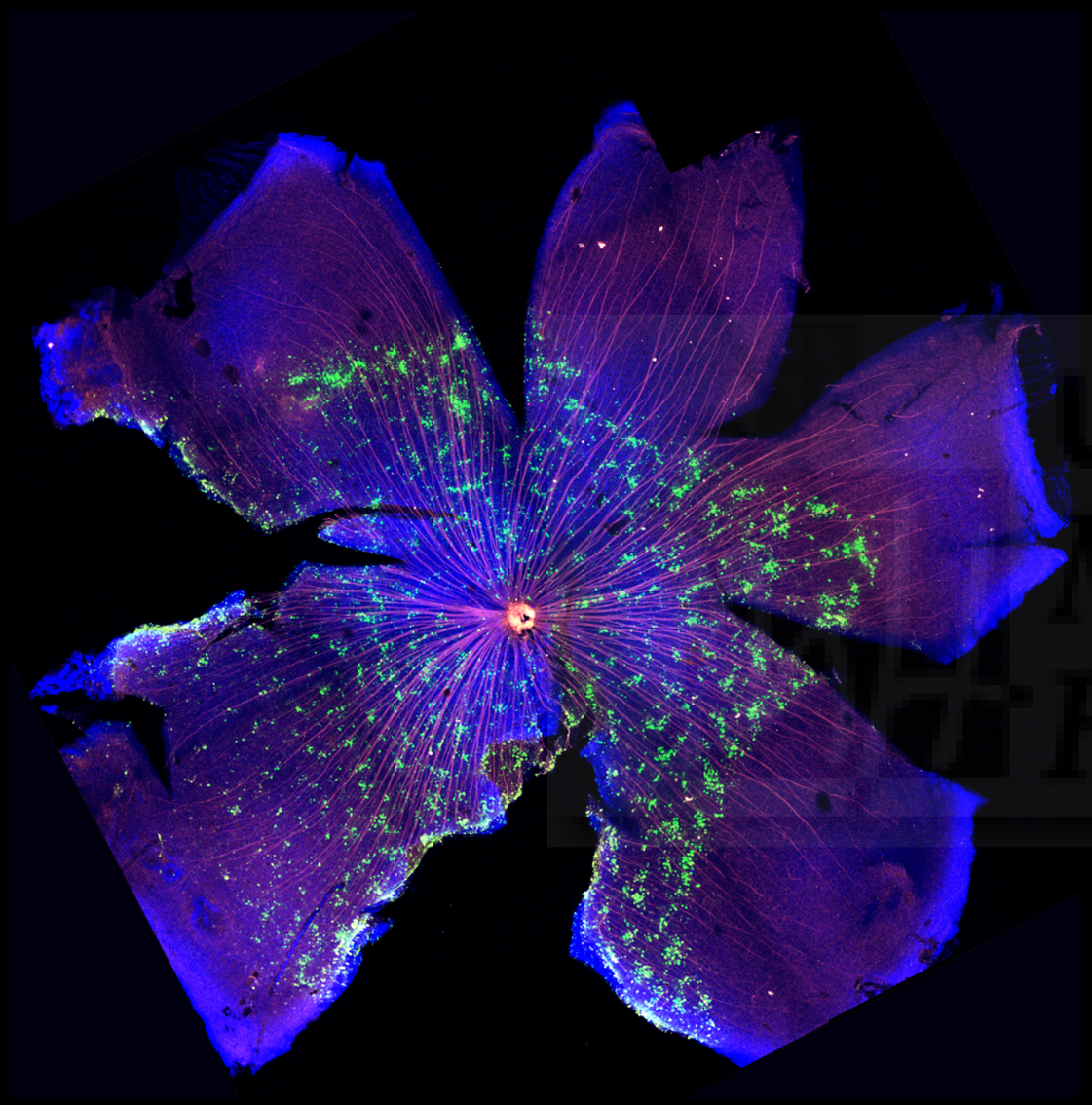
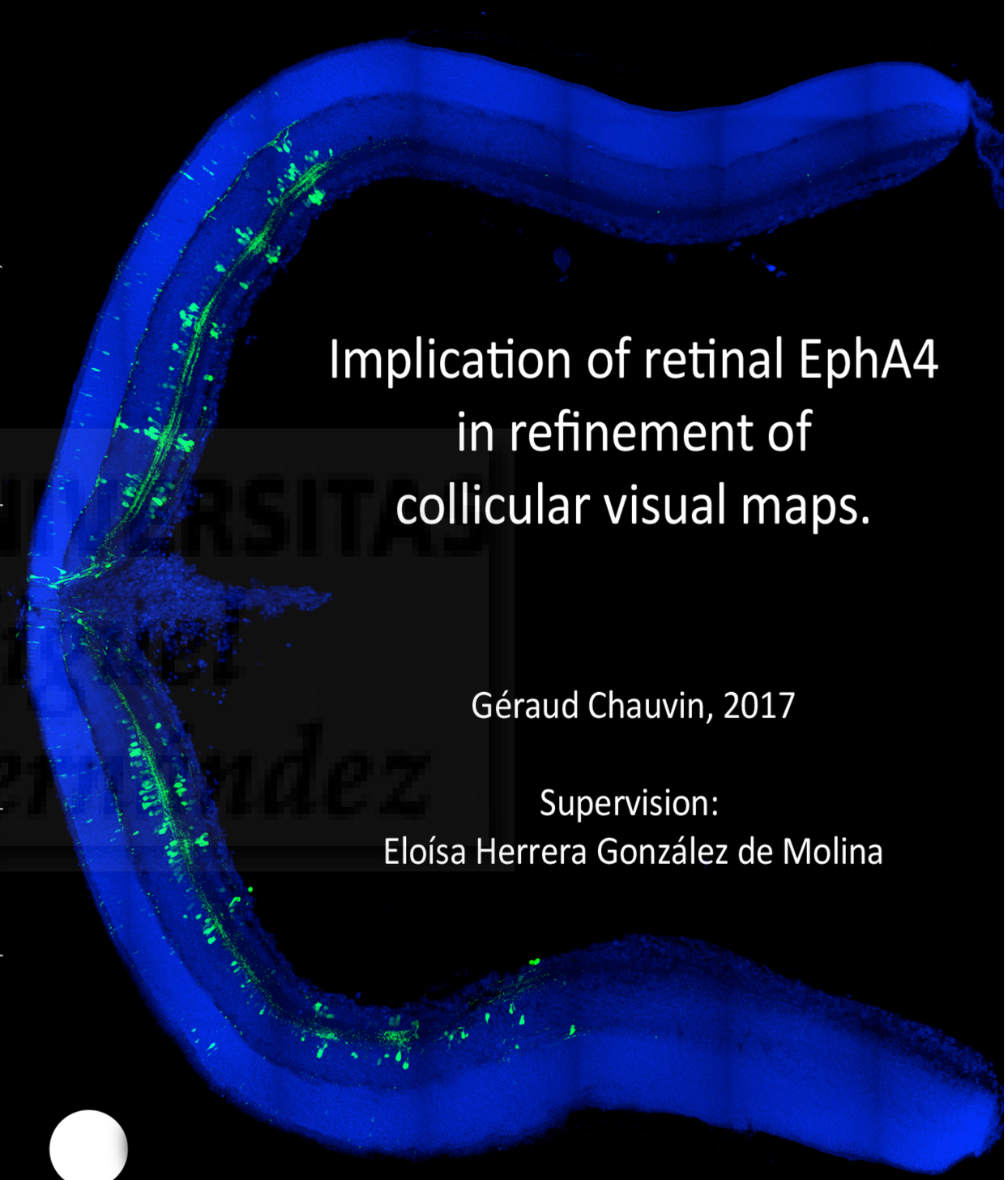


Implication of retinal EphA4 in refinement of collicular visual maps. Géraud Chauvin, 2017

Implication of retinal EphA4 in refinement of collicular visual maps.



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Supervision:
Eloísa Herrera González de Molina

Cover picture: P10 retina electroporated with EphA4 (green) and counterstained with DAPI (blue) by Géraud Chauvin
Backcover: Whole mount retina electroporated with EphA4 (green), immunostained against L1CAM (red) and DAPI (blue) by Yaiza Coca

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IMPLICATION OF RETINAL EPHA4

IN REFINEMENT OF

COLLICULAR VISUAL MAPS

Géraud Chauvin

Supervisor: Eloísa Herrera González de Molina

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CERTIFICA :

Que la Tesis Doctoral titulada: *" Implication of retinal epha4 in refinement of collicular visual maps"* ha sido realizada por D. Géraud Chauvin (NIE Y0808186A) bajo la dirección de la Dra. Eloísa Herrera González de Molina, y da su conformidad para que sea presentada a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste a los efectos oportunos, firma el presente certificado en San Juan de Alicante, a 7 de septiembre de 2017

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D. ª Eloísa Herrera González de Molina, Investigador Científico del Consejo Superior de Investigaciones Científicas, AUTORIZA:

La presentación de la tesis doctoral titulada *"IMPLICATION OF RETINAL EPHA4 IN REFINEMENT OF COLLICULAR VISUAL MAPS"* realizada por **D. Géraud Chauvin**, bajo su inmediata dirección y supervisión en el Instituto de Neurociencias (UMH-CSIC) y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que así conste y surta los efectos oportunos, firmo el presente certificado en San Juan de Alicante a 7 de septiembre de 2017.

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Gé!

INDEX

ABBREVIATIONS

Ach	Acetylcholine
B2AchR	Beta2 Acetylcholine Receptor
C	Caudal
CAG	Chicken Actin Globin
CNS	Central Nervous System
CSPG	Chondroitin Sulfate Proteoglycan
D	Dorsal
dLGN	dorsal Lateral Geniculate Nucleus
E#	Embryonic Day #
FL	Fiber Layer
GCL	Ganglion Cell Layer
INM	Interkinetic Nuclear Migration
INL	Inner Nucleus Layer
IPL	Inner Plexiform Layer
L	Lateral
M	Medial
M-L	Medio-Lateral
M>L	High Medial to Low Lateral
N	Nasal
OD	Optic Disc
ON	Optic Nerve

ONL	Outer Nucleus Layer
OPL	Outer Plexiform Layer
P#	Post Natal Day #
P>C	High Peripheral to Low Central
PE	Pigmented Epithelium
PRL	Photo Receptors Layer
R	Rostral
R-C	Rostro-Caudal
R>C	High Rostral to Low Caudal
RGC	Retinal Ganglion Cell
RPC	Retinal Progenitor Cell
RTK	Receptor Tyrosine Kinase
SAM	Sterile Alpha Motif
SC	Superior Colliculus
Shh	Sonic Hedgehog
T	Temporal
T>N	High Temporal to Low Nasal
TZ	Terminal Zone
V	Ventral
V-D	Ventro-Dorsal
V>D	High Ventral to Low Dorsal

ABSTRACT

EphA/ephrin-A signaling has been demonstrated to mediate the establishment of the topographic visual map: In mammals EphA5 and EphA6 are expressed in a high-temporal to low-nasal gradient in the retina while ephrin-A5 is expressed in a high-caudal low-rostral gradient in the superior colliculus. In contrast, EphA4, another member of the EphA family, is homogenously expressed in retinal ganglion cells (RGCs) and its role in retinal development has not been clarified.

In this study we investigated the role of EphA4 during the development of the visual system in mice. We found that EphA4 mutant mice show larger and extended ramifications of RGC arbor terminals in the visual targets compared to control mice. Conversely we determined that ectopic expression of EphA4 in the retina induces aberrant adhesion of axon terminals at the visual targets, but also of RGCs in the retina. We demonstrated that EphA4/ephrin-B1 forward signalling is responsible for this adhesive phenotype. Together with previous reports, our results strongly suggest that axon terminals coming from the retina activate EphA4 when encountering ephrin-B1 at the visual targets, to modulate axon branches adhesion during the refinement phase of topographic visual mapping.

La señalización de EphA/ephrin-A ha sido demostrada como responsable del establecimiento de las mapas topográficas visuales: En los mamíferos, EphA5 y EphA6 se expresan en un gradiente alto-temporal hacia bajo-nasal en la retina, cuando ephrin-A5 se expresa en un gradiente alto-caudal bajo-rostral en el colículo superior. En contraste, EphA4, un otro miembro de la familia EphA, se expresa homogéneamente en las células retínales ganglionares (RGCs) y su papel en el desarrollo retinal no ha sido aclarado aún.

En este estudio, hemos investigado el papel de EphA4 durante el desarrollo del sistema visual del ratón. Hemos encontrado que los ratones mutantes de EphA4 padecen de axones retínales con ramificaciones más grandes y más extendidas en las dianas visuales, comparados con ratones controles. Al contrario, hemos determinado que la expresión ectópica de EphA4 en la retina induce una adhesión aberrante de los terminales axonales en las dianas visuales, y también entre las RGCs de la retina. Hemos demostrado que la señalización directa de EphA4/ephrin-B1 es responsable de este fenotipo adhesivo. Junto con previos artículos, nuestros resultados indican que los axones de la retina, al encontrar ephrins-B1 en el colículo, activan EphA4, para modular la adhesión de los terminales durante la fase de refinamiento del establecimiento de la mapas topográficas visuales.

INTRODUCTION

Most mammals, including humans, rely on vision to sense the environment. The main function of the mammalian visual system consists in generating object representations of the external world and integrating them with cognitive and contextual information from the rest of the sensory modalities in order to generate adequate behavioural responses to interact with the environment (Ramon y Cajal, 1894).

The eye, the entry point of visual perception, is a superficial structure integrated by the sclera, the lens, the iris and the retina. The retina is also the only neural part of the eye, is a thin layer covering the back of the sclera responsible for the transduction of light in relevant signals into the brain to mediate vision. The retina is the only part of the central nervous system in direct contact with the external world and, because its accessibility to genetic manipulation, together with the nerves that connect the visual axons to the brain, the retina has served as a useful model to study general principles underlying the molecular, cellular and physiological processes controlling brain development and functions.

The visual system has been studied by neuroscientists for many centuries and today its anatomy has been vastly documented and its physiology well described. However, many aspects of its development, including the molecular mechanisms underlying how retinal axons make precise connections with the visual targets in the brain to establish a mature and functional sensory circuit, are still not completely understood. In this thesis, we aimed to analyse the role of the tyrosine kinase receptor EphA4, a membrane protein expressed in the developing retina, with a dual objective: 1/ To precisely determine its function in visual system development and 2/ To decipher the mechanisms of action of this receptor, which could help to better understand its function in other processes and systems where it is also expressed.

General anatomy of the mammalian retina

In mammals, the adult retina is a thin laminated sheet of neural tissue located on the inner surface of the eye. Its role is to convert light into electrical impulses and transmit this information to the main visual processing centres in the brain in order to be interpreted as vision.

This laminated structure is formed by three layers of cell bodies from different types of neurons that alternate with other three layers corresponding to the processes of these neurons. The most outer layer of the retina, underneath the non-neural pigmented epithelium (PE), is the photoreceptor layer (PRL). This layer is formed by the outer segments of photoreceptors (rods and cones) that are responsible for photon reception.

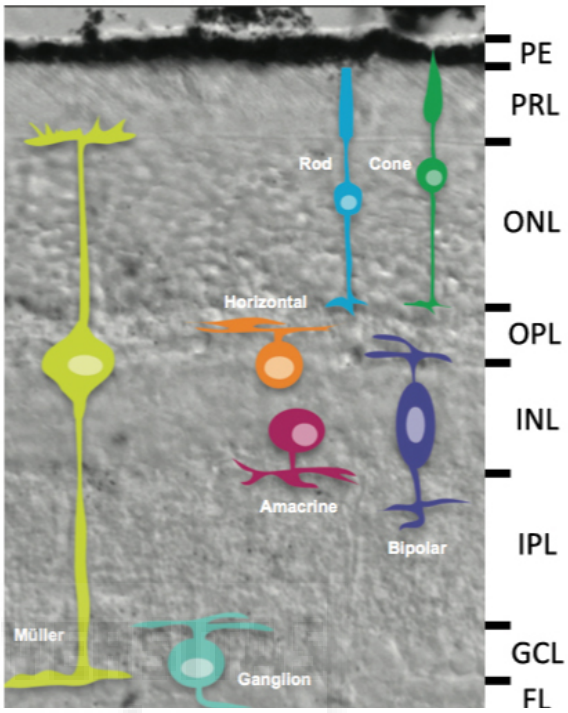

Figure 1: Retinal cell types in the adult mouse retina.

