 6 was tested had their eyes enucleated for use in other experiments so the expression of caspase 6 was not possible to observe in the same sections that were used to study the expression of caspase 6 in the RGC axons. But cell bodies in the brains of embryos electroporated with GFP showed positivity for Caspase 6 in other brain regions at the three antibody dilutions that were performed. These results confirm that the conditions selected to perform the immunohistochemistry for these two primary antibodies were optimal for detection of these epitopes in the embryonic tissue we studied.

Activated Caspase 3 is expressed along the optic tract.

First we verified the expression of activated Caspase 3 in the optic tract and observed strong labeling for activated Caspase 3 along the optic tract (n=2 embryos) that did not allow us to understand whether the epitope of activated Caspase 3 was expressed intra-axonally in the RGC axons labelled with GFP or in other cells, such as glia, along the optic tract (Fig. 1B). Still, this expression of activated Caspase 3 could represent dynamic remodeling of the cells in the optic tract, such glial cells, during the development of the projection into the optic tract.

Caspase 6 expression could not be detected in the optic tract.

While the immunohistochemistry for Caspase 6 revealed expression in cell bodies of cells of the brain and tissue surrounding the OT (yellow arrow in Fig. 1D), it did not reveal any expression in the electroporated ipsilateral RGC axons from the central retina at three antibody dilutions

used (Fig. 1C-E). The fact that this immunohistochemistry was not able to detect the axonal expression of Caspase 6 could mean that Caspase 6 is not expressed in the ipsilateral RGC axons from the central retina, or the expression of Caspase 6 is so low that it is below the threshold of detection of the antibody we used.

The morphology of the projecting axons and degenerating axons is difficult to distinguish. While the results of our experiments for the expression of Caspase 3 and Caspase 6 in RGC axons were inconclusive, we could potentially still address the question of whether the ipsilaterally projecting RGC axons from the central retina undergo degeneration using classical methods of histological analysis of axonal morphology during degeneration. During axonal degeneration axons first form "beads" along their projection, then fragment in multiple segments, and later disappear. Nevertheless, the "beading" stage of axonal degeneration is similar to the "beading" of axonal transport of cell cargo that is present even in growing axons in normal development (Fig. 2).

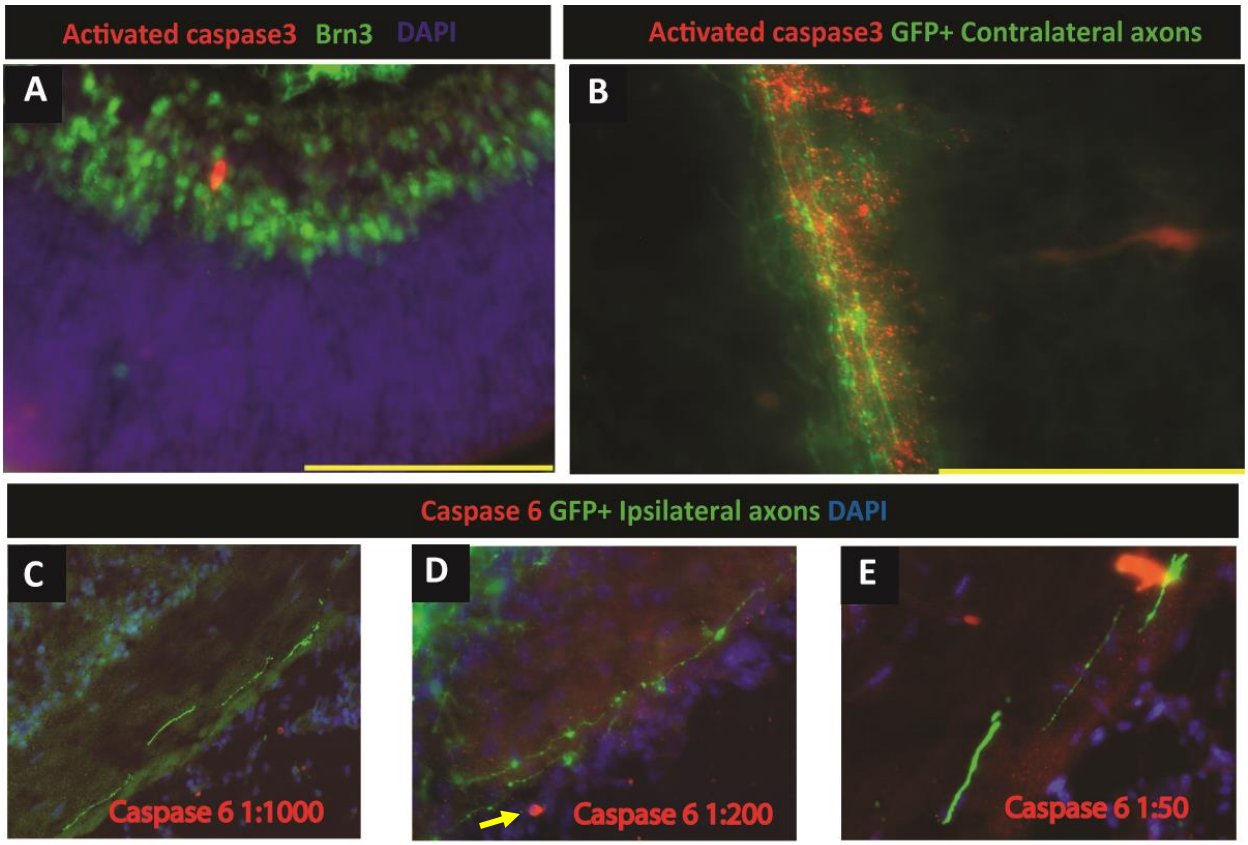
Discussion

The degeneration of axonal projections during development is still a process that remains elusive with few molecular pathways associated with the mechanism of this fundamental property of neural circuits. Unfortunately in our set of experiments we were unable to advance knowledge in the field concerning the role of caspases in the process of elimination of transient axonal projections during development. Nonetheless, we verified high expression of activated caspase 3 in the optic tract at E17.5 that could be an interesting subject for further experiments. It is also interesting to note that while we found the expression of the initiator caspase 3 in the optic tract we did not observed the expression of its effector Caspase 6 in the optic tract. The absence of Caspase 6 could be due to the lack of expression of Caspase 6 in the optic tract or its expression could be below the threshold of detection of the antibody we selected. The expression of Caspase 3 in the optic tract could represent the elimination of cells in the optic tract to create space for incoming RGC axons, or it could represent a dynamic process of projection remodeling as seen by Campbell and Okamoto, 2013, using probes in live tissue that detected the activity of caspases. Additionally, it would be interesting to study in

further experiments which cells are expressing activated Caspase 3 in the optic tract and whether the inhibition of Caspase 3 in those cells would stall the progression of RGC axons in the optic tract.