The adult mouse retina is comprised of three cellular layers separated by two synaptic layers. Underneath the non-neuronal pigmented epithelium (PE) reside the external segments of rods and cones, in the photoreceptor layer (RPL). The cell bodies of rods and cones reside in the outer nuclear layer (ONL) and form synaptic contacts in the outer plexiform layer (OPL) with horizontal and bipolar cells, both of which reside in the inner nuclear layer (INL). In addition, amacrine cells and the cell bodies of Müller glia are found in the INL. Synaptic contacts between bipolar, amacrine and ganglion cells are present in the inner plexiform layer (IPL). Ganglion cells reside in the ganglion cell layer (GCL) and extend their axons in the fiber layer (FL). Adapted from Zhang et al (2011)

The outer plexiform layer (OPL) is the one medially located. This layer integrates the dendrites of Bipolar and Horizontal cells which connect with the feet of photoreceptors. The inner plexiform layer (IPL) is the layer where amacrine cells arborize and connect to the dendrites of retinal ganglion cells (RGC) nested in the ganglion cell layer (GCL). Finally, the closest layer to the

vitreal surface is called the optic fibre layer (FL) and contains axons of RGCs that are all oriented towards the optic disc (OD) (Sefton et al., 2017) (Figure 1).

There are also three different types of glial cells: i/ The Müller cells, with their cell bodies located in the INL and extending their ramifications throughout the entire thickness of the retina. ii/ Astrocytes, located in the FL, and iii/ Microglia, found in the FL, GCL, IPL and OPL, are not following the same layer-specific organization as neurons. While the different retinal neurons populations differentiate at early stages of development, all three types of glial cells differentiate at late retinal stages, when the layered organization of the retina is nearly completed. It is however important to notice that only Müller cells are born in the retina. Astrocytes are invading cells coming from the optic nerve, and microglia comes from the immune system.

Photoreceptors in the ONL

The ONL is populated by two types of photoreceptors, cones and rods (Figure 2), which are the cells responsible for phototransduction. Most mammals have both types of photoreceptors but the proportion between them varies wildly among species. In highly diurnal species such as humans, the cones are dominant with relative lack of rods while in nocturnal species, such as mice, cones only represent 3% of all photoreceptors

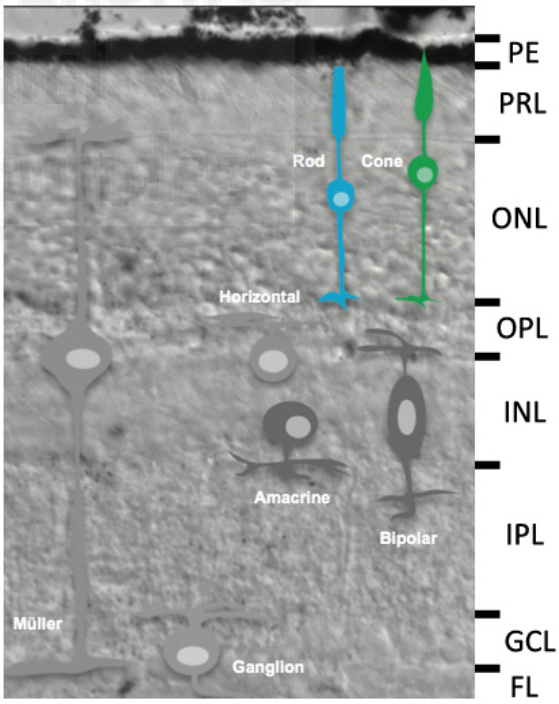

Figure 2: Retinal cell types in the ONL.

The cell bodies of rods and cones reside in the outer nuclear layer (ONL). Rods and Cones are responsible for light transduction into neuronal signal. Their outer segments, expressing photopigments, extend in the photoreceptor layer (PRL). They connect to retinal interneurons through their end-feet in the outer plexiform layer (OPL). Adapted from Zhang et al (2011).

(Carter-Dawson and Lavail, 1979).

Mice have dichromatic vision mediated by two types of cones recognizable by the kind of photopigment they contain: the UV-Cones absorb light at 350nm while the M-Cones do it at 510nm (lyubarsky et al., 1999). Cones have a typical morphology of a short conical outer segment, a large mitochondria-filled inner segment and a long axon projecting to the OPL. In most species, cones are concentrated in a specialized central temporal area of the retina called the area centralis. In some species the area centralis has evolved into an elongated horizontal zone across the retina called the visual streak. This area of high cone concentration pushes the rods to the peripheral parts of the retina and specializes in high visual acuity, movement detection and form discrimination. Species with very high visual acuity present a foveal-based visual system. The fovea is an area located in the center of the retina composed of closely packed cones and without rods. The fovea is characteristic of highly diurnal primate species, like humans, where cone vision has evolved high spatial acuity and trichromatic colour vision. However, mice do not have fovea and even the presence of a visual streak is still debated (Salinas-Navarro et al., 2009).

The other type of photoreceptor is the rods. In mammals, there is only one kind of rods and they constitute about 97% of retinal photoreceptors (Carter-Dawson and Lavail, 1979). Rods contain rhodopsin, which absorbs light maximally at 498nm (lyubarsky et al., 1999). In most mammalian species rods have small cell bodies and a long and slender inner and outer segment architecture. The outer segment is particularly long and thin to reach the non-neuronal pigment epithelial layer. From an evolutionary perspective, rods derive from cones, however many mammalian species, including mice, have developed rod-dominated retinas with little differences in topography from centre to periphery (lyubarsky et al., 1999). Despite their differences in morphology and function, rods and cones share some features. For example, they are both

responsible for phototransduction and they extend their axons down into the OPL where they connect via a specialized type of synapse, called the ribbon synapse, onto second order neurons, the horizontal and the bipolar cells.

It is of note that a third type of photoreceptor exists: the intrinsically photosensitive retinal ganglion cells (ipRGCs), which are not involved in image-forming vision, but instead, in resetting the circadian clock by detecting general levels of light (Hattar et al., 2006).

Horizontal, bipolar and amacrine cells in the INL

The INL contains cell bodies from three different classes of interneurons: bipolar, horizontal and amacrine cells (Figure 3). Horizontal neurons interconnect photoreceptor terminals laterally across the retina. These cells exhibit different morphologies depending on the particular cohort of photoreceptors they connect to. In rodents there are two types of horizontal cells: Those with a long axon and those with a very short axon, but in mice most horizontal cells are long axon-type (Peichl and González-Soriano, 1994). Horizontal cells may be identified with antibodies

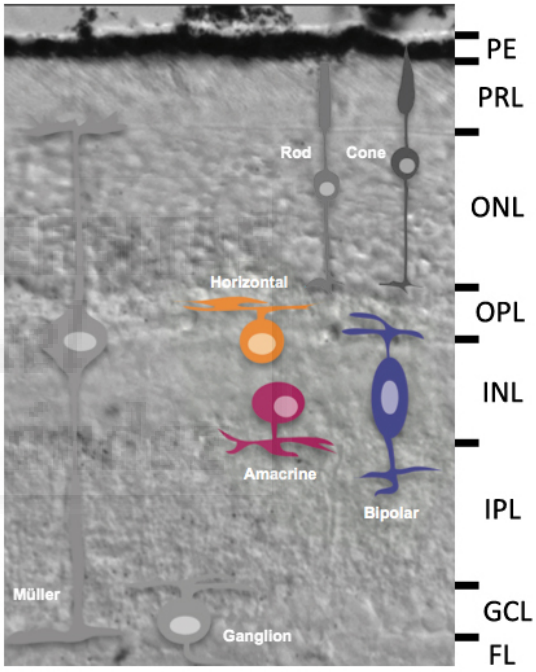

Figure 3: Retinal cell types in the INL.

Three types of interneurons populate the inner nuclear layer (INL): Horizontal, bipolar, and amacrine cells. Each type has a specific morphology adapted to its function. Horizontal cells extend neurites in the OPL to contact groups of photoreceptors. Amacrine cells arborize in the inner plexiform layer (IPL) to modulate RGC's dendritic trees signal integration. Bipolar cells are connected to photoreceptor through their dendrite in the OPL, while their axons connect RGCs in the IPL. Adapted from Zhang et al (2011).

against calcium binding proteins, calbindin or calretinin. In addition, horizontal cells may be anatomically identified by their large ramified dendritic trees that make direct contact with photoreceptors end-feet.

Bipolar cells mediate vertical communication between photoreceptors and ganglion cells. The number of different types of bipolar cells depends on the species. In mice, 10 morphologically distinct types have been described. They may be distinguished by, at least, three different features: i/ Their synaptic connections to rod or cone photoreceptors; ii/ The depth of the axon into the inner plexiform layer and, iii/ their response to increased (ON) or decreased (OFF) light intensity. The combination of all these characteristics makes them a very large and complex cell population.

Most amacrine cells are inhibitory interneurons. Similar to interneurons in the rest of the central nervous system (CNS), their function is to control the excitatory pathways of bipolar and ganglion cells. Amacrine cells, as a population, have very diverse morphologies, and are subdivided into more than 20 subtypes based on both morphology and function (MacNeil and Masland, 1998). For instance, a certain type of Amacrine cells, called AII, is involved in constructing a strong inhibitory surrounding on the receptive fields of ganglion cells, a feature necessary for correct image processing. Others like Narrow and Wide Field Amacrines, promote or reduce RGC excitability respectively, while the main function of Starburst Amacrines, is to inhibit specific parts of RGCs dendritic trees to create orientation selectivity. These are just some examples of the vast implication of amacrine cells in early visual processing (Cook and McReynolds, 1998).

Retinal Ganglion Cells in the ganglion cell layer

The most superficial layer of the retina known as the retinal ganglion cell layer (GCL) is mostly populated by ganglion cells (Figure 4) but also contains displaced amacrine cells, in a number that varies depending on the specie. Retinal ganglion cells (RGCs) are excitatory neurons that collect the visual information received by photoreceptors after being partially processed by the rest of the retinal cell types. Then, RGCs send visual information to the brain, where it will be later processed and integrated with the other sensory modalities.

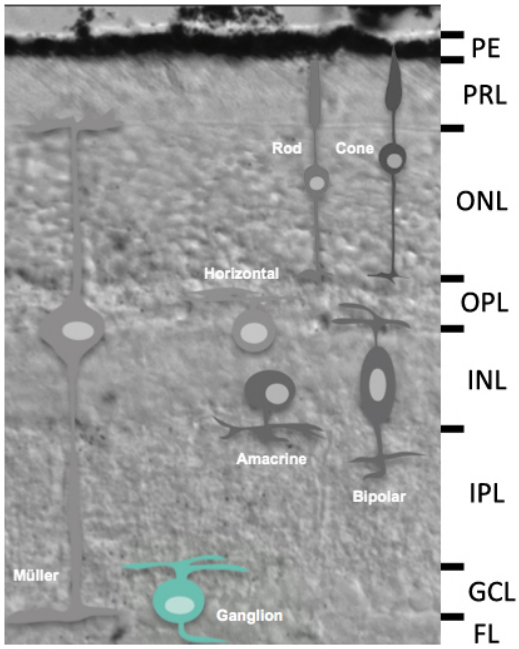

Figure 4: Retinal cell types in the GCL.

Ganglion cells reside in the ganglion cell layer (GCL). Their dendritic trees span the IPL, receiving information from both amacrine and bipolar cells. RGCs are the only output neurons of the retina: All the information perceived and processed in the retina exits it through the axons that RGCs extend in the fiber layer (FL) towards the optic disc (OD). Adapted from Zhang et al (2011).

Over the last 50 years, physiological, genetic and anatomical studies have described many particularities and features of RGCs and their function. RGCs typically exhibit important dendritic trees that cover large spaces forming both a centre and a surrounding representation of the visual field right above their cell bodies. RGCs dendrites collect relevant information from a large number of cones via bipolar and amacrine cells. RGCs involved in high acuity visual tasks usually have narrower dendritic trees.

There are about 20 different subtypes of RGCs (Masland, 2012), although the precise number depends on the specie. Each RGC type is highly specialized in specific visual modalities such as orientation and direction of motion (Oyster and Barlow, 1967), contrast (Cook and McReynolds, 1998), or even serve as atypical photoreceptors, detecting general levels of light (Hattar et al.,

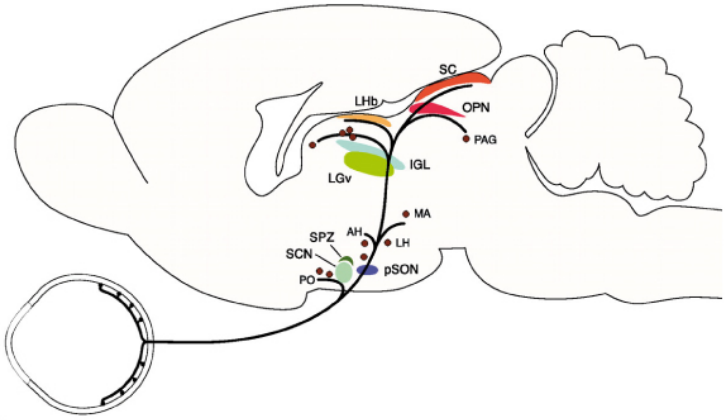

Figure 5: Targets of RGC axons.

A schema of the mouse brain in a sagittal view showing the regions innervated by RGCs. PO, preoptic area; SCN, suprachiasmatic nucleus; SPZ, subparaventricular zone; pSON, peri-supraoptic nucleus; AH, anterior hypothalamic nucleus; LH, lateral hypothalamus; MA, medial amygdaloid nucleus; LGv, ventral lateral geniculate nucleus; IGL, intergeniculate leaflet; BST, bed nucleus of the stria terminalis; LGd, dorsal lateral geniculate nucleus; LHb, lateral habenula; SC, superior colliculus; OPN, olivary pretectal nucleus; PAG, periaqueductal gray. Adapted from Hattar et al (2006).

2006). The list of features encoded by RGC subtypes is still ongoing. Despite this variety of functions, all RGCs hav e the common function of sending visual information to visual centres in the brain (Figure 5). More than a dozen visual nuclei have been described in the mammalian brain (Sefton et al., 2017). However, many details about the precise projection pathways of RGCs to these brain nuclei remain undefined (Wilks et al., 2013).

Early Retinal development

The eyes begin to develop as a pair of optic vesicles on each side of the forebrain. Following eye field formation, the neuroepithelium of the ventral forebrain invaginates resulting in the formation of bilateral optic vesicles. The distal portion of the vesicle makes contact with the overlying lens ectoderm to form the lens placode. This interaction results in invagination of the lens placode and distal optic vesicle leading to the formation of a bilayered optic cup.

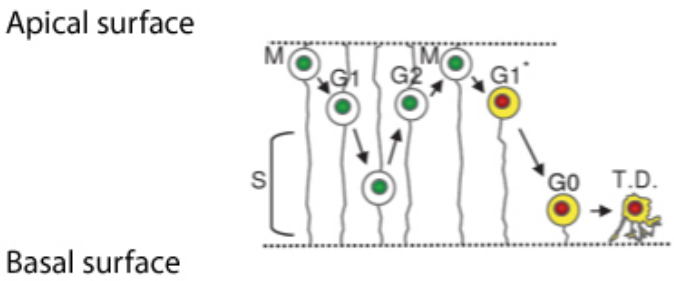

Figure 6: Neurogenesis in the retina.

The nucleus/cell body of Retinal Progenitor Cells (RPC) change its position as the cell progress through the cell cycle. This process is termed interkinetic nuclear migration (INM). Following cell birth at the apical surface, the apical process begins to retract as the new post-mitotic cell moves to its final destination and undergoes terminal differentiation (TD). Green and red nuclei depict whether the cell is dividing or post-mitotic respectively. Yellow cytoplasm indicates induction of a distinct transcriptome in the post-mitotic cells. The different parts of the cell cycle are annotated as mitosis (M), Gap 1 phase (G1), Synthesis phase (S), Gap 2 phase (G2), and Resting phase or Gap 0 (G0). Adapted from Bremner and Pacal, (2011).

Retinal neurogenesis begins in the inner layer of the optic cup, with cell cycle exit and cell-type determination followed by cell differentiation. In mouse, retinogenesis progresses in a wave-like manner that spreads from central areas to the peripheral retina (Malicki, 2004). A subset of neuroepithelial cells produces postmitotic neurons while the remaining cells keep dividing as retinal progenitors (RPC) (Figure 6).

RPCs are organized in a layering-like manner: RPCs in interphase are observed throughout the epithelium, while mitotic RPCs are only found at the apical surface. RPCs migrate into the developing retina in a process called interkinetic nuclear migration (INM) (Baye and Link, 2007). During retinal development, the nucleus of each RPC undergoes INM and migrates back and forth across the proliferative layer during the cell cycle. RPCs nuclei are first located at the basal surface. Then, as they progress through S-phase, move to the apical surface where mitosis will happen. After cytokinesis a daughter cell will be born. This recently differentiated neuron migrates back to its final position depending on the fate it has acquired. RPCs up and down migration in the retina is different than radial migration of cortical neurons because they do not need radial glia to migrate.

After separating from their cycling precursor, neuronal dispersion is highly controlled (Reese et al., 1995). Even if the molecular mechanisms responsible for this dispersion has not yet been elucidated, it is well documented that rods, Bipolar and Müller cells will remain spatially associated with the position of their precursor, creating a column within the same clonal identity through the retina. In contrast, cones, RGCs, amacrine and horizontal cells will undergo tangential migration, mixing up with neighbouring cells independently of the original position of their progenitor (Fekete et al., 1994). Each RPC undergoes several rounds of division migrating back and forth until its stemness capability is over. As RPCs continue to proliferate, retinal thickness increases and new postmitotic neurons are added to each layer.

Multipotency of RPCs and time frame of differentiation

The six different types of retinal neurons and the Muller glia are all generated from the same pool of pluripotent RPCs (Turner and Cepko, 1987; Wetts and Fraser, 1988). More than 40 different transcription factors have been implicated in RPCs differentiation (Figure 7 Left Panel) through different cascades of repression and promotion of each other. We will not explore in detail these processes, as it is not the main focus of this thesis, but the list of transcription factors implicated is large and still growing (for review(Bassett and Wallace, 2012)). RPCs generate different cell types by going through irreversible non-cell-autonomous competence states (Young, 1985). The first differentiating retinal neurons are the RGCs, then horizontals, cones and most amacrine cells. Finally, late born RPCs produce rods, bipolar cells and Müller glia (Rapaport et al., 2004) (Figure 7 Right Panel). Although RGCs are the first cell type starting to be generated, it is important to emphasize that there is considerable overlap in the production of retinal neurons at any given time

and that, birth order does not correlate with laminar position. This strongly suggests that some external factors bias RPCs toward a particular fate during development. The exact balance between both intrinsic and external cell fate determinant mechanisms is still unclear but many transcription factors have been reported as key cell-autonomous regulators of RPC cell fate. There are also many

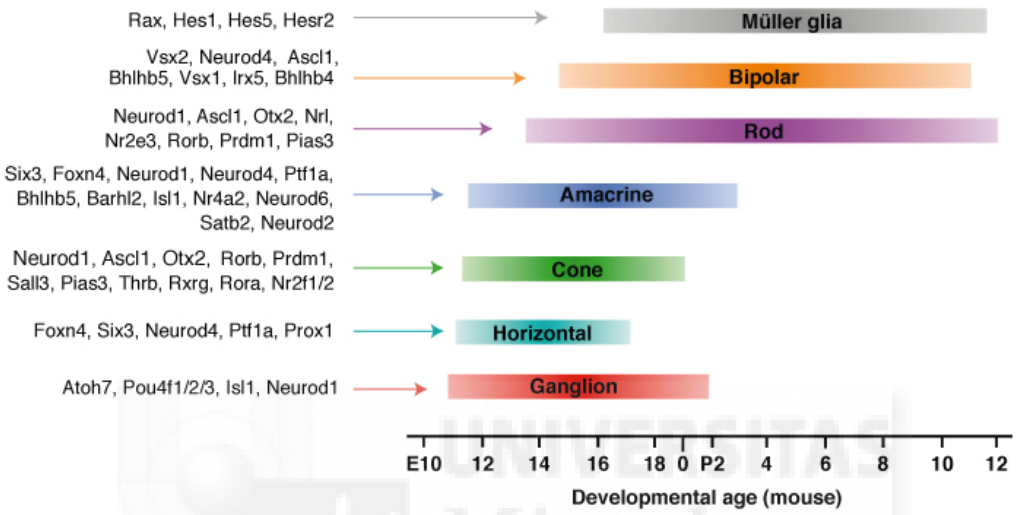

Figure 7: Balance between intrinsic and non-cell-autonomous factors, during determination of the different retinal cell fates.

Left panel: Some of the transcription factors reported as intrinsic regulators of RPC cell fate.
Right panel: Time course of cell genesis in the developing mouse retina. Retinal cell types are listed on the Y-axis, developmental time on the X-axis. Birth of the animal is indicated as 0, embryonic development is represented at the left of 0, postnatal development is at the right. The time course of cell genesis is indicated by the bar adjacent to each cell type. Adapted from Zhang et al (2011), based on Young RW, (1985).

other factors implicated in this process, including the dynamic behaviour of RPCs as they move through INM, orientation of the spindle during division, as well as cell-autonomous factors like contact inhibition of proliferation or secreted factors that can greatly influence cell fate specification (Livesey and Cepko, 2001).

Basic anatomy of the visual pathway

As we describe above, the image perceived by the retina is first transduced into electrical signals by photoreceptors and further processed by local circuits of neurons inside the retina. After this first filtering, visual information is sent to the brain by the only efferent cells of the retina, the RGCs. To this purpose RGC axons exit the retina through the optic disc and they continue growing to form the optic nerves. During their journey to the brain, RGC axons encounter a choice point at the optic chiasm level. There, some RGC axons cross the midline to project into the contralateral brain hemisphere while a variable number of visual fibers, which correlates with the degree of binocular vision and depends on the specie, avoid the midline and turn to finally project into the ipsilateral side of the brain with respect to the eye they come from. For instance, in humans, that have frontal eyes and therefore the visual fields overlap greatly, approximately 40% of RGC axons project ipsilaterally while the rest of them project to the opposite side (Prieur and Rebsam, 2017). However, in species with very poor binocular vision, such as mice, only around 3 to 5% of all RGCs axons project to the same hemisphere (Dräger and Olsen, 1980).

Once RGC axons leave the optic chiasm region they form the optic tracts, going deeper into the brain and looking for their targets. RGC axons project to a dozen of different nuclei in the brain (Sefton et al., 2017), but the two main targets implicated in vision are the dorsal lateral geniculate nucleus (dLGN) of the thalamus and the superior colliculus (SC).

The connections of RGCs with their targets are not randomly established. RGC axons coming from neighbour cells project to neighbouring regions in the target nuclei, creating a point-to-point representation of the retina in the target called retinotopic maps. Not all visual nuclei exhibit the same degree of topography. The SC is an example of a highly topographic target. In the SC, RGC axons from the nasal (N) retina project to the caudal (C) region, whereas RGCs in the temporal (T) retina project into rostral (R) areas. RGCs located into the dorso-ventral (D-V) axis of the retina map

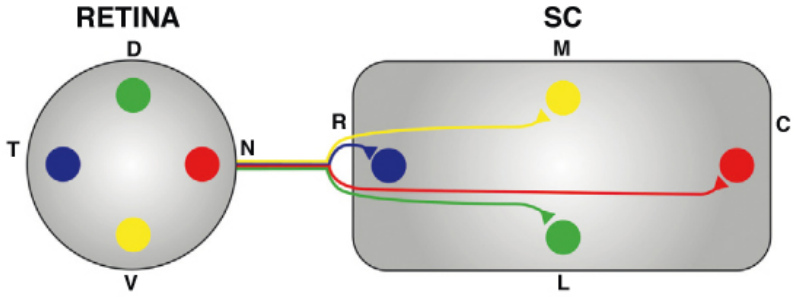

Figure 8: Organization of the retinocollicular map

The retina can be divided into the dorsal–ventral (D–V) and temporal–nasal (T–N) axes, which map along the lateral–medial (L–M) and rostro-caudal (R–C) axes of the SC respectively. As such, the spatial relationships of cell bodies in the retina are maintained in their terminations in the target. Adapted from Triplett JW (2014).

along the latero-medial (L-M) axis of the SC, with dorsal RGCs projecting into the lateral SC and ventral RGCs into the medial areas (Figure 8).

Molecular mechanisms controlling the formation of the visual pathway

The visual pathway is established during embryonic development, in a process in which RGC axons grow over long distances guided by a myriad of different molecular cues transitorily expressed in specific locations known as decision points. At the different choice-points RGC axons decide whether to turn one way or another, stop growing or branch out to reach multiple targets. The direction that each axon is going to take in a particular decision point is instructed by the expression of a set of molecular cues in the surrounding tissue that are sensed by a specific repertoire of receptors expressed at the membrane of the growth cone (the frontal part of the axon) in a particular moment. RGC axons are chemorepelled or chemoattracted by different guidance cues. Guidance cues may be short or long-range signals, depending on whether they are cell-bound or diffusible respectively.

Many different families of guidance cues, such as Netrins, Semaphorins, Slits or Ephrins, have been described as implicated in the formation of the visual pathway (Figure 9). When RGCs start to extend their axons, they express Robo receptors and are responsive to Slit signaling. Slit1 and Slit2 are present in the inner retina to prevent RGCs axon to grow inside the outer retina, ensuring that axons extend directly into the fiber layer (Thompson et al., 2006). Growth of RGC axons toward the optic disk is highly controlled: High-peripheral to low-central (P>C) gradients of Slit2 (Plump et al., 2002) and Chondroitin Sulfate Proteoglycans (CSPGs) (Brittis et al., 1992) are found in the developing retina together with a countergradient (C>P) of Sonic Hedgehog (Shh) help to drive axons towards the optic disk (Kolpak et al., 2005). At the OD, glia cells express Netrin-1, a guidance cue essential for the growth of retinal axons out of the eye (Deiner et al., 1997) (Figure 9 Eye Blowup). Once they exit the eye, RGCs axons are maintained into the optic nerve thanks to the action of repulsive molecules such as Slit2 and Sema5A (Plump et al., 2002). When RGCs axons reach the optic chiasm they are guided by diffusible Slit molecules that delineate a repulsion-free corridor to grow through. Axonal divergence between ipsilateral axons (green axons in Figure 9 Left panel and Chiasma blowup), coming from the ventrotemporal retina and contralateral axons (red axons in Figure 9) from the rest of t he retina, occurs at the midline because ipsilateral axons express the tyrosine kinase receptor EphB1, which expression is induced by the transcription factor Zic2 (Herrera et al., 2003). At the midline, EphB1-expressing axons are repelled by glial cells that express ephrin-B2 (Williams et al., 2003). As a consequence, ipsilateral axons turn about 90° to project to the same hemisphere. In contrast, contralateral axons do not express EphB1 and will ignore ephrin-B2. Contralateral RGCs express Neuropilin1 and therefore they will be attracted by VEGF-A also expressed at the midline (Erskine et al., 2011). Positive interactions between NrCAM and PlexinA1 on contralateral axons and PlexinA1 and NrCAM together with Sema6D at the midline also help to promote midline crossing (Erskine et al., 2011).

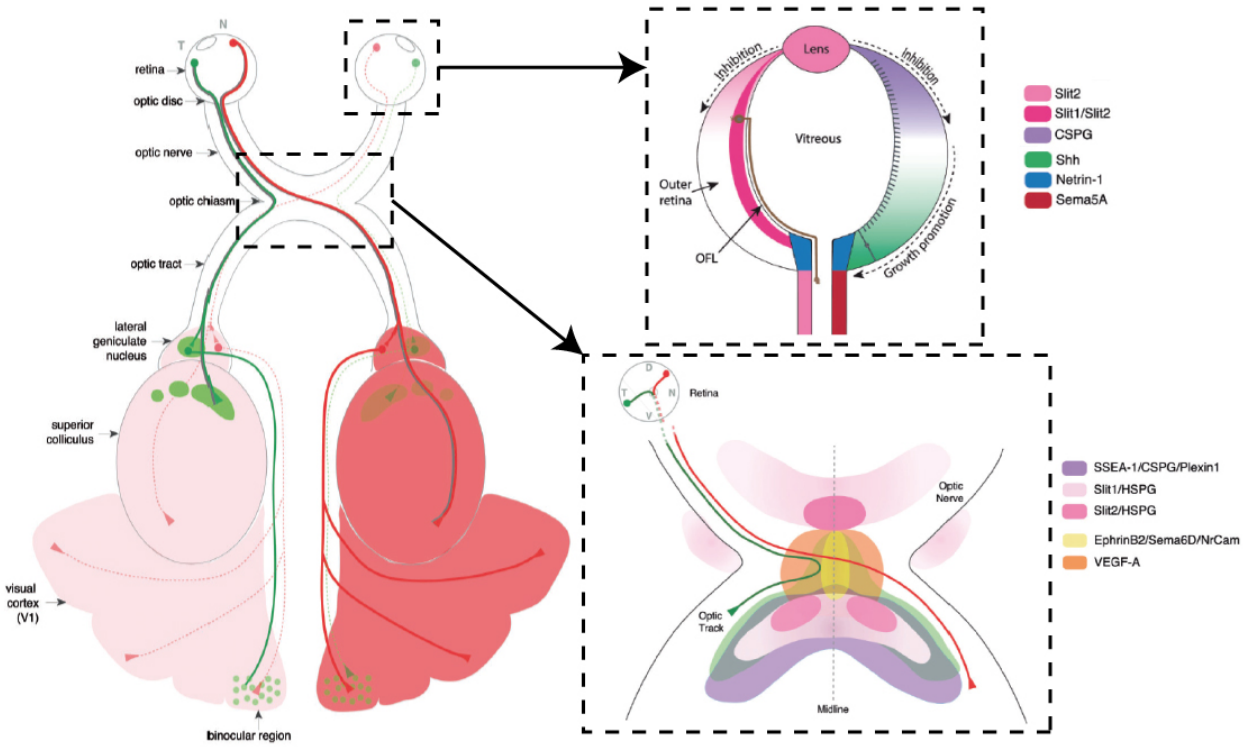

Figure 9: Schematic representation of the visual pathway in the mouse and main guidance families involved.

Left panel: RGC axons transverse the eye to exit the retina through the optic disk. The axons then travel via the optic nerves to the optic chiasm where they cross or avoid the midline to project ipsilaterally or contralaterally into the optic tracts towards the main visual targets: the lateral geniculate nucleus (LGN) in the thalamus and the superior colliculus (SC). Ipsilateral and contralateral projections follow distinct projection patterns at the visual nuclei. While ipsilateral axons form confined patches at the rostral LGN and SC (green), the contralateral terminals fill the rest of the tissue (red/pink).
Eye blowup: Expression pattern of key molecules that direct intraretinal RGC axon guidance. For clarity most molecules are shown only on one side of the retina but will be symmetrically distributed *in vivo*.
Chiasma blowup: Expression pattern of key molecules that direct RGC axon navigation at the mice optic chiasm. Dorsal (D), Nasal (N), Ventral (V), Temporal (T). Adapted from Erskine and Herrera (2014).

Once RGCs axons leave the optic chiasm, contralateral and ipsilateral fibers meet at the optic tracts. There, they will keep growing together forming a tight bundle. This fasciculation of axons has been shown to be orchestrated by adhesion molecule DSCAM (Bruce et al., 2017). Axons will keep growing ventrally along the surface of the diencephalon to finally reach the LGN in the thalamus, and the brachium of the colliculus. There, they will perform a 90° turn caudally toward the SC, a structure that they will finally invade. Netrin-1 is expressed in the dorsal diencephalon in an area that is non overlapping with, but adjacent to, growing retinal axons, suggesting that it

might act as a repellent cue to prevent RGC axons from leaving the optic tract (Shewan et al., 2002). In *Xenopus*, Sema3A, expressed along the boundary of the optic tract, has been proposed as a repulsive cue forcing RGC axons to turn caudally (Campbell et al., 2001). In rodent, interaction between Slit and Robo has been shown critical to prevent axon-pathfinding defects and defasciculation all along the optic tract (Ringstedt et al., 2000).

Finally, upon arrival to their targets, RGC axons establish connections according to a topographic map. The transmembrane proteins Ephs/ephrins are main players in the establishment of proper retinotopic maps at the visual targets.

Eph/ephrins

The Eph receptors are the largest family of tyrosine kinases (RTK) described in vertebrates. This family is integrated by at least 15 members, named after the erythropoietin-producing hepatocellular carcinoma (EPH carcinoma) where they were described for the first time (Hirai, 1987). Based on sequence homology and binding affinity there are two main types of Eph receptors, A and B (Gale et al., 1996). There are 9 class A receptors (EphA1 to -A8 and EphA10) and 6 class B receptors (EphB1 to -B6). The ligands for Eph receptors are the ephrins (Eph receptor Interacting proteins). Ephrins are also subdivided into A and B classes on the basis of sequence homology. There are 5 ligands for EphAs (ephrin-A1 to –A5) and 3 for EphBs (ephrin-B1 to –B3) (Figure 10 Left panel). In general, ephrin-A proteins bind to EphA receptors, while ephrin-Bs bind to EphB receptors. There are however some exceptions to this rule: For instance, EphB2 may bind to ephrin-A5 (Himanen et al., 2004) and EphA4 may bind to both ephrin-As and ephrin-Bs (Gale et al., 1996). Independently of the subfamily they belong to, all Eph receptors have a similar structure

(Figure 10 Right panel). They are transmembrane RTK proteins composed of an ephrin binding domain on the N-terminal region, which determines their affinity to ephrins, a Sushi and EGF like domain responsible for Eph/Eph binding, two fibronectin type II domains, a transmembrane domain, an intracellular kinase domain responsible for most of their signalling, a Sterile Alpha Motif (SAM) in charge of protein-protein interactions and finally, on the C-terminal they have a PDZ domain (Zisch and Pasquale, 1997). In contrast, the ephrins have structural differences depending on the family they belong to: ephrin-As are short membrane tethered protein composed by an extracellular Eph specific domain. Ephrin-Bs also feature a N-terminal Eph specific domain but are transmembrane proteins with a short GPI anchor on the C terminal side (Gale et al., 1996).