The caspase cascade is a surprising mechanism for mediating this degenerative process during development since it was associated for years with the PCD with its high expression in the cell body. Simon et al. 2011 showed that in mice lacking Caspase3 or Caspase6, the retraction/degeneration of inappropriate axonal ramifications in the superior colliculus is delayed. Since some degree of elimination of inappropriate projections still occurs, it is possible that additional molecules are involved in the process of refinement of the RGC axonal projections to the target, or compensatory mechanisms could occur in the knockout mice.

Figures



Section5 - Figure 1 Optimization of the immunohistochemistry for activated Caspase 3 and Caspase 6.

(**A**) The immunohistochemistry to activated Caspase 3 was able to detect its expression in the conditions tested. In this case, a Brn3 positive RGC is expressing activated Caspase3. (**B**) While it is not possible to distinguish the expression of activated Caspase3 in single axons it is possible to identify its expression along the optic tract; the positive staining could be labeling in glial cells or axons. (**C-D**) The expression of Caspase 6 was not detected in the RGC axons in the conditions tested while it was detected in brain cells in the same samples and cell near the optic tract (yellow arrow). Scale bar: 100µm.

Section 5 - Figure 2 Electroporated RGC axons at E12.5 projecting in the optic tract at E17.5.

Axonal beading can be an indirect correlate of axonal degeneration but during development of the axonal projections the very active cargo transport from the cell body to the growing tip can mimic the configuration of beading axons. In this sample, all contralaterally projecting RGC axons electroporated with GFP display beading while still having a growth cone.

IV. Discussion

The development of neuronal circuits is a dynamic process involving the formation of projections that do not persist in mature functional circuits (Luo and O'Leary, 2005). The elimination of these transient projections ranges from small-scale events such as synapse elimination and local pruning of dendritic and axonal branches, as in the vertebrate neuromuscular junction (Tapia et al., 2012), to large-scale axon elimination of major axon collaterals in subcortical projections (Luo and O'Leary, 2005; Stanfield and O'Leary, 1985), and extensive elimination of axons (Simon et al., 2016) and/or neurons themselves (Linden et al., 1999). While transient projections are a common feature of the development of neural circuits the mechanism of their elimination remains elusive. Another remaining question it is why and how these stereotyped projections are formed and conserved in evolution. An example of a transient projection during development is the transient RGC projection from the central retina to the brain.

The fate of the transient ipsilateral RGC projection from the retina to the brain has been an elusive aspect of the establishment of retinal projections. This thesis aims to contribute to a better account of the timing of this projection and its targeting.

The novel labeling technique developed in this thesis, in combination with other approaches discussed here, contribute to answering to some of the questions in the literature. Nevertheless, many additional questions were raised from our results.

In Section 1, both DiI labeling and GFP *in utero* electroporation were used to track the earliest ipsilateral fibers from retina to brain. The latter method was an advance compared to DiI or other conventional labeling methods, since the GFP label was retained in developing RGCs for days, so that the axons could be “fate-mapped”. It showed that while the first RGC axons emerging from dorsocentral (DC) retina that project ipsilaterally enter the optic tract first, they do not progress as far as the contralateral axons from the DC retina, from E13.5 to E15.5. The number of ipsilateral RGC axons increases until E16.5 and sharply decreases thereafter. Second, a few remaining ipsilateral RGC axons from the central retina project to the superior colliculus (SC) to an area corresponding to the permanent contralateral territory. Third, although a few early-growing ipsilateral RGC axons enter the SC and elaborate arbors at

postnatal ages, RGC axons do not make substantial projections to the more proximal dorsal lateral geniculate nucleus (dLGN). Finally, at the time that the early ipsilateral RGC axon cohort from DC decreases, by E17.5, most have apparently never entered their target, suggesting that their disappearance may not be related to target-derived factors.

Using innovative tracing techniques I found that most of the transient central retina RGC axons do not project to a target, suggesting that not all the mechanisms of elimination of transient projections are target-dependent. This was an unexpected result that brought new questions on the disappearance of transient projections and whether cell autonomous mechanisms play an additional role in their disappearance. Further experiments using *in utero* electroporation of plasmids with candidate genes, such neurotrophic factors, or RNAi could study the cell autonomous mechanisms of the disappearance of transient projections.

The low number of central retina ipsilateral RGC axons that actually reach a brain target raises the issue on why this transient population disappears and challenges the model of a pure competition of axonal projections at the target. The results also suggest that the process of disappearance of this transient visual projection might be more complex than expected, with multiple mechanisms assuring the elimination of this inappropriate projection.

In parallel to the anterograde labelling of the central retina RGC with *in utero* electroporation of GFP and DiI presented in the Section 1, two other tracing and 'fate mapping' techniques were used in preliminary experiments, to track the transient projection. An Adeno (Ad) 5 viral vector in Section 2 and Brn3b conditional knock-out (CKO) mice in Section 4 each aimed to trace the central retina ipsilateral RGCs but produced sub-optimal labelling of the central retina RGCs in early development when compared with method performed in Section 1. The Ad5 viral vector labeled a broad and a non-contiguous area in the retina and cells in the brain, and the Brn3b CKO mouse produced dense labeling of RGCs that did not allow the identification of individual axons or distinguishing ipsilateral vs contralateral RGCs. Nevertheless, the studies in the Brn3b CKO showed a possible transient expression of Brn3b at the ventral midline that was previously unknown.

In Section 3 a combined method of retrograde labeling with DiI in fixed tissue and immunohistochemistry was developed that should be useful for further expression experiments

in this population of RGCs. The technique optimized in this Section could be applied to further experiments on the verification of the expression of candidate genes in central retina ipsilateral vs contralateral RGCs.

In Section 5, a possible mechanism for the elimination of the transient ipsilateral RGC projections was explored through immunohistochemical labelling of caspases in RGC axons that were electroporated at E12.5. The immunohistochemistry for activated caspase 3 and caspase 6 was not conclusive; nevertheless, possible elevated expression of activated caspase 3 was identified along the optic tract.

Fate of the central retina ipsilateral retinal ganglion cell axons

In the Prospectus of this thesis three models on the fate of the central retinal ipsilateral RGC axons were presented. Model A proposes that central retinal ipsilaterally-projecting RGCs project along the OT but do not extend as far as the SC or dLGN, and then disappear. In Model B the central retinal ipsilaterally-projecting RGCs project to the SC or dLGN, but do not arborize at the target and then disappear. In the last hypothesis presented, Model C, the central retinal ipsilaterally-projecting RGCs project to the SC or dLGN, arborize within the target and then disappear.

Model A is a target-independent model that proposes the hypothesis that the selection of the ipsilateral RGC axons from the central retina might be dependent on cues at decision points or in the optic tract or axon-intrinsic factors, such as the endogenous supply of neurotrophic factors as proposed by Harvey and collaborators, and others (Cohen-Cory et al., 2010; Harvey et al., 2012). These factors would normally support growth toward targets but the central retina ipsilateral axons lack receptors to these cues. Thus, as they never reach the target they could not ever rely on target-derived neurotrophic factors for survival (Marshak et al., 2007; Spalding et al., 2004).

In Model B, the disappearance of the central retina ipsilateral RGC axons could be target-dependent. The central retina ipsilateral RGC axons would not express adhesion molecules to 'fix' their position at the target or they might lack neurotrophic receptors to recognize the target-derived neurotrophic factors (Cohen-Cory et al., 2010).

In Model C, the disappearance of the central retina ipsilateral RGC axons only occurs after the axons arborize and potentially after trying to form synapses with neighbor cells. Adhesion molecules involved in synapse formation and/or activity dependent mechanism could play a role in this model of disappearance of RGC axons (McLaughlin and O'Leary, 2005).

The results of Section 1, in which the early central retina RGCs were labeled with the electroporation of GFP, support both Model A and Model B. While most of the central retinal ipsilateral RGC axons disappear after E16.5 before reaching a target, supporting Model A, some ipsilateral RGC axons reach the SC, as in Model B, even if not forming broad arborizations as in Model C. The disappearance of the central retina RGC axons labeled by electroporation at E12.5 is probably independent of activity since the activity-dependent selection of projections seems to occur later. It is still possible that later projecting ipsilateral RGCs from the central retina are dependent on activity-dependent mechanisms. Most of the ipsilateral RGCs which disappear in our experiments in Section 1 projected to an area below the dLGN in the OT, so a potential 'decision point' could be located there. But since some central retinal ipsilateral RGC axons project farther than that point, it may be that many of the central retina ipsilateral axons lack receptors to the cues at that hypothetical point. Since growth cone shape becomes more complex near decisions points, whether the anatomy of the RGC growth cones gets more complex at any point of the OT could be chronicled to identify potential unknown 'decision points' in the optic tract.

Targeting of the central retinal ganglion cells electroporated at E12.5

The molecular identity of the central retina ipsilateral remains elusive. The targeting described above implicates that they respect the matching EphA/ephrinA gradients between the retina and the targets, targeting the same area of the contralateral RGCs electroporated in the same retinal territory. Another hypothesis would be that the central retinal ipsilateral RGCs have properties in common with the permanent ipsilateral RGCs from the ventrotemporal retina and they would target the same area as the permanent ipsilateral RGCs. My observations on the Section 1 of the Result of this thesis would not allow clearly validating/refuting these hypotheses. The few ipsilateral RGCs electroporated at E12.5 that project to the SC after E17.5

do not complete their process of targeting; they project to the contralateral territory of the SC but do not form complex arborizations in an area at the target. To understand the extent to which they target, it would be necessary to chronicle the process by which ipsilateral RGCs overshoot, arborize and eliminated inappropriate projections outside the final target to identify their final targeting area.

Development of *in utero* electroporation with GFP for "prospective" labeling.

The development of the brain is a complex and dynamic process in which neuronal cells are born, axonal and dendritic projections are formed, synapses are established, and only some of these survive into the adult, mature and functional nervous system. The transiency of these cells, projections or synapses makes them challenging to study. The ideal method to study transiency in neurodevelopment is live imaging that is feasible in simpler models such as zebrafish (Harris et al., 1987). In mammals, the execution of live imaging during *in utero* development is difficult. Methods of live culture of full embryos or slices of tissue allow live imaging and studying of the development dynamics of neural projections but the duration or survival of embryonic tissue in culture does not go beyond a few days. The most common methods to study the process of formation of the neural projections in mammals is the collection of the embryonic tissue at different ages and compare the samples through the different ages, with the number of samples able to statistically dilute the individual differences in the stage of development (Taylor and Guillery, 1995). Traditional methods like the use of dyes to label a population of neurons does not allow making a consistent and reliable conclusion on the evolution of the transient projections. These 'retrospective' or "snap-shot" studies label already formed projections. Neurons can also be labelled 'prospectively' by electroporation of a plasmid with a marker during the development or the transfection of a dividing cell with a viral vector carrying a plasmid as marker. Prospective labelling allows following the progression and evolution of a population of neurons through time to understand the fate of their progression, such as the work presented here, or the migration of cells during development (Miyata et al., 2010). Ideally, markers such as a fluorescent protein or enzymes such as alkaline phosphatase, should include a motif that harbors the marker in the cell membrane if the aim is to label neuronal projections. It is possible to label neuronal projections

without a marker attached to the cell membrane, nevertheless, the quality of the labelling might be uneven along the projection. In our studies in Section 1 we used a GFP plasmid containing a palmitoylation sequence of GAP43 of the GFP's N-terminus that harbor the GFP protein in the cell membrane, to improve the quality of the neuron's labeling (Matsuda and Cepko, 2004, 2007).