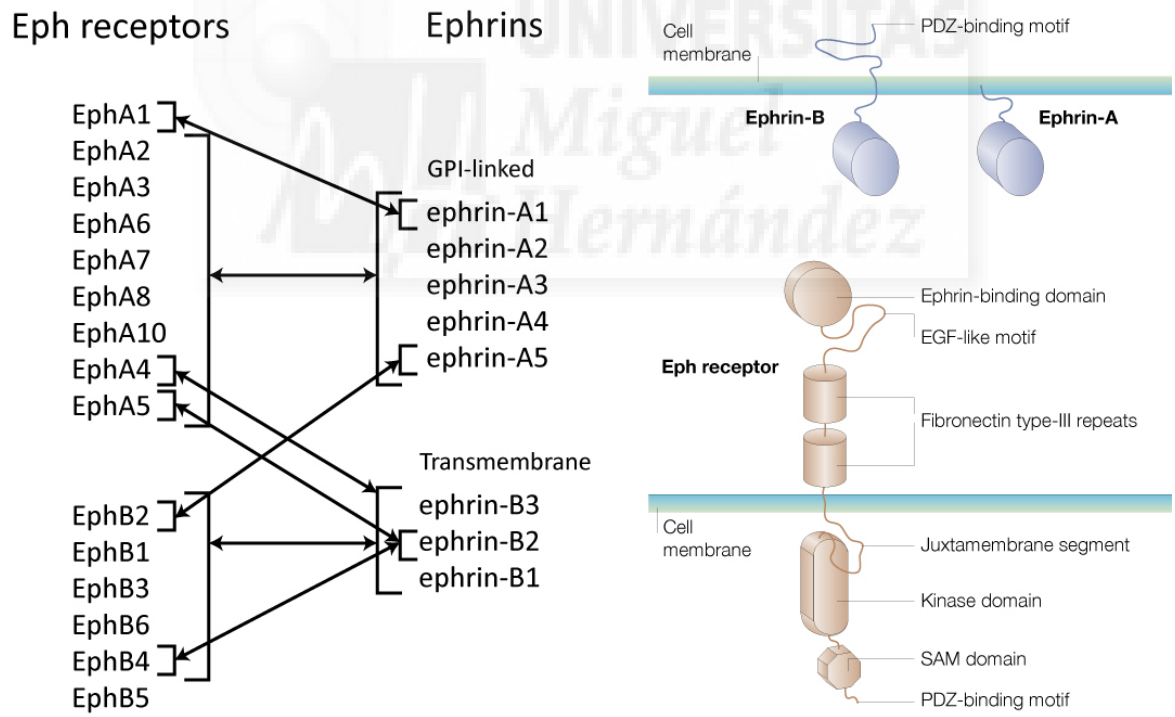

Figure 10: Structure and binding preferences of Eph and ephrins.

Left panel: Double-sided arrows, interactions between the specific Eph receptors and the respective interacting ephrins. GPI, glycosylphosphatidylinositol. Adapted from Wilkinson DG (2000).
Right panel: The EphA and EphB receptors have a conserved domain structure. The ephrin-A ligands are attached to the cell membrane by a GPI anchor. The ephrin-B ligands are transmembrane proteins. PDZ, post-synaptic density protein-95. Adapted from Pasquale EB (2005).

Upon detection of a ligand, Ephs change their configuration, allowing their SAM to interact with others Eph receptors starting to cluster together (Davis et al., 1994). This association will allow their own tyrosine kinase domains to phosphorylate each other. Under this new configuration, Eph receptors will be able to phosphorylate second messenger proteins, hence starting a signalling cascade. Activated Eph/ephrin complexes will tend to form even larger clusters by recruiting other Ephs, forming lipid rafts inside the membrane of the cell (Marquardt et al., 2005). These clusters can contain multiple types of Eph receptors, and may serve as an additional mechanism for crosstalk between –A and –B subclasses (Janes et al., 2011).

While all the members of both families are associated with cell membranes, the extracellular domain of the proteins can be cleaved by metalloproteases of the ADAM family (Janes et al., 2005). This cleavage is believed to be the main mechanism to terminate signalling of the

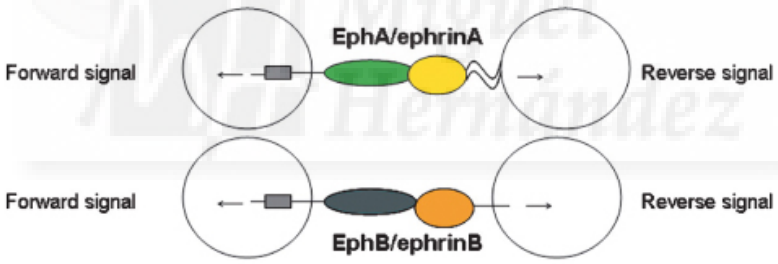

Figure 11: Bidirectional signaling through Ephs and ephrins.

Bidirectional signaling may occur between an Eph receptor-expressing cell and an ephrin-expressing cell. Forward signals are propagated into the Eph receptor-expressing cell, and reverse signals are propagated into the ephrin-expressing cell. Adapted from Pasquale (2003)

receptor and allow cell repulsion. Another mechanism important in Eph/ephrin signalling is the endocytosis of the entire Eph/ephrin complex. Endocytosis may happen either in the Eph expressing cell (Marston et al., 2003) or in the ephrin expressing cell (Zimmer et al., 2003) and it is essential to allow a repulsive response.

Another important issue about the Eph/ephrin mediated signalling is that ephrins are capable of acting as a receptor instead of as a ligand (Figure 11). The canonical signal transduction, called forward signalling, is mediated by Ephs proteins acting as receptors: upon contact with an ephrin, Ephs initiate an internal signalling cascade that leads to modifications in the cell they are expressed in. Ephrin/Eph reverse signalling has also been described. In that case, upon contact with an Eph protein, ephrins signalize intracellularly acting as a receptor (Holland et al., 1996). It was initially thought that Eph/ephrin interactions only elicited repulsion through forward signalling, however it has now been shown that both forward and reverse signalling are active during axon guidance, and it has been also proposed that some of these interactions may be attractive (Xu and Henkemeyer, 2011).

The role of Eph/ephrin signalling in the establishment of the visual topographic map

Topographic mapping is established in different steps. In mice this stepwise process takes place during the first postnatal week (Figure 12). In the early phase of topographic mapping, RGC axons grow deep inside the SC to reach the most caudal part. After this initial phase of expansion, interstitial branches start to form around the future termination zone (TZ). At the same time, the primary axon starts retracting towards its TZ (Simon and O'Leary, 1992).

After the establishment of a rough topography around the TZ, a step of refinement and remodelling of the axonal arborisation takes place. Axon terminals typically form side branches and arborize exuberantly in a region that includes, their correct TZ, then a rapid remodelling of the early

diffuse projections occurs finally restricting the arbours to the correct TZ (Nakamura and O'Leary, 1989). The profuse extension of axon terminals before the refinement process facilitates axons to explore the vicinity of their TZ in order to establish connections with the proper neighbouring cells. The refinement process involves both the development of arborisations at topographically correct TZ and the removal of branches from inappropriate positions. One week after birth the branching pattern at the TZ in the SC of mice begins to look similar to the ones observed at maturity. Further refinement leads to an adult like topographic ordering of axonal arborisations (Simon and O'Leary, 1992).

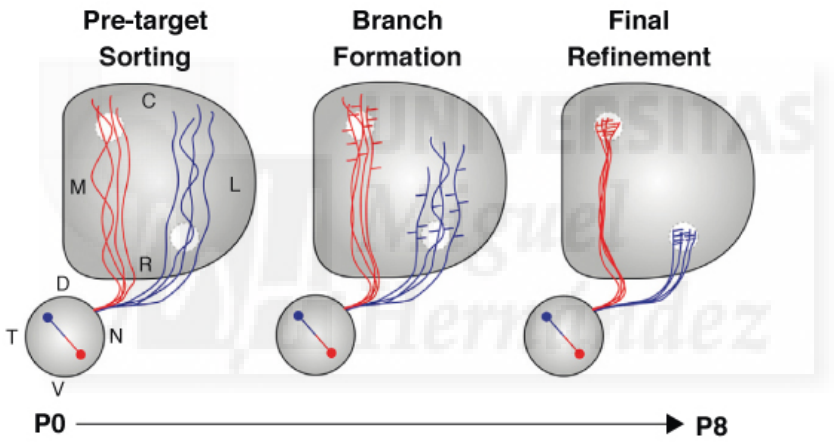

Figure 12: Developmental time course of retinocollicular map formation

Rather than projecting directly to their termination zones, retinal ganglion cells (RGCs) innervate the SC and refine to a final topographic map over the first postnatal week in the mouse. RGC axons are present in the SC at birth (postnatal day 0, P0), where they already display pre-target sorting along the L–M axis. Over the next week, RGCs extend interstitial branches in the area of their future termination zone (white circle), which are directed by molecular cues expressed in gradients along each axis of both the retina and SC. During the final stages, correlated activity patterns direct the final refinement of RGC branches into a tight termination zone. Adapted from Triplett JW, 2014.

As above stated, the establishment of the visual topographic map in the SC is mainly controlled by Eph/ephrin signalling. The function of these proteins in topographic maps is

conserved through evolution. In fact, their role in topographic mapping was described in chicken (Drescher et al., 1995) and in mammals (Cheng et al., 1995) during the same year.

The specific members of the Eph/ephrin family involved in topographic mapping vary between species, but the general principles mediating this process are highly conserved. In mice, RGCs express a high temporal to low nasal gradient (T>N) of EphA5 and –A6 while the SC expresses a high rostral to low caudal (R>C) opposing gradient of ephrin-A5 (Feldheim et al., 1998). In addition, EphB2 (Barbieri et al., 2001) and -B3 (Hindges et al., 2002) are expressed in a high ventral to low dorsal gradient (V>D) in the retina and ephrin-B1 and -B2 are expressed in a gradient along the medial-lateral (M>L) axis in the SC (Hindges et al., 2002)(Figure 13). For a complete overview of the different members of the Eph/ephrin family and their expression pattern in the developing retina, see the annex at the end of this study. In some cases, these protein gradients are counterbalanced by opposite gradients in the same tissue: in the mouse retina, ephrin-A5 is found in a high nasal to low temporal gradient (N>T)(Marcus et al., 1996), while ephrin-B1 and –B2 are expressed in a high dorsal to low ventral gradient (D>V)(Hindges et al., 2002). Similarly, in the SC gradients of ligands are countered by opposite gradients of receptors: EphBs are expressed in a high lateral to low medial gradient (L>M)(Hindges et al., 2002) and EphAs in a high rostral to low caudal fashion (R>C) (Feldheim et al., 1998). The precise function of these counter gradients is still highly debated, but the current consensus is that they help to shape the steepness of the other protein's gradient.

Manipulation of endogenous levels of both Ephs and ephrins is a classical tool to study the role of these proteins, but the existence of Eph and ephrins in the same tissue, the counter gradients, the redundancy between different members of the family and the complex signalling mechanisms of these molecules, complicates the interpretation of the results. Numerous studies have reported that genetic disruption of Ephs and/or ephrins in the mouse retinocollicular system

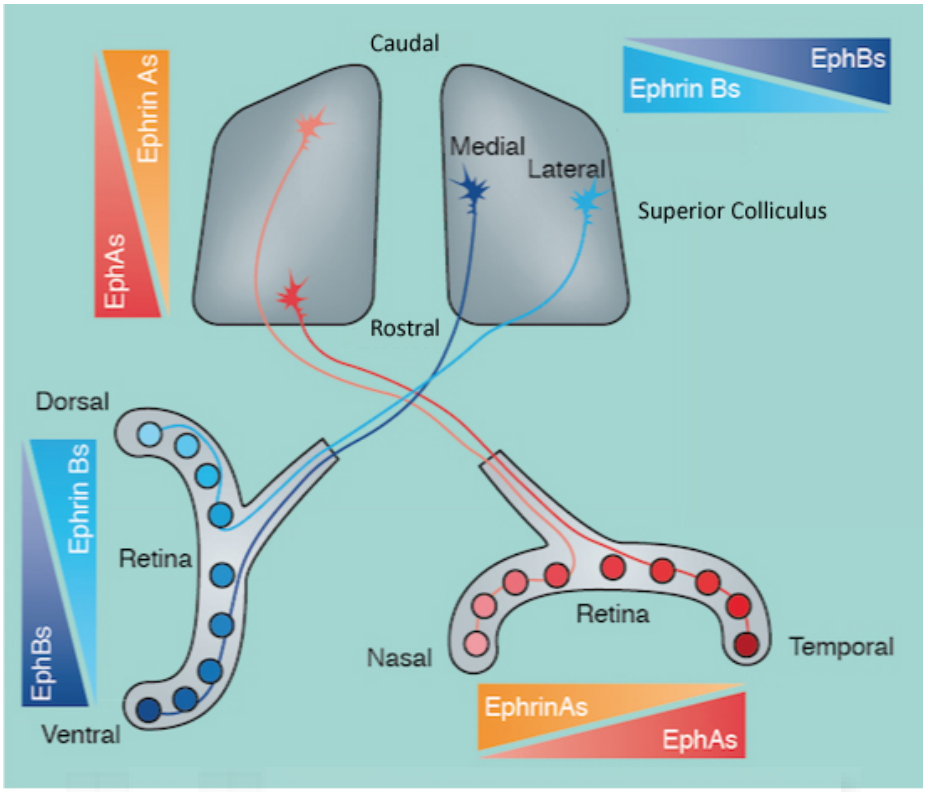

Figure 13: Eph/ephrin mediate the formation of the retinocollicular map.

Dual gradients of ephrins and countergradients of Eph receptors in the SC guide retinal axons. Axons with high levels of EphAs target regions with low levels of ephrin-As in the SC, consistent with repulsion. By contrast, the mediolateral map is determined by an ephrin-B gradient that can act as a repellent or attractant, depending on the ephrin-B abundance relative to EphB levels. Adapted from Klein R (2012).

leads to abnormal topographic mapping. Genetic deletion of EphA5 leads to a caudal shift of the axons in the SC (Feldheim, 2004), while ectopic expression of EphA3 in a retinal T>N gradient lead to a caudal shift (Brown et al., 2000). EphB2/B3KO mice exhibit an aberrant lateral shift of the axons in the SC (Hindges et al., 2002), similar to mice lacking EphB1 and –B2 (Thakar et al., 2011). Genetic deletions of ephrin-As, either ephrin-A5 mutant mice (Suetterlin and Drescher, 2014), ephrin-A2/A5 double mutant mice (Feldheim et al., 2000), or ephrin-A2/A3/A5 triple mutant mice (Pfeiffenberger et al., 2006), all result is a caudal shift of retinal axons in the SC, directly proportional to the number of protein eliminated. Also, ephrin-B1/B2 mutants have a lateral topographic error of retinal axons in the SC (Thakar et al., 2011). All those studies together demonstrate the crucial roles of Ephs and ephrins in visual map topography.

After establishment of a rough topography, the late phase of fine-tuning refinement during the formation of the visual circuit depends, among other mechanisms, on correlated spontaneous activity initiated at the retina. Prior to visual experience, the developing retina spontaneously generates retinal waves. Waves initiate in the ventrotemporal retina (Ackman et al., 2012) and from there, fire correlated bursts of action potentials propagate randomly across the retina (Feller et al., 1996). In mice, a certain type of wave (Stage II waves) emerges around the time of birth and coincides with retinotopic and eye-specific refinement. Those waves are driven by acetylcholine (Ach) released from starburst amacrine cells (Feller et al., 1996). The propagation of stage II waves allows them to relay information about the retinotopic relationship of RGCs to the SC in an activity-dependent manner (Butts, 2002). In mutant mice lacking the Beta2 Acetylcholine receptor (β2AChR), RGCs still exhibit spontaneous activity, but do not have Stage II waves. In these mutants, RGCs remain active but rather than firing in a correlated fashion, they produce spontaneous spikes of activity that are not correlated among neighbouring RGCs and do not propagate (Bansal et al., 2000). β2AChR KO mice exhibit a nearly normal topography but dramatically enlarged axonal arbors that fail to refine in the SC (Dhande et al., 2011). It has been debated for years whether spontaneous activity interferes with Eph/ephrin signalling during the establishment of the retinotopic map. Overexpression of the potassium channel Kir2.1 blocks activity (Burrone et al., 2002) and electroporation of this channel in the retina leads to a blockade of spontaneous activity, which affects axonal pruning of axon terminals once they are located in the correct TZ. However, it does not affect axon pathfinding, EphA/ephrin-A signalling or the location of the TZ. These observations suggest that retinal spontaneous activity is critical for refinement once axon terminals reach their correct topographic position independently of Epha6 signalling (Benjumeda et al., 2013).

Nowadays it is widely accepted that Ephs and ephrins play a critical role on the establishment of topographic visual maps in mammals. However, the function of one of those retinal Eph receptors, EphA4, is still poorly understood. Despite belonging to the Eph family, both EphA4 expression pattern and its ability to bind multiple ephrin-A/-Bs ligands, points to this receptor as an unlikely candidate to play a role in topography. This study aims at elucidating the role of EphA4 during the development of the mouse visual system.

MATERIALS AND METHODS

Mouse Lines

Most of the experiments involving electroporation have been performed on wild type mice. To ensure a stable genetic background we crossed two inbred mice lines, namely the C57BL/6J and the DBA/2J, to obtain a first generation of B6DBAF1/J hybrid line, from which we selected the females to back cross with C57BL/6 males, obtaining our line of wild type mice: B6B6F2 (later referred as B6 or WT).

We also used several transgenic mouse lines in this study, the EphA4$^{lox/lox}$ (later on EphA4cKO) kindly provided by Dr.Rüdiger Klein's lab at Max Planck Institut für Neurobiologie in Martinsried, Germany. In this line the third exon of the EphA4 gene has been flanked with LoxP sites, allowing us conditional silencing of EphA4 upon Cre Recombinase expression (Herrmann et al., 2010; Paixão et al., 2013). We also used the EphA4$^{-/-}$ (later on EphA4KO), also provided by Dr.Rüdiger Klein's lab. This line has been previously described (Kullander et al., 2001; Paixão et al., 2013). Briefly, this is a knock-in mouse line, where the third exon of the EphA4 gene has been disrupted by a targeted insertion, leading to the shortening of the mRNA product, which will be degraded before translation, leaving the cells unable of producing any EphA4 protein.

DNA plasmids

For all electroporations DNA plasmids were mixed with 1% Fast Green for easier visualization and diluted in TAE to obtain the desired concentration. pCAG is a commonly used mammalian expression vector, where the expression of the gene of interest is under the control of CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site (CAG), a synthetic strong general promoter (Niwa et al., 1991). Plasmids have been described previously: pCAG.eGFP (Garcia-Frigola et al., 2008), pCAG.EphA6 (Carreres et al., 2011a), pCAG.EphA4DC, pCAG.EphA4DSAM and pCAG.EphA4KDDSAM (Kullander et al., 2001), pCAG.EphA4 (gift from Joaquim Egea's Lab), pCAG.Cre (gift from Avihu Klar's lab). pCAG.ephrin-B1 and pCAG.ephrin-B2, have been design in the lab, by cloning the coding sequence of the gene of interest via PCR.

In utero electroporation

Timely pregnant B6DBAF1 female mice were taken at 13.5 days post fertilization (E13.5), deeply anaesthetised with isofluorane, placed face up under a binocular dissection microscope, after insuring the sterility of the area by cleaning the abdominal fur with ethanol 70%, a 2cm surgical incision was performed along the midline of the abdominal skin, starting approximately 2mm above the vagina. Skin was then carefully separated from the muscle, and another incision was performed along the linea alba, taking care not to damage any of the inferior epigastric artery ramifications. Both horns of the uterus were then carefully taken out of the abdominal cavity allowing us to orientate the embryos for retinal injection of plasmidic DNA. Borosilicate glass pipette, previously pulled to obtain elongated glass needles, were filled with 5uL of plasmidic DNA mix and mounted on a rubber mouth pipetting tube. The desired volume of DNA mix was then injected by mouth

pipetting inside the eye of the embryo, ideally between the neural retina and the retinal pigment epithelium, the fast green in the mix allowing us to visually confirm the injection site. Electroporation was performed with a CUY21edit current generator (Nepa Genes) by delivering 5 electrical pulses of 45V for 50ms each, through the uterus walls, by placing a 5mm diameter positive electrode on the side of the injected eye, and the negative one on the other side of the head. After electroporating all the embryos, the uterus was carefully put back in the abdomen cavity, muscles and skin stitched up, and the mother was allowed to recover on a 37ºC incubator before been put back in its cage with food and water ad libitum.

Electroporation of single RGC were performed in a similar way, however, pups were not electroporated in utero but 12 to 24h after birth (P0.5). Animals were anaesthetized on ice for 5 minutes. The eyes were opened by surgical incision of the eyelid. Since eyes were bigger than at E13.5, injection of plasmid DNA was performed using an autoinjector (Tritech Research) allowing us a better control of volumes injected in the retinal region of interest. Electroporation was performed using the same protocol. Eyelids were then closed back in place and covered with ophthalmologic cream facilitating healing of the wound. Pups were allowed to recovers on a 37ºC incubator before being put back in their cage with their mother.

Tissue preparation

After electroporation pups were allowed to develop postnatally for 10 (P10) or 20 days (P20), and were then perfused with a solution of paraformaldehyde 4%. Organs of interest were carefully removed, post-fixed by immersion in PFA 4% over night at 4ºC with constant agitation. After post-fixation, retinas and brains were sectioned into 50um section with a Leica vibratome before being processed for immunofluorescence. For *in situ* hybridization, organs were cryo-protected and cut

into 15um sections using a cryostat. Some organs were not sectioned to analyse in whole mount preparation, as describe in the immunostaining section.

***In situ* hybridization**

This protocol was used on both vibratome and cryostat processed tissue, mounted on pre-treated glass slides (SuperForst plus Extra, ThermoFisher): After air drying the tissue for 1 hour at 37ºC, slides were circled with an hydrophobic marker and placed in vertical incubation boxes and post-fixed with 4% PFA in PBS for 10 minutes (all steps were performed at room temperature unless specified otherwise.) Then, slides were washed 3 times in PBS for 3 minutes. Then they were incubated for 5 minutes in a Proteinase K solution in order to permeabilize and neutralize any enzymatic activity in the tissue (See below for details on solutions). A second incubation of 5 minutes in 4% PFA was necessary to inactivate the proteinase K. Then, slide were washed 3 times 5 minutes in PBS and transferred to horizontal incubation boxes soaked with Wash Solution to start the pre-hybridization phase. 300uL of previously heated Hybridization solution was added onto each slide and let to pre-hybridize at 62° C for 1 hour in the oven. Riboprobes were diluted at 10ug/mL in hybridization solution, denatured at 85°C for 5 minutes and quickly quenched on ice for 5 minutes, in order to assure linearization. After pre-hybridization, the solution onto the slides was replaced with 400uL of probe solution and hybridized at 62° C overnight. The incubation box was sealed to prevent evaporation during this process. On the next day slides were taken out of the horizontal incubation box and placed back in a coupling for washing the non-specifically hybridized probe. 5 washes of 30 minutes each were performed at 62°C with warm Wash Solution. Then the slides were transfer back to a horizontal incubation box soaked with water at room temperature for the immune detection steps. 300uL of Blocking Solution was added onto the slides to block any

non-specific reactivity with the primary antibody, during 40 minutes, then it was discarded and replaced by an Anti-Digoxigenin Alkaline Phosphatase conjugated antibody (ROCHE) solution at 1:3500. The slides were then place for antibody incubation over night at 4°C. Once again the incubation box was sealed to avoid evaporation during the night. On the third day the antibody solution was discarded, the slides put back in a Coplin jar, washed 10 times with MABT solution for 5 minutes each, to ensure the complete elimination of any free antibody. Then the slides were incubated in freshly made NTMT solution 2 times, 10 minutes to buffer the pH of the tissue. The coupling was protected from light with folding paper before starting the revelation phase. The slides were then incubated with Reaction Solution until the ratio expression level to noise was judged acceptable. The reaction time can vary greatly from probe to probe due to difference in endogenous expression but we empirically determined that in the case of our EphA4 probe the best reaction time was 9 hours. When the reaction was considered done, the slides were washed twice in NTMT for 10 minutes, then in MABT for another 10 minutes, and finally 10 more minutes in 4% PFA solution to neutralize any more enzymatic reaction. The slides were then processed for immunohistochemistry if needed, or directly mounted with cover slips in Mowiol and then stock for later microscopy. Sequences of primers used for the EphA4 riboprobe are GGGCCACTGAGCAAGAAA and RGCCTGGACCAAAGCAATG (Forward and Reverse respectively) and have already been published (Escalante et al., 2013).

***In situ* hybridisation solutions composition:**

ISH ProteinaseK Solution:	1% ProteinaseK 10mg/mL ; 3% Tween20 in PBS. Prepare on ice to avoid denaturation of the enzyme.
ISH 10x Salts Stock:	11,4g NaCl ; 1,4g TrisHCl ; 0,134g TrisBase ; 7,8g NaH2PO4x2H2O ; 7,1g Na2HPO4 ; 60mL dH20. Adjust pH to 7,5 and complete to 100mL with dH2O. Stock at room temperature.
ISH Hybridization Buffer:	0,45g Dextran Sulfate ; 2,25mL Dionized Formamide ; 450uL 10x Salts Solution; 120uL tRNA 25mg/mL ; 90uL 50x Derhardt's ; 1,59mL dH2O. Volumes adapted for about 5 slides.
ISH Wash Solution:	5mL 20x SCC pH7 ; 100mL Formamide ; 1mL Tween20 ; 94mL dH2O.
ISH 5x MAB:	23,2g Maleic Acid ; 15,2g NaOH ; 17,2g NaCl ; 250mL dH2O. Adjust pH to 7,5 and complete to 400mL with dH2O. Volume adapted for about 50 slides. Stock at room temperature.
ISH MABT:	40mL 5x MAB; 0,2mL Tween20 ; 160mL dH2O.
ISH Blocking Solution:	3,6mL MABT; 450uL Sheep Serum ; 450uL Blocking Reagent.
ISH NTMT:	3mL 5M NaCl ; 15mL 1M TRIS HCl ph9,5 ; 7,5mL 1M MgCl2 ; 0,75mL Tween20 ; 123,75mL dH2O. Always prepare fresh.
ISH Reaction Mix:	0,45uL/mL NBT (Roche); 3,4uL/mL BCIP (Roche); in fresh NTMT.

Immunostaining

After slicing and mounting on glass slides for cryostat-processed samples, or directly after slicing in floating condition, for vibratome-processed samples, all tissues were blocked for one hour at room temperature using PBS with 0,5% tween 20 and 10% gelatine. Then the samples were washed in

PBS and let to incubate over night in a primary antibody solution made of PBS with tween 0,5% and 10% gelatine under constant rocking at 4ºC. On the next day, primary antibody solution was recycled for later use, then the samples were wash in PBS before being incubated with the relevant secondary antibodies for 2 hours at room temperature with constant rocking. Samples were then washed in PBS containing 0,01M DAPI for nuclear counterstaining, before being mounted onto glass slide with Mowiol and let to dry over night before being analysed.

Whole retina were too thick to allow a good antibody penetration so, for flat mounted preparation we had to adapt our protocol as following: Retinas were permeabilized in PBS with 0,5% TritonX for half an hour at room temperature with rocking, then froze at -80°C for 15 minutes, thawed, and incubated in PBS with 2% TritonX at 4°C over night with constant rocking. The next day samples were incubated with the antibody of interest in a solution of PBS TrintonX 2% and BSA 2%, first on hour at room temperature, then 24 hours at 4°C under constant rocking. Then the antibody solution was discarded, the samples washed in PBS TritonX 2% and incubated with the relevant secondary antibody for 2 hours at room temperature. Then washed again five times, 30 minutes each, and finally flat mounted onto microscope glass slide with mowiol for later analysis.

Brain wholemount immunodetection was performed at P10. After fixation with PFA 4% brain were washed in PBS and dissected in order to obtain the smallest piece possible containing both the SC, as well as the IC and the most rostral part of the cerebellum for orientation. Those samples were then dehydrated by immersion into Methanol solutions of increasing concentrations (25% Methanol in PBS, 50% in H2O, 80% in H2O, 100% Methanol), and then bleached with 3% H_2O_2 solution for one hour, before being gradually rehydrated (80% Methanol in PBS, 50% in PBS, 25% in PBS, and finally PBS). The samples were then blocked for one hour in a PBS solution containing 5% BSA and 1% Tween20, before being incubated 48 hours at 4°C with constant rocking in a solution of PBS with 1% BSA, 1% Tween and anti-GFP antibody. After 48 hours the brains were washed 5 times,

one hour each, in PBS Tween 1% at 4°C with rocking, before being incubated for another 48 hours with the relevant secondary antibody. After incubation the samples were washed again five times, one hour each in a PBS 1% Tween solution before being analysed.

Antibody	Reference	Concentration
GFP	Aves Lab, #GFP-1020	1:2000
EphA4	Santa Cruz, #SC921	1:10
Pan Axonal Marker	Covance, #SMI-312	1:1000
ChAT	ATS, #AB-N3AP	1:1000
E-Cadherin	BD, #610181	1:1000
N-Cadherin	BD, #610920	1:500
NCAM	DSHB, #5A5	1:500
L1CAM	Millipore, #MAB5272	1:1000
Calbindin D-28K	Swant, #CB38A	1:2000
Islet1/2	DSHB, #39.4D5	1:500
Chicken Alexa 488	Invitrogen, #A11039	1:1000
Mouse Alexa 546	Invitrogen, #A11003	1:1000
Rabbit Alexa 546	Invitrogen, #A11010	1:1000
Mouse Alexa 647	Invitrogen, #A31571	1:1000
Rabbit Alexa 647	Invitrogen, #A21244	1:1000
Rat Alexa 633	Invitrogen, #A21094	1:500
Rabbit Alexa 405	Invitrogen, #A31556	1:100

***In vitro* culture of retinal explants**

Cultures were performed on poly-lysine pre-treated glass cover slips that we coated with laminin for better explant adhesion (ThermoFisher). E14.5 electroporated retina were quickly extracted and

dissected into small square pieces of about half a millimetre length, of either dorsal or ventral retina. Explants were mounted onto round pretreated cover slips and allowed to grow at 37°C for 24 hours. Quantification of axons entering the ephrin-B1 containing zone was performed based on a method previously described ((Herrera et al., 2003)): The total area covered by axons was quantified using ImageJ analysis software, calculating the total fluorescence of axons immunostained with a pan-axonal antibody. To normalize for the variability in distance that explants settled from the border, we drew a circle, with the radius from the centre of the explant extending to the border, just up to where axons crossed or avoided the border, and did not include any axons or cell bodies within this circle. The fluorescence outside this circle was defined as the fluorescence of total axons. In the second step only fluorescence of axons inside the ephrin-B1 region was measured. The percentage of axons entering the ephrin-B1 zone (as pixels of fluorescence) was then calculated as the fluorescence of axons in the ephrin-B1 zone over fluorescence of total axons.

Imaging

Sections were imaged with a fluorescent Leica DM2500M microscope setup with a DFC350FX Leica camera, or with Zeiss LSM880 Confocal microscope. Whole mount preparations of retinas were also imaged with Olympus FV1000 Confocal, while brain whole mounts were capture using a Leica MZ10F microscope with DFC7000T Leica camera. All raw images processing and quantification were performed with SCV software or ImageJ software for Mac.

RESULTS

EphA4 is homogenously expressed in the RGC layer at perinatal stages

To investigate the function of EphA4 in the development of the visual system we started by carefully analysing its spatiotemporal expression pattern by *in situ* hybridization. We checked the expression of *EphA4 mRNA* in mouse retinas from embryonic day 14.5 (E14) to adult stages using a specific probe against EphA4 previously generated in our laboratory (Escalante et al., 2013).

At early stages *EphA4 mRNA* was not detected in the ganglion cells layer (GCL) (Empty arrowheads in Figure 14). EphA4 expression in the GCL was first detected around E18, and maintained until at

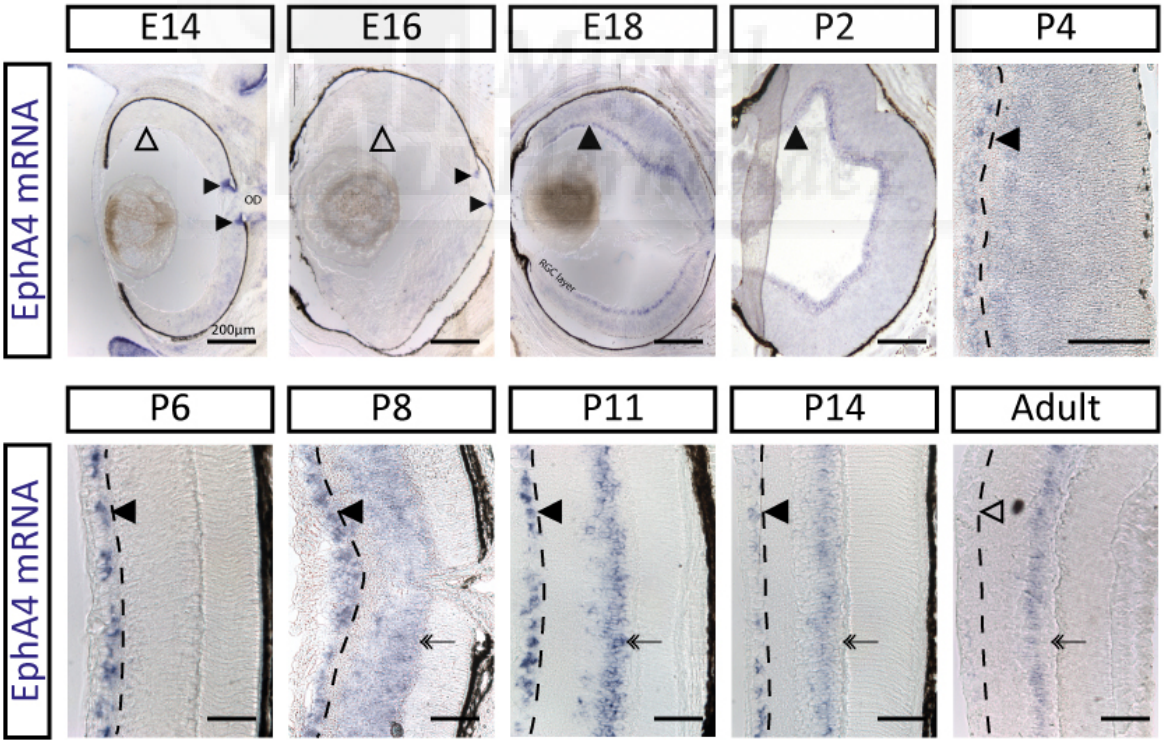

Figure 14: Spatiotemporal expression of EphA4 in the developing retina.