The progression of the transient RGC ipsilateral projection in chick and mice.

In the Introduction of this thesis, the development of the retinal projection to the tectum in chick was described. It is interesting to note that some of the characteristics of the transient ipsilateral RGC projection in chick is similar to the transient RGC projection we describe in mice. In the experiments presented in Section 1, the ipsilateral RGC axons labelled with GFP project to the SC later than the contralateral RGC axons electroporated on the same day. Only around P0 were a few ipsilateral RGC axons from the central retina seen to project to the SC, while at E17.5 the contralateral RGC axons were already occupying and branching in the SC. In chick, the transient ipsilateral RGC axons arrived at the ventroanterior tectum at day 7, one day later than the contralateral axons. Since, ipsilateral and contralateral axons arrived at the midline at the same time, at incubation day 3.5-4, this delay of the ipsilateral projection might not be a consequence of different timing in exiting the retina in ipsilateral versus contralateral RGC axons but rather the consequence of a slower progression of the ipsilateral RGC axon growth or a stall/"waiting period" at some point of their projection from the chiasm to the tectum.

The transient ipsilateral central retina RGC projection as a pioneer projection.

It has been hypothesized that the first RGCs to project to the OT pioneer that neuronal track (Raper and Mason, 2010). Since the transient ipsilateral RGCs are the first to reach the OT, they have been called "pioneers" that would facilitate the progression of "follower" axons. The data to support this hypothesis in mammals is still missing, while in fish the work described in the Introduction of this thesis suggests that the first RGCs to project to the brain are pioneers (Pittman et al., 2008).

In the studies presented in Section 1 the DiI/DiA labeling of the OT showed that the first ipsilateral and contralateral RGC axons to reach the OT around E13.5 occupy segregated

territories in the OT with the ipsilateral axons more medial and the contralateral axons more lateral. Earlier studies (Godement et al., 1984) and others performed by Austen Sitko in the Mason Lab (in preparation) showed that at later stages of development the contralateral RGC axons occupy a broad area in the OT but predominantly medial, while the permanent ipsilateral RGCs from the VT retina course more dorsolaterally in the OT. The apparent segregation between the early central retina ipsilateral RGC axons and their contralateral counterpart decreases the opportunity for axon-axon interaction between these two populations, and lessens the probability that the central retinal ipsilateral RGCs axons pioneer the OT facilitating the progression of the "follower" contralateral RGC axons. Still, the early RGC projection to the brain, both ipsilateral and contralateral RGC axons, could have special properties that facilitate the progression of axons that project later. These properties could be axon-axon interactions, or fasciculation, mediated by adhesion molecules (Jaworski and Tessier-Lavigne, 2012) or interactions with the environment, as described in the Introduction of this thesis, with the preformed pathways hypothesized in the inner ear development (Carney and Silver, 1983). For the purpose of understanding the fasciculation properties of the early central retina RGC, some experiments are proposed in the Further Experiments section of this thesis.

Central retinal ipsilaterally projecting RGCs in rhesus monkey

Chalupa and Meisserel traced the early projections from the whole retina to the brain in rhesus monkey and described a transient population of ipsilateral RGC axons that are positioned in the medial most portion of the optic tract (Meissirel and Chalupa, 1994). In light of our data that the early projecting central retina RGC axons occupy the medial most position in mice's optic tract, we hypothesized that the population of transient ipsilateral RGC axons in the medial OT described by Chalupa and Meisserel in rhesus monkey might be a population of RGC axons from the central retina that was conserved in evolution. Further studies in rhesus monkey with a tracer injected specifically in the central retina, or early retrograde tracing in embryonic fixed tissue, could contribute to verify whether these transient ipsilateral axons projecting to the medial OT project from the central retina.

V. Further experiments

Profiling of central retina ipsilateral RGCs.

This thesis elucidated several questions on the fate of the central retina ipsilateral RGCs showing the progression of the central retina RGC axons through time. Nevertheless, the molecular mechanism of elimination of the ipsilateral RGCs axons remains unknown. Also, it is still unclear whether the central retina ipsilateral and contralateral RGC axons have different genetic profile expressing diverse differentiation factors and adhesion molecules or whether the transient central retina ipsilateral RGC express factors common to the permanent VT ipsilateral RGCs. Genetic profiling of these populations of RGCs would contribute to clarify these aspects.

There are two major strategies for gene profiling of cells. On the candidate approach the expression of a candidate gene is verified in the population of interest. In the context of understanding the differences of central retina ipsilateral versus contralateral RGCs, neurotrophic factors and theirs receptors are candidates of interest. In the Model A presented earlier, the central retina RGC axons do not reach a target and their disappearance is target-independent. One mechanism that could explain the disappearance of the transient ipsilateral central retina RGC axons is a lower level of endogenous neurotrophins or a lower level of expression of neurotrophins receptors. The levels of neurotrophins such as brain-derived neurotrophic factor, neurotrophin-4/5, ciliary neurotrophic factor, leukemia inhibitory factor, glial cell-derived neurotrophic factor, insulin-like growth factor-1, the fibroblast growth factors and/or hepatocyte growth factor and of neurotrophin receptors, such as tyrosine receptor kinases (Trk): TrkA, TrkB, TrkC and p75 neurotrophin receptor (Harvey et al., 2012) could be quantified by qPCR in the RGCs.