In situ hybridization for EphA4 mRNA in coronal retinal sections from wild-type mice shows three distinct patterns of expression along time: In the optic disc (OD) from E14 to E18, in the RGC layer from E18 to P14 (Solid arrowheads); and in the ONL from P8 through adulthood (Double arrows). The spatiotemporal pattern of EphA4 mRNA in the GC layer does not match those of others EphA receptors in the retina, because it is not expressed in any appreciable gradient, or at the ages previously described for other Ephs.

least P11, but it was completely gone in adult retinas (Solid arrowheads in Figure 14). It has been previously reported that EphA5 and EphA6 are expressed in a gradient in the mouse retina (Carreres et al., 2011b). However, we found that EphA4 is homogenously expressed in the RGC layer throughout the entire retina. Another notable difference with the expression of other EphAs in the retina, is that EphA4 expression starts around E17, while expression of other EphAs is detected as earlier as E13.5 (Diaz et al., 2003; Reber et al., 2004). We also confirmed previous reports describing the expression of *EphA4 mRNA* in astrocyte progenitors located at the optic disc area from E14 to E18 (Petros et al., 2006) and in Müller cells (Joly et al., 2014).

EphA4 is required for the correct arborization of axon terminals at the targets

Since EphA4 is highly and homogeneously expressed in RGCs, we decided to analyse the projections of RGCs in EphA4 mutant mice in order to understand the role of this receptor in the visual system. To visualize a putative phenotype of RGC axons all along the visual pathway we electroporated plasmids encoding the fluorescence protein GFP (pCAG-GFP) alone or pCAG-GFP plus plasmids encoding for the Cre recombinase (pCAG-Cre) into the retinas of EphA4 conditional mutant mice (EphA4cKO) (Paixão et al., 2013) and control E13.5 embryos and analysed the visual system of electroporated mice at P30. This approach allows the visualization of both cell bodies and axons of targeted RGCs that after the action of the Cre recombinase have lost EphA4.

EphA4cKO mice electroporated with pCAG-Cre and pCAG-GFP (Figure 15E and 15A) showed no obvious phenotype in the retina compared with the controls. RGCs and amacrine cells were evenly distributed in their correct respective layers. Dendritic arborisations in the IPL of conditional mutant mice electroporated with pCAG-Cre and pCAG-GFP did not show any differences compared

to those electroporated with pCAG-GFP alone. Some photoreceptors were also targeted, but no differences were detected either between the electroporated with pCAG-Cre plasmids and the control conditions. In addition, retinal thickness was similar in both conditions. Overall, the selective loss of EphA4 at E13.5 did not produce obvious retinal disorganization in P30 mice.

We then analysed the projections of targeted RGCs into the brain of EphA4cKO mice. As GFP-expressing axons, axons lacking EphA4 correctly reached the dorsal part of the lateral geniculate nucleus in the thalamus (dLGN). Axon fasciculation was maintained all along the path and no differences inside the dLGN were detected between the arborisations of RGCs lacking EphA4 and the controls (Figure 15D and 15H). In the superior colliculus (SC) arborisations of electroporated cells were also similar in both conditions. We did not observe topographic mistakes in the axons of GFP or Cre/GFP expressing RGCs along the R-C (Figure 15B and 15F) or the M-L axis of the SC (Figure 15C and 15G). As the controls, axons lacking EphA4 were found navigating through the deep

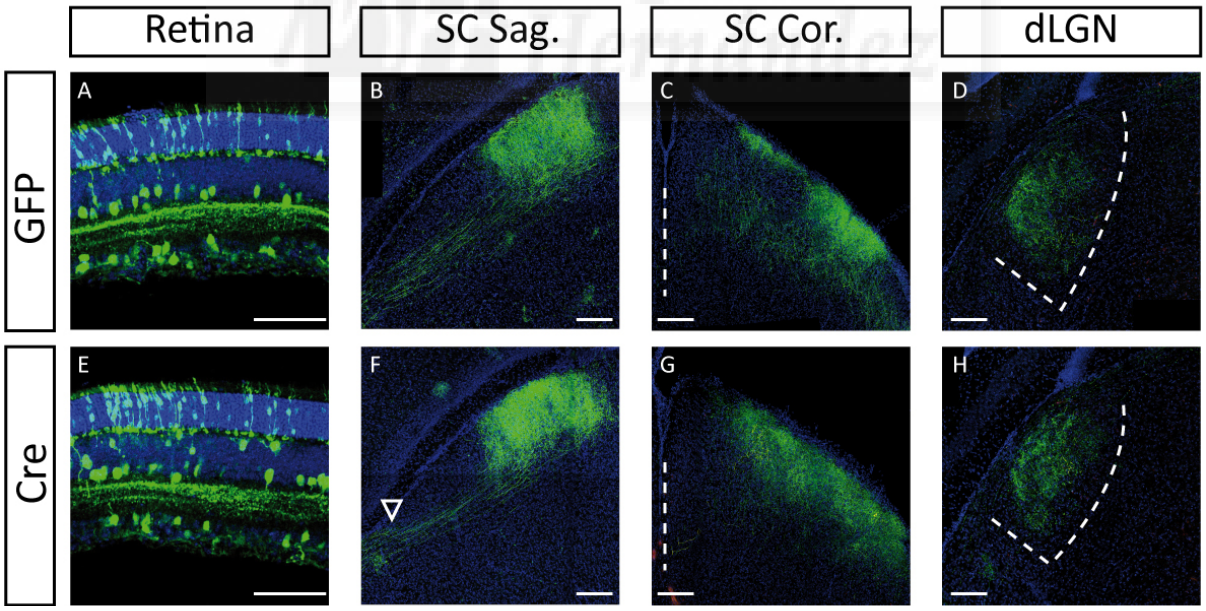

Figure 15: EphA4 conditional KO mice do not present any detectable phenotype at the retina.

A and E, P30 retinal sections, electroporated at E13 with pCAG-GFP or pCAG-Cre respectively show no significant differences. **B and F,** sagittal sections through the SC of electroporated mice reveal no gross alterations in the axon guidance of rostro-caudal projections at P30. Rostral is left**. C and G,** coronal sections of electroporated mice at P30 show no significant alteration of the medio-lateral guidance. The dotted line indicates the midline. **D and H,** coronal sections through the dLGN of electroporated mice show that axon guidance is not affected by the absence of EphA4. The dotted line indicates the contour of the nucleus. Scale bars, in A and E, 200µm, in B and F, 250µm, in C and G, 150µm, in D and H, 100µm.

layers before arborizing into superficial layers (Figure 15G).

Therefore, the electroporation of pCAG-GFP and pCAG-Cre in conditional EphA4 mice demonstrated that EphA4 is not necessary for the guidance of RGCs axons along the visual path or for the general topographical mapping of axon terminals along the R-C or L-M axis at the visual targets. In utero electroporation of pCAG-GFP into the retinas of EphA4cKO embryos at E13.5 led to a massive number of GFP-labeled RGCs making it difficult to detect potential phenotypes in the arborisations of individual RGC terminals at the targets. However, visualisation of single RGCs arbors at the visual targets is possible by performing single cell electroporation, which is possible if electroporation is performed between P0 and P1. As we have demonstrated, EphA4 is highly express in the GC layer just before birth. Therefore, single cell electroporation of pCAG-Cre plasmids in EphA4cKO mice would not be convenient, as EphA4 might have started its function a day before removal. For this reason, we decided to analyse the arbors of individual RGCs in EphA4 full KO mice instead of using EphA4cKOs. EphA4KO mice lack EphA4 expression from oocytes stage. They are viable but have a severe locomotor phenotype, preventing them from walking properly (see rear paws in Figure 16A). Although this line was generated more than 15 years ago (Kullander

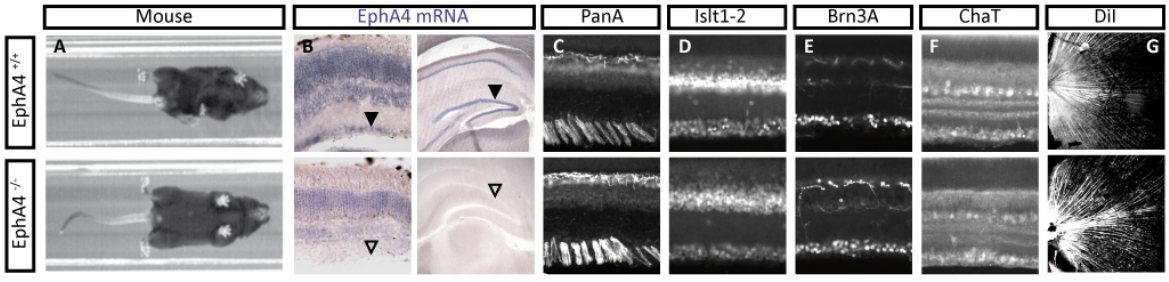

Figure 16: EphA4 is not essential for proper retinal development.

A. Mice walking on a treadmill. As previously described, EphA4KO mice present a heavy locomotor phenotype. They are not able to walk alternating their hindlimbs, instead they show synchronization of the rear paws, leading to a "hoping" locomotor phenotype. **B.** *EphA4 mRNA* is absent from the RGC layer in the adult retina of the KO (empty arrowheads) compared to the WT (Solid Arrowheads). Hippocampus is shown as control. **C.** PanA immunostaining in retinal sections shows that intraretinal RGC axons are not affected in EphA4KO mice. **D, E.** IHC of Islet1/2 and Brn3a in retinal sections show no alteration in the gross number, or layering of RGCs. **F.** IHC of Chat in retinal sections labels amacrine cells and show no gross difference in the IPL layering, where RGC dendrites arborize. **G.** Intraretinal RGC axons in whole mount retrogradely labeled from the optic nerve show no differences between EphA4KO and the WT mice.

et al., 2001), the visual system of these mice has never been reported.

First we confirmed, by *in situ* hybridization, that this mouse line lacks EphA4 expression in RGCs (Figure 16B). Then, we analysed the morphology of RGC axons into the retina by visualization of whole mount retinas previously labelled by retrograde DiI tracings applied at the optic nerve level (Figure 16G). In addition, we also performed immunolabelling with the axonal marker PanA in coronal retinal sections (Figure 16C). No differences were found between the axons of retinas of EphA4KO mice and the control littermates. To further investigate a potential role of EphA4 in the retina we analysed the retinal cytoarchitecture by checking the expression of specific markers for RGCs (Brn3a and Islt1-2) and amacrine cells (ChAT). Labelling of Brn3a and Ist1/2 (Figure 16D and 16E) revealed no misallocation of RGCs outside their layer. ChAT labelling did not show defects in the IPL where RGC extend their dendrites or in the INL (Figure 16F). Taken together these results suggested that the general morphology of the adult retina in the absence of EphA4 is not altered.
Once we analysed the retinas of EphA4KO mice and did not detect defects in RGC proliferation, differentiation or stratification, we decided to analyse RGC axon terminals in the SC of EphA4 and control mice. Retinas of both EphA4KO and control littermates were electroporated with pCAG-GFP at P0 and axonal arborisations in the SC were analysed 10 days later in whole mount immunostained brains (See Material and Method and Figure 17A).

GFP labelled axons coming from both the ventral and the dorsal retina reached their correct target inside the SC by P10 in EphA4KO and in control littermates, confirming again that EphA4 is not necessary for axon growth, guidance or to reach the correct terminal zone (TZ) (Figure 17B). However, despite targeting the correct TZ, we observed that axonal arborisations occupied a statistically significant wider area in the SC of EphA4KO than in control mice (Figure 17B and 17C;

p=0,0407). Individual WT axons arborized in a restricted TZ, with very few branches extending away. In contrast, EphA4KO axons showed a significant large spanning of their collaterals (Arrowheads in Figure 17B). All together these results revealed that EphA4 does not participate in RGCs axon guidance to the targets but instead, is involved in fine-tuning refinement once axons have found the correct TZ in the SC.

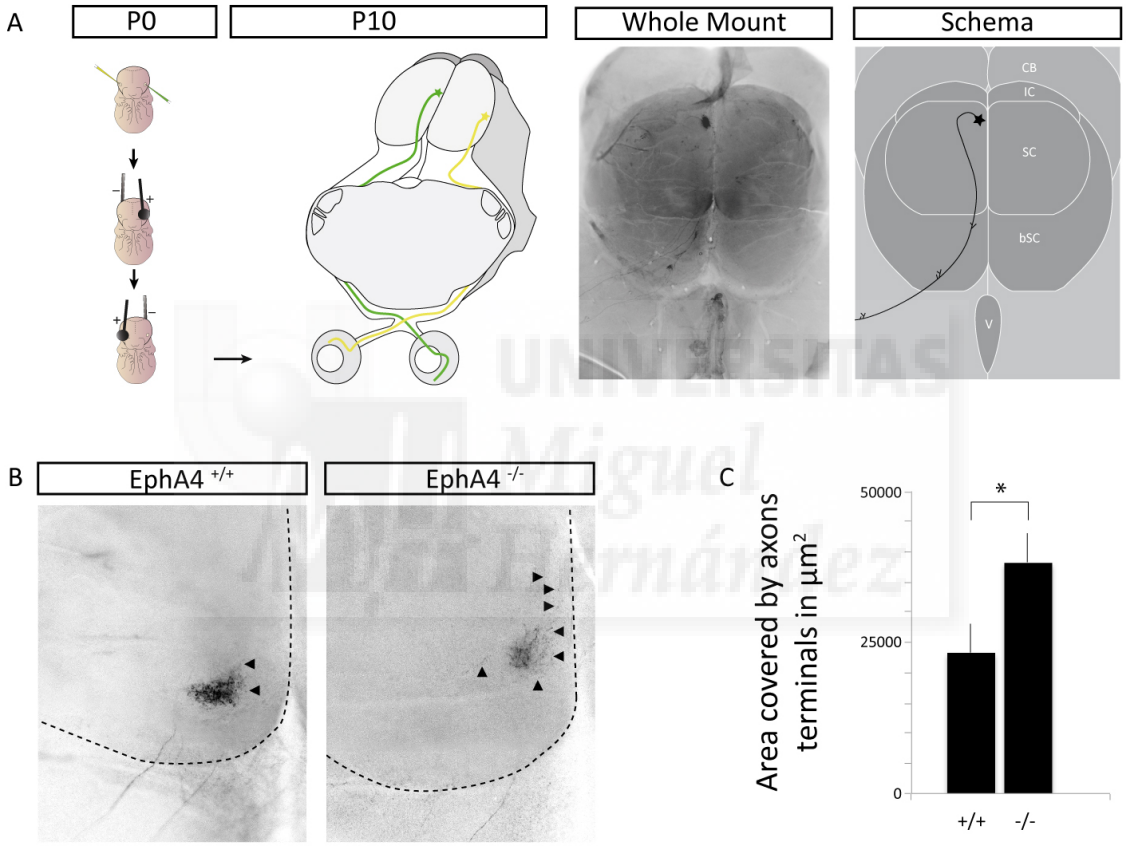

Figure 17: EphA4 KO mice show larger axons arborisations in the SC.

A. Experimental design. P0 pups were electroporated in both eyes, either dorsally or ventrally to obtain single cell labeling. Pups were allowed to grow until P10, age at which retinal axons have reached their TZ in the SC. Whole mount preparations of whole P10 SC were obtained and analyzed. A schematic representation of the SC is provided for clarity purpose. Cerebellum (CB), Inferior Colliculus (IC), Superior Colliculus (SC), brachium of the SC (bSC), Ventricle (V). **B.** Representative example of a single cell arborisation in the SC. In WT mice arbors are restricted to a well-defined zone, with very few ramifications out of it. In contrast, a single cell arborisation in the EphA4KO mice covers a larger space, and produce more extended ramifications (black arrowheads). **C.** Quantification of area covered by axonal arborisations in μm^2. Axonal arborisations of individual RGCs in the EphA4KO mouse show a significant difference compared to those of WT animals. N=12 for each group. p value < 0,05 as asserted by ANOVA.