In alternative to a candidate gene approach is the unbiased screen approach to find candidate markers for a population with no or minimal assumptions made before the screening. An example of an unbiased screening was performed in the Mason Lab by Wang (Columbia Graduate Thesis 2013. See Figures in Annex B). To identify new genes that would distinguish ipsilateral from contralateral RGCs at the peak phase of ipsilateral RGC production, Wang performed retrograde labeling of the RGCs from the OT at E16.5 with rhodamine-dextran in live tissue, performed cell sorting by fluorescence-activated cell sorting (FACS) of the rhodamine-

dextran positive cells and submitted the contralateral and ipsilateral RGCs to microarray screening. Performing this technique before E16.5 or E15.5 would be extremely challenging since the embryos are much smaller. Nevertheless, since there are some remaining ipsilateral central retinal RGCs projecting to the OT at E16.5 or E15.5, a similar labelling technique could be used to label the ipsilateral and contralateral RGC from the central retina. Later the central retina could be dissected to obtain the ipsilateral and contralateral RGCs from the central region in the retina, separated by FACS and an unbiased screening technique such as microarray or RNA-seq of the population of labeled cells or single cell sequencing could be performed on the ipsilateral vs contra cells from central retina. To validate the results of such a screen like this, labelling and immunohistochemistry techniques developed and discussed in Section 3 could be useful, to confirm the expression of the candidate in the ipsilateral or contralateral RGCs in the central retina. If a microarray was performed, the qPCR of candidate genes could be performed in the RGC labelled with rhodamine to validate the results of the microarray.

Otx1 as a candidate mechanism of elimination of transient neuronal projections

In the Introduction of this thesis the transient subcortical projections of the layer 5 neurons were described (Stanfield and O'Leary, 1985). The elimination of the transient collaterals of the layer 5 neurons is mediated by Otx1. Otx1 KO mice the collaterals that normally disappear during development fail to do so (Weimann et al., 1999). Since Otx1 was hypothesized to play a role in the refinement of layer5 neurons in the cortex, this gene could be an interesting candidate to study further with respect to the disappearance of the central retina transient ipsilateral RGCs. The expression of Otx1 and the role of Otx1 during the development of the retina was described by Martinez-Morales et al. (2001) (Martinez-Morales et al., 2001). Otx1 is expressed in the early eye field and at E13.5 and E16.5 the intra-retinal area surrounding the optic nerve head. Otx1 KO mice lack ciliary body processes and lachrymal glands and have other brain defects (Acampora et al., 1996). To address the role of Otx1 in the disappearance of the transient ipsilateral RGC axons, further studies should be performed to verify the expression of Otx1 in RGCs and more specifically in the ipsilateral central retina RGCs. The technique

developed in Section 3, using retrograde labelling with DiI, is useful for identifying the ipsilateral RGCs. The RGCs labelled with DiI could be photoconverted with DAB, as described by Soares (Master thesis in Medicine, 2014), and later combined with *in situ hybridization* for Otx1 (Martinez-Morales et al., 2001). To verify the expression of the Otx1 protein in the central retina RGCs, the technique optimized Section 3 could be useful to confirm the expression of Otx1 by immunohistochemistry in the central retina RGCs labeled with DiI. Afterwards, the techniques performed Section 1 could be repeated in the Otx1 KO mice. If Otx1 is involved in the disappearance of the transient ipsilateral projection it would be expected that in the knock-out mice, this projection might persist longer than found Section 1. Nevertheless, Otx1 deletion could be compensated by other molecules. It is also possible that Otx1 is only relevant at a specific stage of the process of disappearance and that in subsequent stages it would guarantee the disappearance of the inappropriate projection. Nonetheless, a careful analysis of the RGC axonal projection throughout development could assure that minor phenotypes are identified. Since Otx1 is also expressed in the brain in mice and humans (Acampora et al., 2001; Larsen et al., 2010) the Otx1 KO mice might not be the ideal model to perform these experiments. A conditional knock in which Otx1 can be deleted after the expression of Cre would be a better experimental setting by which to understand the role of the expression of Otx1 in RGC. Cre could be delivered to the retinal cells by *in utero* electroporation of a Cre-plasmid or by crossing the conditional Otx1 mice with cell- and tissue-specific Cre transgenic mouse lines expressed exclusively in the retina and not in the brain (Lu et al., 2013).

Further studies on the mechanism of disappearance of the transient ipsilateral RGC projection

The development of methods to identify the ipsilateral central retina RGCs would be useful to understand whether the disappearance of these RGC is dependent of the apoptotic cascade and, more interestingly, at what stage of the axonal projection the cell death mechanisms are activated. If the RGCs undergoing apoptosis still keep their intact axonal projection while expressing apoptotic markers, it would be possible to perform a retrograde labeling of the RGC from the OT to the retina at different ages and verify what RGCs nuclei express apoptotic

markers. A potential caveat of the use of axonal tracing to identify ipsilateral vs contralateral RGCs is the early degeneration of the axonal projection of a cell undergoing cell death, which would not allow labelling of the cell body with the axonal tracer. Different assays can be performed to identify cells undergoing apoptosis, such as immunohistochemistry to activated caspase 3 (Gashegu et al., 2007) or a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay (Pequignot et al., 2011; Shin et al., 2014). If a genetic marker is available to identify the ipsilateral and contralateral RGCs in the central retina during development, the same studies could be performed without the need of a axonal tracer, since the ipsilateral and contralateral RGCs could be identified by *in situ* hybridization, immunohistochemistry or using a mutant mice that express a fluorescent protein under the control of the promoter of the known marker.

Further studies on the fasciculation of the early projecting RGC axons.