Ectopic expression of EphA4 but not other Eph receptors induces adhesion

EphA5 and EphA6 have been shown to be responsible for the establishment of the R-C topography in the SC. However, both spatiotemporal expression pattern and loss of function experiments suggest a different role for EphA4. To further explore the role of EphA4 in visual system development, we manipulated the levels of EphA4 in RGCs by a gain of function approach. E13.5 WT embryos were electroporated with plasmids encoding for EphA4 (pCAG-EphA4) or for EphA6 (pCAG-EphA6) as a positive control because it is known that EphA6 drastically affects topography. These plasmids were co-electroporated with pCAG-GFP for visualization, and pCAG-GFP alone was used as control. Arborisations of targeted RGCs were then analysed in the SC of P10 mice.

As expected, electroporation of GFP encoding plasmids in the central retina of WT embryos consistently led to arborisations in the central part of the SC (Figure 18A). As previously reported, electroporation of pCAG-EphA6 in the same conditions resulted in a massive shift of RGCs axons to the rostral part of the SC (Figure 18B and (Carreres et al., 2011a)) confirming that overexpression of EphA6 is sufficient to change the topographic map. However, electroporation of pCAG-EphA4 in the central retina did not lead to such massive shift in R-C topography (Compare arrowheads in Figure 18B and 18C). Although a small number of axons showed a rostral shift indicating that, when expressed at very high levels, EphA4 might be able to affect mapping (Brackets in Figure 18C), most of the EphA4-overexpresing axons reached the correct topographical TZ in the centre of the SC.

These gain of function results once again confirmed that EphA4 does not play a major role in the establishment of topographic mapping along the R-C axis of the SC. In contrast to the phenotype observed in EphA4KO mice in which RGCs axons occupied a wider area than the

controls, EphA4-overexpressing axon terminals were adhered to each other forming patches (Compare Figure 18A and 18C).

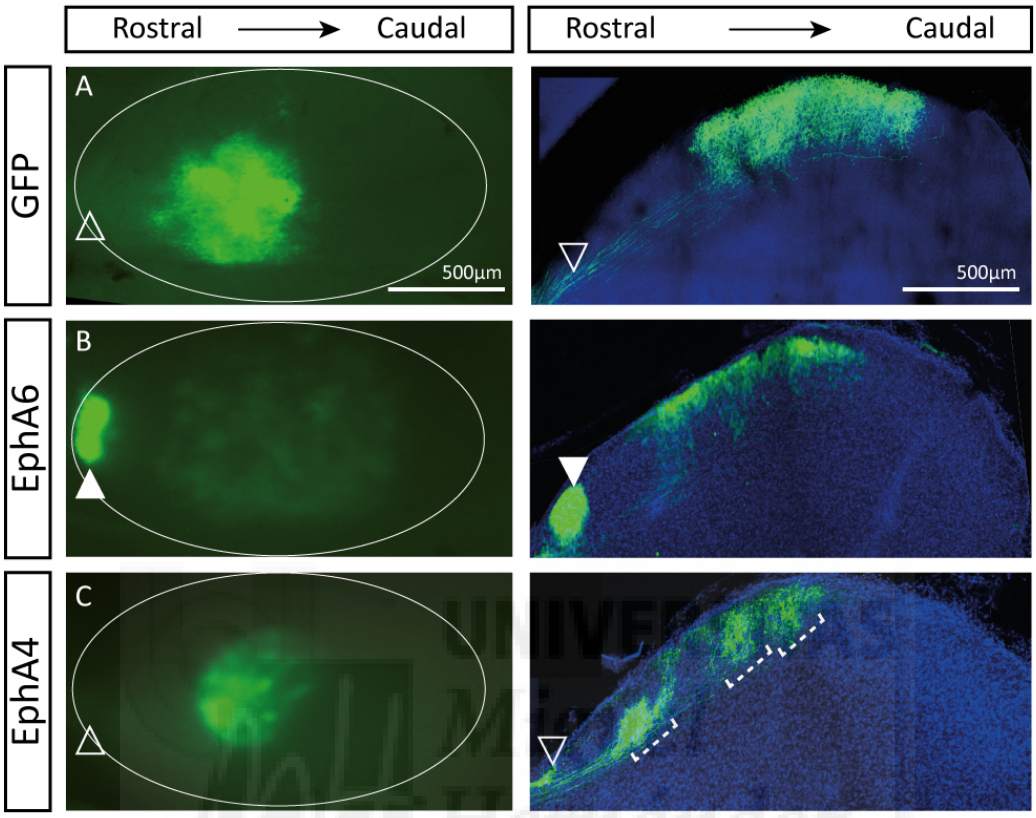

Figure 18: Ectopic activation of EphA4 does not shift the topographic map of RGCs but produces patches of axonal arborizations at the SC

Top view of the entire SC (left) and sagittal sections of the SC (right) from P10 mice electroporated at E13 with pCAG-GFP (**A**), pCAG-EphA6 (**B**) or pCAG-EphA4 (**C**). While ectopic expression of EphA6 produces a rostral shift the of RGC projections, EphA4 does not induce a massive rostral shift of the axons (Empty arrowheads). In contrast, EphA4-expression axons formed patches in medial locations of the SC (Brackets).

A similar adhesion phenotype was also found in retinas of pCAG-EphA4 electroporated mice. Retinas electroporated with pCAG-EphA4, pCAG-EphA6, pCAG-EphB1 or pCAG-GFP were analysed in whole mount and in coronal sections (Figure 19). Retinas electroporated with pCAG-EphA6 or pCAG-EphB1 showed an even distribution of targeted cells both in sections and in wholemount retinas (Figure 19A, 19C and 19E). Retinal sections also showed that targeted cells were correctly distributed in their corresponding layers: RGC in the GCL, and amacrine cells both in the GCL and in the INL (Figure 19B, 19D, and 19F). However, overexpression of EphA4 resulted in a massive

alteration of cellular distribution into the retina (Figure 19G and 19H). Laminar organization was not altered but the distribution of the cells into each particular layer was not homogenous (Figure 19H). Electroporated cells in the GLC and the IPL were grouped leaving non-electroporated cells in between patches of EphA4 overexpressing cells (Brackets in Figure 19H). In flat mounted preparations, cells appeared aggregated forming nearly equidistant ectopic patches (Figure 19G and 19G'). This result indicated that, despite being members of the same receptor family, retinal EphA4 is not implicated in the same biological processes than its relatives EphA6 and EphB1.

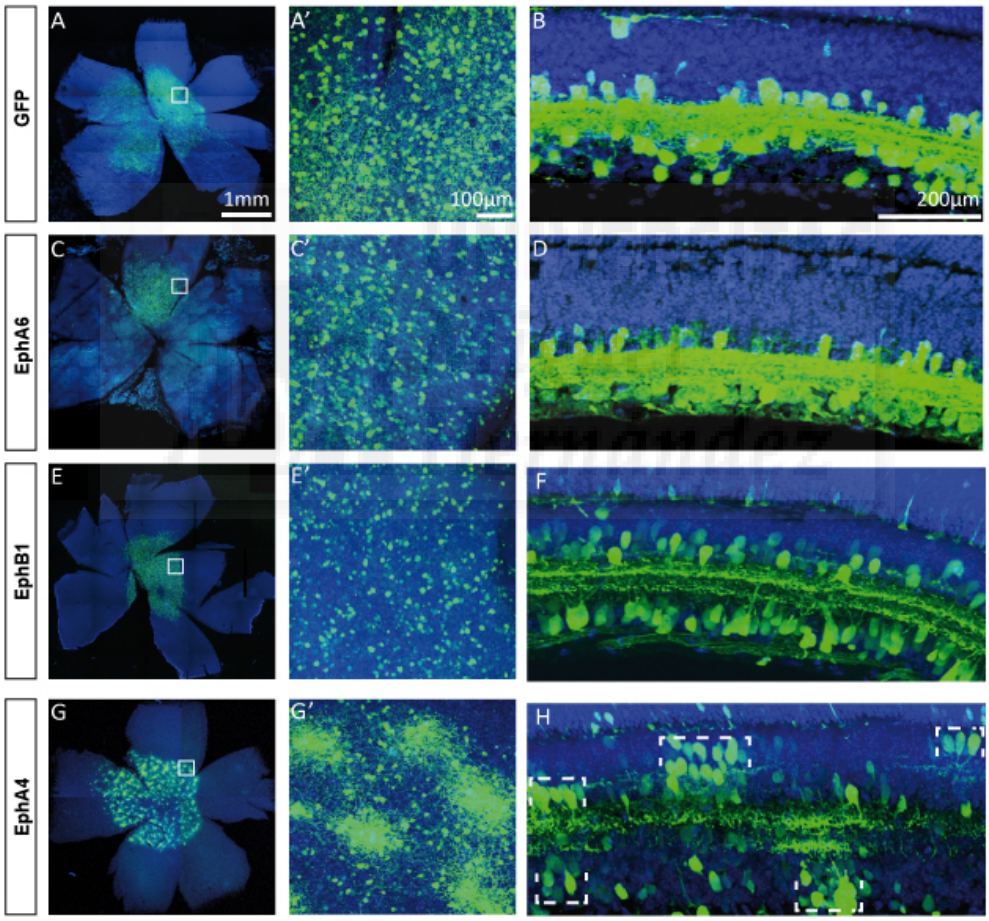

Figure 19: Ectopic activation of EphA4 disturbs retinal organization.

A, C, E, G. Flat mounted views of P10 retinas, electroporated at E13 with the plasmids indicated in each case. **A', C', E', G'**. Magnifications of squared areas in A, C, E and G respectively. **B, D, F, H**. Retinal sections through the zone of the retina electroporated with the indicated plasmid. Electroporation of pCAG-GFP, pCAG-EphA6 and pCAG-EphB1 does not disturb the cellular architecture of the retina. Overexpression of EphA4 induces ectopic patches leading to cell reorganization (Brackets).

In deconvoluted confocal pictures of flat-mounted electroporated retinas we measured mean fluorescence intensity in the different layers in electroporated retinas (Figure 20) and observed that overexpression of EphA4 induces ectopic patches throughout all the retinal layers (Green zone of EphA4 in Figure 20C), affecting cells independently of their cell type. This cell type independent phenotype suggests an adhesion mechanism, rather than an axon guidance phenomenon. As electroporation of pCAG-GFP shows, retinal neurons do not endogenously form patches in WT retinas.

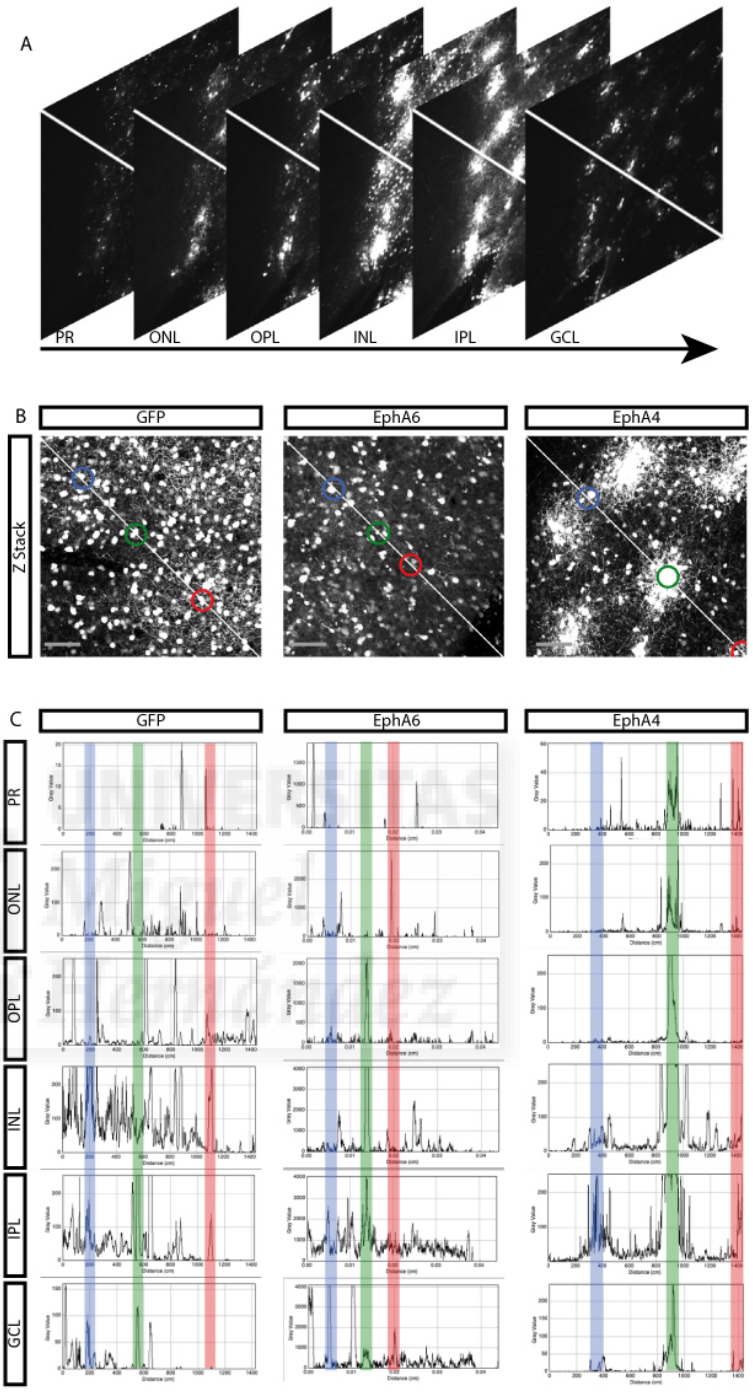

Figure 20: Quantification of ectopic patches produced by EphA4 through the different retinal layers.

A. Example of deconvolution of confocal stacked images of flat mounted retinas. Stacks were deconvoluted to extract 6 single plane images each at the level of a different retinal layer. **B.** Confocal stacked picture of flat mounted retinas electroporated with the indicated plasmids. Quantification of mean fluorescence intensity was performed along an arbitrary axis (white diagonal). Circles of colors are indicating the corresponding zones on single planes analysis in part C. Scale bars, 100µm. **C.** Individual plane quantification of mean fluorescence intensity in AU, across the diagonal line in B. No particular organization is observed under electroporation of pCAG-GFP or pCAG-EphA6, while accumulation of cells through most retinal layers can be observed in retinas with EphA4-induced patches (Green and Red zone in EphA4) and very little fluorescence was observed in-between patches, except in the IPL (Blue zone in EphA4).

These results indicate that EphA4, but not EphA6 or EphB1, might induce cell adhesion. They also support our previous conclusions that EphA4 plays a different role than the other Eph receptors expressed in the retina.

The EphA4- adhesive phenotype is induced by forward signalling

Multiple studies have demonstrated that tyrosine kinase proteins have ligand independent kinase activity when overexpressed (Inaki et al., 2012); (Runeberg-Roos et al., 2007); (Tanaka et al., 2003). Thus, it is likely that electroporated-EphA4 is already active and signalizes independently of any ligand. Self-activation of the receptor would induce forward signalling, resulting in the observed adhesive phenotype. However, it is also possible that the phenotype observed is the result of reverse signalling, induced by ectopic EphA4, in ephrin-expressing surrounding neurons. To clarify this issue and the further understanding of the mechanism by which EphA4 induces cell adhesion, we electroporated a

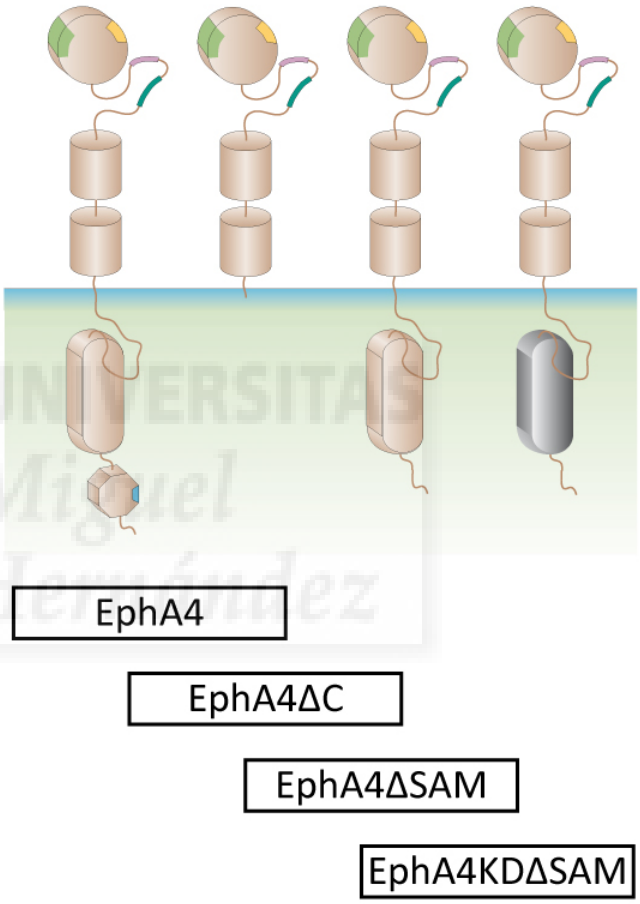

Figure 21: EphA4 structure in the cell membrane.