To understand whether the earliest projecting central retina ipsilateral RGC axons, or even both ipsilateral and contralateral RGCs, have characteristics that could facilitate the progression of follower axons by cell-cell interactions, fasciculation assays could be performed (Jaworski and Tessier-Lavigne, 2012; Muhleisen et al., 2006). In an ideal experiment it would be possible to sort the central retina ipsilateral and contralateral RGCs at an early stage of development so fasciculation assays could test specific populations of cells. Another possibility would be to identify the central retina ipsilateral RGCs through the rhodamine-dextran methods described above. The RGC labelled with rhodamine-dextran could be FACSorted or cultured as an explant of the central retina. These rhodamine-dextran positive RGCs could be put together in a petri dish culture to verify how the ipsilateral RGC axons labeled with rhodamine-dextran behave in fasciculation assays with another retinal explant, as is being performed in the Mason Lab by Austen Sitko and as performed with motor neuron explants (Jaworski and Tessier-Lavigne, 2012). The fasciculation assays could also be performed with explants from the early central retina interacting with explants from the peripheral retina that are born and project later. The explants with specific populations of RGCs would reveal whether the RGCs in the central retina fasciculate more readily among the axons in the same explant (self-association) or avoid axons in the heterotypic explant, and could demonstrate that the early retina RGC have different

fasciculation properties than later-growing axons and not a role as pioneers of a tract. One caveat of studying fasciculation in retinal explants it that it is not possible to replicate the dynamics of the interaction of the projecting axons with their extracellular environment, such as the optic chiasm or optic tract. These assays may not reveal the properties of axons that might enable some to be pioneers, but could be used as an assay to probe molecular basis of avoidance or fasciculation of unlike or like axons, respectively.

VI. Conclusion

This thesis project proposed to examine the long standing question on the fate of the central retina RGC projections. Different approaches were used and different methods were optimized. Even if some methods did not allow answering questions in the present thesis they will be useful for further experiments. Some of the experiments that were started on neurogenesis of RGCs (presented as a Master Thesis 2015) are already ongoing in the Mason lab.

Overall, this thesis shows that the disappearance of transient projections might not be dependent on interactions of the projections with the target, but that additional target-independent interactions might play a role in their disappearance.

Analysis of the development and disappearance of transient projections during development is crucial to analyzing the formation of accurate neuronal circuits. This thesis on disappearance of transient projections brought new questions and directions to a better comprehension of the development of neuronal circuits.

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Annex A - Brn3b CKO Protocols

In this Annex it is described the protocol of induction in the 46 Brn3b conditional knock-out mouse embryos sent to the Mason Lab from Tudor Badea's Lab, National Eye Institute, National Institute of Health. Their phenotype is described in the Section 4 of the Results. The level of labeling of the mutant mice was classified as 'negative' when no labeling was present; 'sparse' when a few cells were labeled or 'strong' when a lot of cells were labeled.

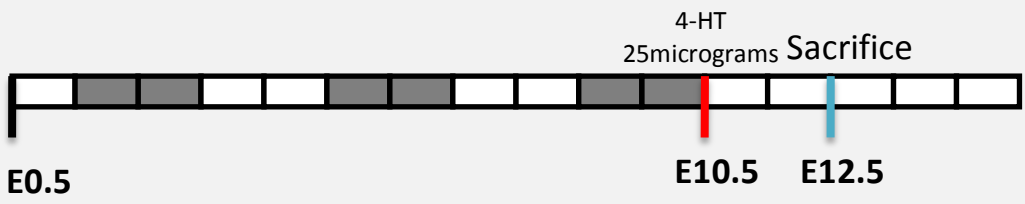
4-HT: 4-hydroxy-tamoxifen

CKO/-: Bn3b CKAP/- ; ROSA26-rtTACreER
CKO/+: Bn3b CKAP/+ ; ROSA26-rtTACreER

: Full day of doxycycline feeding

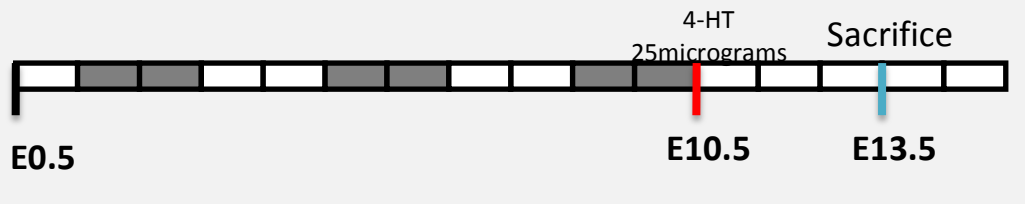
: Half-day of doxycycline feeding

111

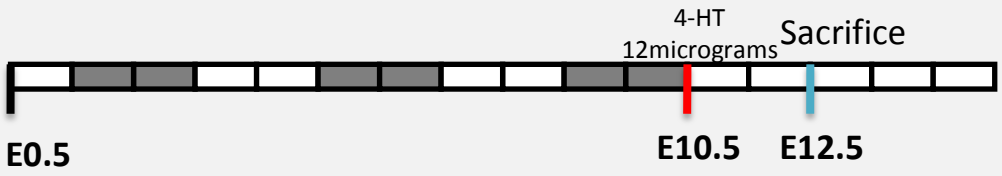


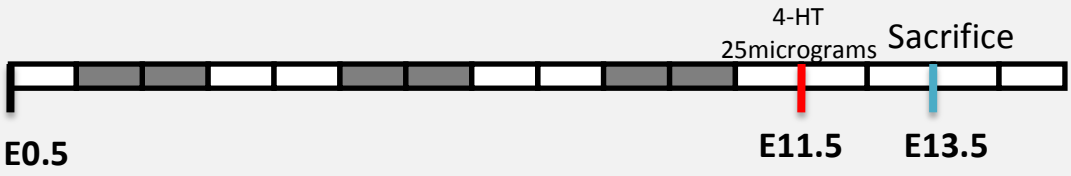
111.6	CKO/-	Sparse
111.7	CKO/-	Negative
111.8	CKO/+	Sparse
111.9	CKO/+	Negative
111.10	CKO/-	Negative in the retina

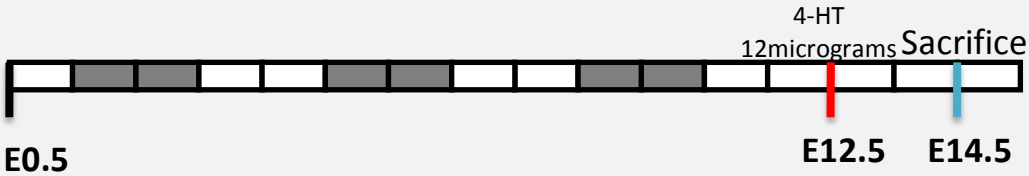
112

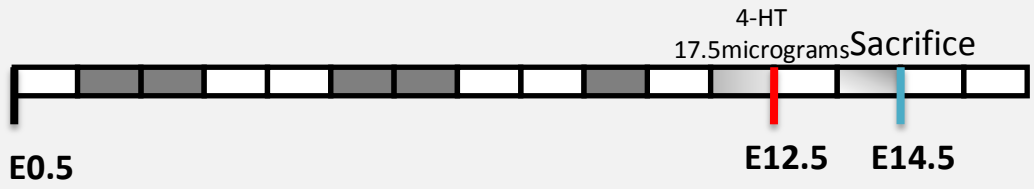


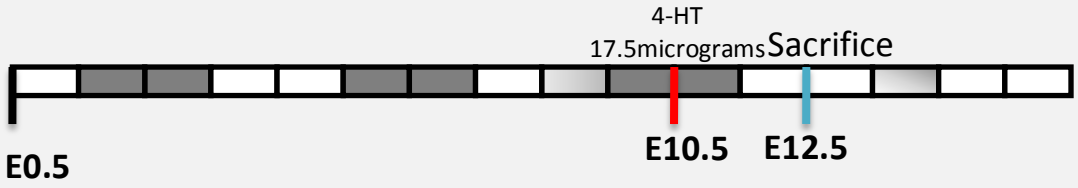
112.4	CKO/+	Strong
112.5	CKO/+	Strong
112.6	CKO/-	Strong
112.7	CKO/-	Negative

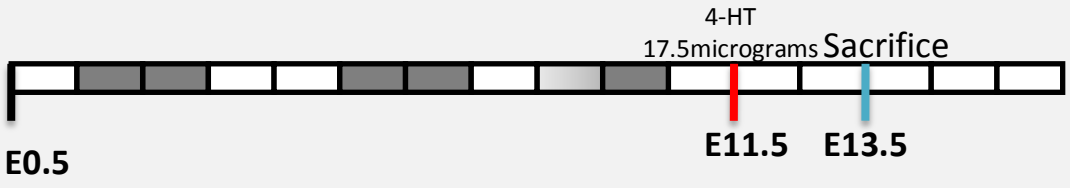
113		
		
113.4	CKO/-	Negative
113.6	CKO/+	Sparse
113.7	CKO/-	Sparse

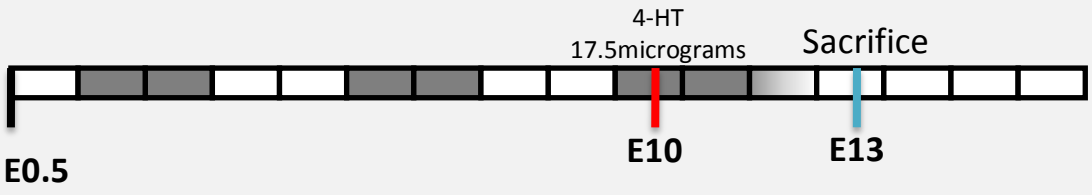
114		
		
114.5	CKO/+	Negative
114.8	CKO/-	Negative
114.9	CKO/+	Negative
114.10	CKO/+	Negative
114.11	CKO/-	Negative

115		
		
115.1	CKO/+	Strong/Sparse
115.2	CKO/-	Negative
115.3	CKO/+	Strong
115.4	CKO/-	Strong
115.6	CKO/-	Few RGCs projecting.

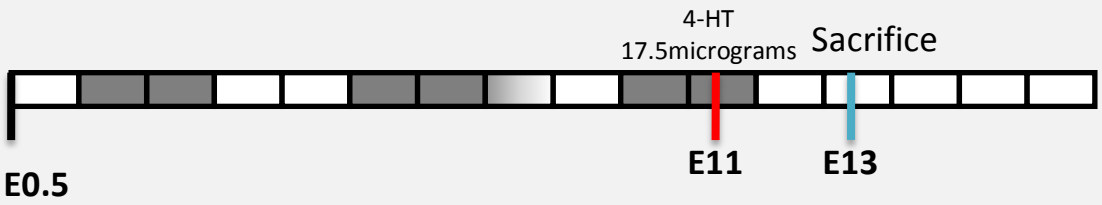
116		
		
116.1	CKO/+	Strong
116.2	CKO/+	Background
116.3	CKO/-	Strong
116.4	CKO/-	Background

117		
		
117.5	CKO/-	**Sparse**
117.6	CKO/-	Strong
117.7	CKO/-	Negative
117.8	CKO/-	Negative
117.9	CKO/+	**Sparse**

118		
		
118.1	CKO/-	Strong
118.2	CKO/+	Background
118.3	CKO/+	Background
118.4	CKO/-	Background

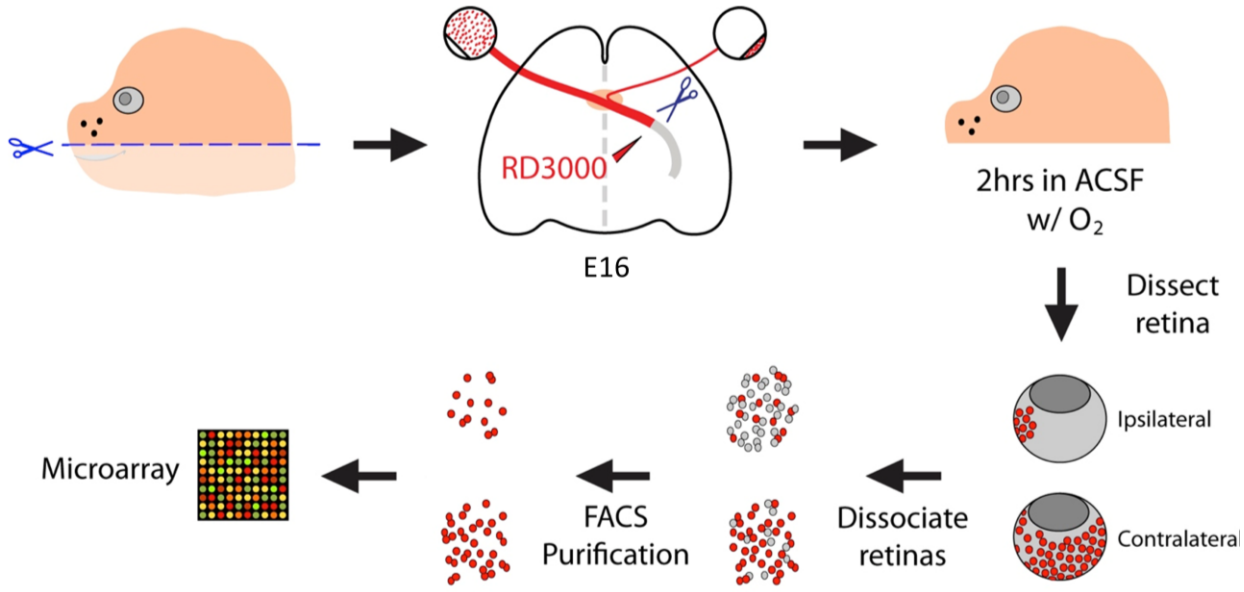
119		
		
119.7	CKO/+	Negative
119.8	CKO/-	Strong
119.9	CKO/+	Negative
119.10	CKO/+	Strong
119.11	CKO/+	Strong

120		
		
120.2	CKO/+	Negative
120.3	CKO/+	Negative
120.4	CKO/-	Strong

121		
		
121.1	CKO/+	Negative
121.2	CKO/+	Negative
121.3	CKO/-	Strong

Annex B - Retrograde labeling of retinal ganglion cells with rhodamine-dextran.

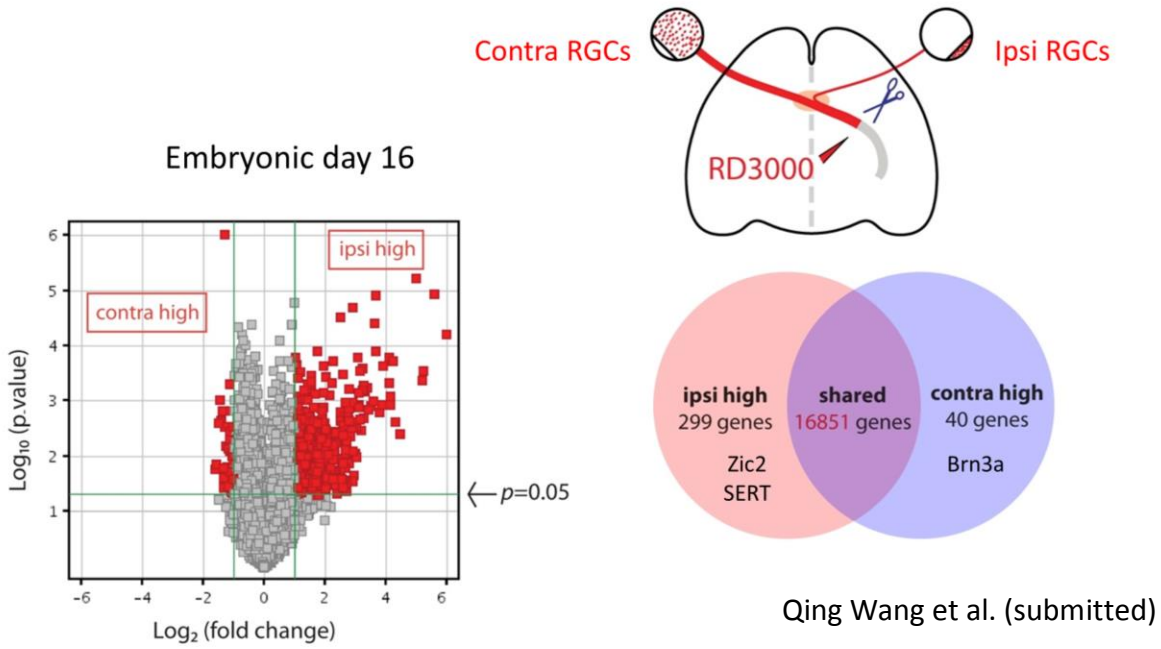
Qing Wang in the Mason Lab developed a new technique for purifying ipsilateral and contralateral RGCs, retrogradely tracing the RGC axons from the optic tract (OT) to the retina using rhodamine-dextran. After labeling the ipsilateral and contralateral RGCs, Wang performed fluorescent activated cell sorting (FACSorting) of the two populations of cell that were later submitted to an unbiased screening approach (Figure 1), to identify further genes that determine the molecular identity of ipsilateral and contralateral RGCs (Figure 2).



Qing Wang et al. (submitted)

Annex B - Figure 1 Purification of ipsilateral and contralateral retinal ganglion cells at E16 by retrograde labeling from the optic tract to the retina using rhodamine-dextran.

ACSF: artificial cerebrospinal fluid. contra: contralateral. E: embryonic day. FACS: Fluorescent Activated Cell Sorting. Ipsi: ipsilateral. RD3000: rhodamine-dextran 3000 molecular weight.



Qing Wang et al. (submitted)

Annex B - Figure 2 Unbiased screening of candidate genes for cell fate determination in purified ipsilateral and contralateral retinal ganglion cells.