EphA4's forward signaling is mediate by the cytosolic part of the protein, which is composed of a juxtamembrane region, a Kinase domain, a Sterile Alpha Motif (SAM) and a PDZ-binding motif. To dissect the role of the different EphA4 domains, we used various mutant versions of this receptor: EphA4ΔC lacks the entire C-terminal domain. EphA4ΔSAM lacks the SAM domain. The EphA4KDΔSAM form lacks the SAM domain and the ability to phosphorylate its substrates. Inactivation of the kinase domain through a point mutation does not induce conformational changes in the Kinase Domain (grey domain).

mutated version of EphA4 lacking the cytoplasmic domain (EphA4ΔC) (Wang et al., 2011) (Figure 21). EphA4ΔC is a truncated form of the receptor that retains its ability to bind the ligand outside the cell but, because it lacks the cytosolic domain, does not convey information through forward signalling.

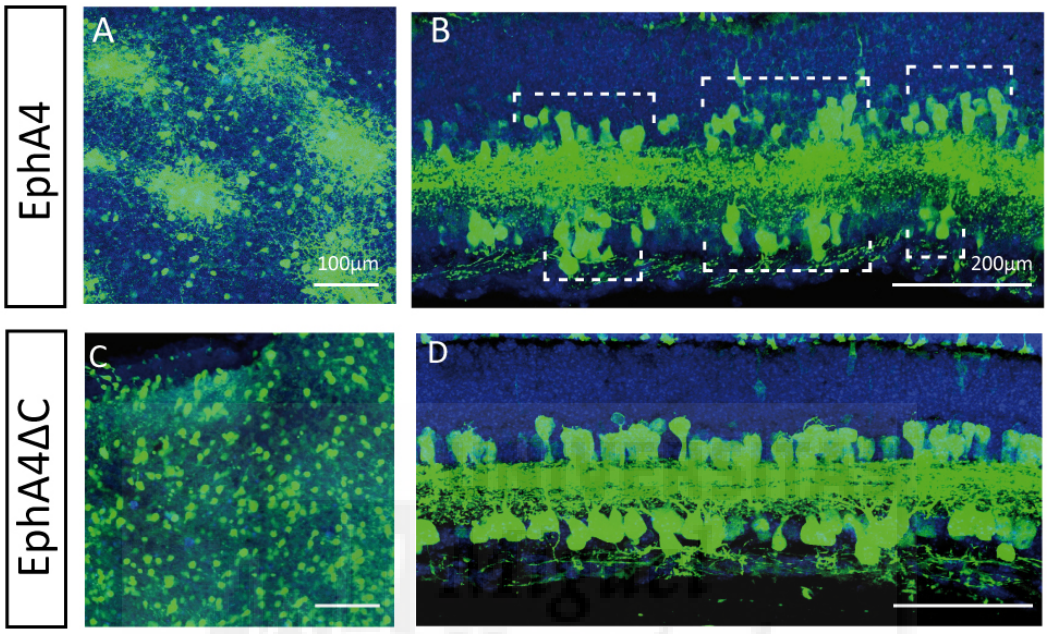

Figure 22: EphA4 forward signaling is necessary to induce ectopic patches.

A, B. Flat mounted views and retinal sections of P10 retinas electroporated at E13.5 with pCAG-EphA4 and pCAG-GFP. Electroporation of pCAG-EphA4 induces a phenotype of adhesion. **C, D**. Flat mounted views and retinal sections of retinas electroporated with pCAG-EphA4ΔC. EphA4ΔC is not able to form patches, showing that forward signaling is necessary to induce the phenotype.

The phenotype observed after electroporation of this mutated form of EphA4 was similar to electroporation of pCAG-GFP, showing no disturbances in the organization of retinal neurons when observed in whole mount (Figure 22C) or in sections (Figure 22D). Therefore, EphA4 needs its cytoplasmic domain to generate cell adhesion, confirming that forward signalling is required for this phenotype to occur.

To further investigate the region of the cytoplasmic domain responsible to generate adhesion, we electroporated two additional mutant forms of EphA4 (Figure 21):

- A mutated form of EphA4 lacking the Sterile Alpha Motif (EphA4ΔSAM) (Kullander et al., 2001). Disruption of this domain is known to prevent the protein from forming complexes with other Eph proteins and/or with mRNA. Retinas electroporated with EphA4ΔSAM form patches in a similar manner to those observed after electroporation of EphA4 full length (Figure 23A and 23B). However, the dendritic arborisations were less dense. When analysed in sections (Figure 23B) patches were evident in both the INL and the GCL (brackets) like the ones formed after electroporation of EphA4 full length. This result indicates that the SAM domain of EphA4 is not necessary for the patchy phenotype to happen.

- A double mutant form lacking both the SAM domain (ΔSAM) and the ability to phosphorylate its substrate (Kinase Dead, KD). In this construct, a point mutation in the sequence of the Kinase domain was induced, changing the lysine residue K653 in the small lobe of the kinase domain to a methionine which results in a full-length kinase domain with all the conformational properties but unable to phosphorylate any substrate.

Electroporation of EphA4KDΔSAM did not induce ectopic patches of retinal cells as clearly as EphA4 or EphA4ΔSAM (Figure 23C and 23D) indicating that EphA4 kinase activity is necessary for this adhesive phenotype to occur.

Taken together, these results demonstrate the involvement of the cytosolic domain of EphA4 in the adhesive phenotype observed. Moreover, the results also demonstrate that EphA4 mediate an adhesive response through forward signalling in a manner independent of the SAM domain but dependent on the classical kinase activity.

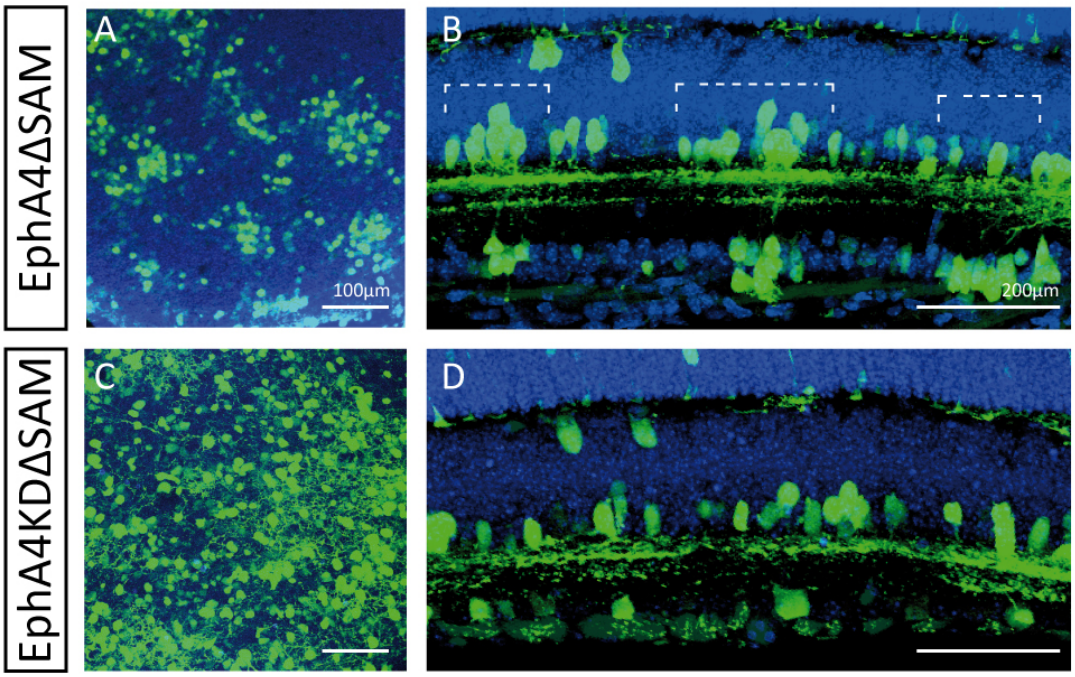

Figure 23: EphA4 kinase domain is necessary to induce ectopic patches.

A, C. Flat mounted views of P10 retinas electroporated at E13.5 with the indicated plasmid. **B, D**. Retinal sections through the electroporated zone for each plasmid. EphA4ΔSAM electroporation leads to a phenotype similar to that observed after ectopic expression of EphA4. It leads to a reorganization of both RGCs and amacrine cells, forming ectopic patches (brackets). However, EphA4KDΔSAM does not induce patches. Inactivation of the kinase activity prevents the formation of ectopic patches.

EphA4-induced cell adhesion is mediated by ephrin-B1 but not by ephrin-B2

Ephrin-B1 and ephrin-B2 have both been reported as ligands for EphA4 (Gale et al., 1996; North et al., 2009). Some evidences indicate that binding of ephrin-B2 to EphA4 mediates repulsion (Hu et al., 2014). However, although it has been assumed that EphA4/ephrin-B1 binding mediates repulsion, the cellular response generated by the binding of these two molecules has not been as much investigated. To test the possibility that EphA4/ephrin-Bs binding mediates adhesion instead of repulsion, we electroporated plasmids encoding for ephrin-B1 or -B2 co-electroporated with pCAG-GFP into the retinas of E13.5 mouse embryos and analysed the retinas at P10 (Figure 24).

Electroporation of ephrin-B2 did not produce any evident retinal phenotype (Figure 24C and 24D). However, ephrin-B1 electroporation partially reproduced the phenotype observed after electroporation of EphA4. Electroporated cells were not distributed evenly across the electroporated zone but agglomerate in ectopic patches as observed in whole mount retinas (Figure 24A). However, the patches were less well defined than in retinas electroporated with full length EphA4 (Figure 22A). Retinal sections analysis revealed that this discordance between these two similar phenotypes is located in the INL (Figure 24B): Electroporation of ephrins-B1 did not have any effect on the amacrine cells layer (Brackets in Figure 24B). However, ephrin-B1-expressing cells formed ectopic patches in the GCL. Since EphA4 is endogenously expressed in the RGCs layer, but not in other retinal layers, these results suggested that ectopic expression of ephrin-B1, but not other ephrin-Bs, was able to activate endogenous EphA4 in RGCs to induce cell adhesion.

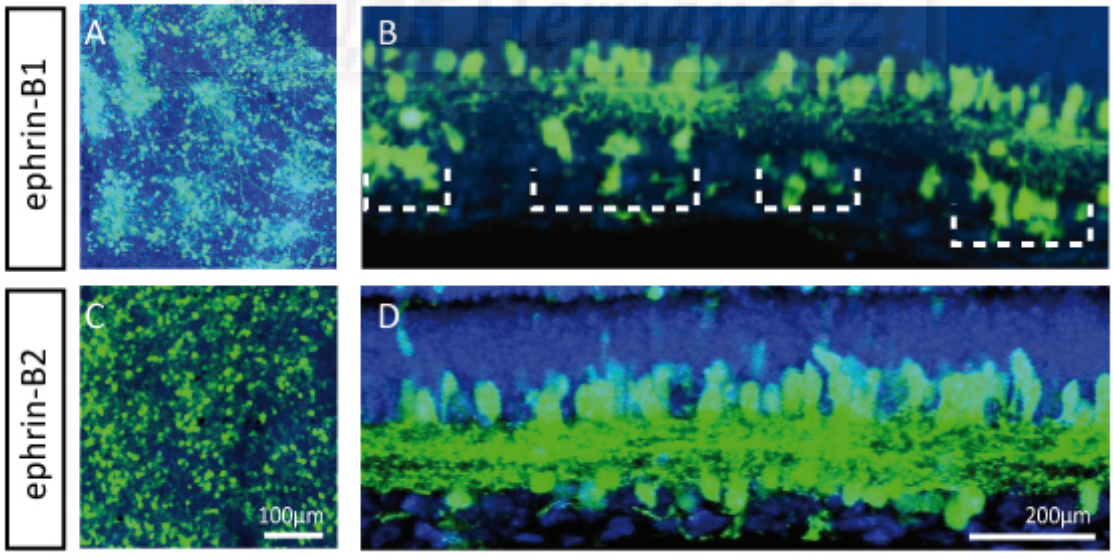

Figure 24: Ectopic expression of ephrin-B1, but not ephrin-B2, reproduces the EphA4 phenotype in the RGC layer.

A, C. Flat mounted views of P10 retinas electroporated at E13.5 with the plasmids indicated in each case. **B, D**. Retinal sections through the electroporated zone for each plasmid. EphrinB1 electroporation leads a phenotype somewhat similar to the one observed in EphA4 electroporation. Overexpression of ephrinB1 in the retina leads to a specific reorganization of the retinal cells in the RGC layer (brackets), but not in other retinal layers.

EphA4/ephrin-B1 signalling in trans does not activate axonal repulsion

We have seen that ephrin-B1, but not ephrins-B2, is able to activate endogenous EphA4 in the retina to produce an adhesive response in RGCs. Ephrin-B1 is expressed in medial SC areas at the moment that retinal axons are entering the SC (Hindges et al., 2002). Ephrin-B1 is also a predicted ligand for EphB receptors. It is known that EphB1 and EphB2 are both expressed in the ventral retina (Williams et al., 2004; Lee et al., 2008) and that RGC axons from ventral retina project to medial collicular areas that express high levels of ephrin-B1 (Hindges et al., 2002). Eph/ephrin signalling usually mediates repulsion, however the fact that EphB1/B2 expressing axons, coming from the ventral retina, project to medial collicular areas expressing ephrin-Bs suggests the contrary. It has been proposed that the relative concentration of ligand and receptor might modulate this repulsion (Reber et al., 2004), but no molecular mechanism mediating this has been suggested. Our functional experiments support the notion that EphA4/ephrin-B1 signalling mediates adhesion. Taken together, these results prompted us to wonder whether competition between EphA4 and EphBs, to bind ephrin-B1, could be responsible for this modulation of repulsion. To answer this question we performed *in vitro* experiments confronting EphA4 expressing axons with ephrin-B1 presented in a substrate. We isolated and dissected E15.5 explants from the dorsal and the ventral retina previously electroporated with pCAG-GFP or with pCAG-EphA4, and confronted these explants with a border of chimeric FC-ephrin-B1 or FC-substrates. Then, we analysed the behaviour of the growing axons after 24h in culture. No effect was observed under FC control conditions (Figure 25A and 25B) confirming no experimental bias on axon guidance due to the chimeric nature of the ephrin protein used. Axons coming from dorsal retinal

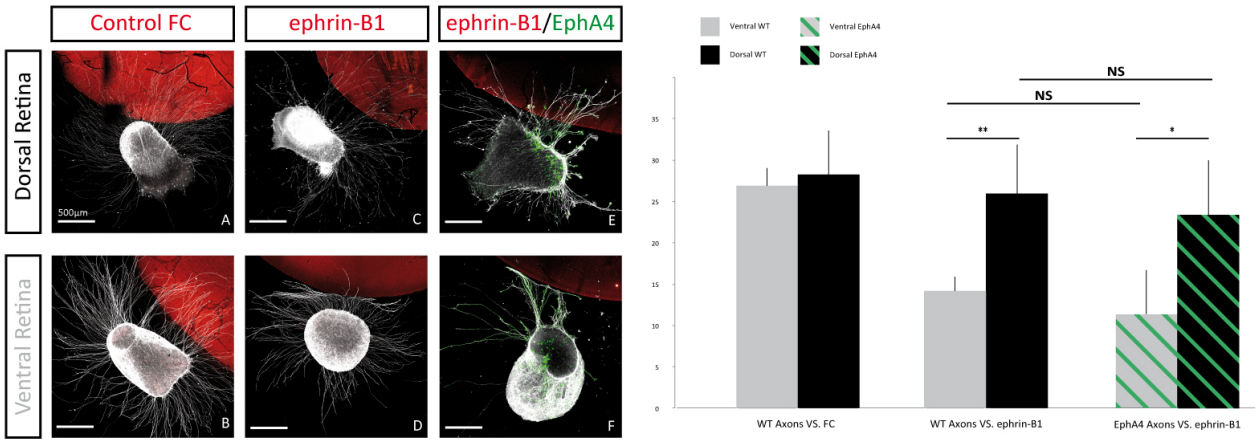

Figure 25: Activation of EphA4 is not able to modulate ephrin-B1 repulsion of ventral axons *in vitro*.

A-F. Retinal explants dissected from ventral or dorsal retina from E15.5 embryos were incubated 24h to allow axons to grow in all directions. When confronted with immobilized protein (in red) the reaction of the axons was observed: **A, B.** Facing control FC protein, both ventral and dorsal axons grow on the protein. **C, D**. When presented to immobilized ephrin-B1, ventral axons are repelled while dorsal axons grow through ephrin-B1 zone. **E, F.** When previously electroporated with pCAG-EphA4 at E13.5 (in green), ventral retinal axons show a clear repulsive response when confronted to ephrin-B and dorsal axons are not affected as control axons do, indicating that activation of EphA4 does not influence the endogenous repulsive response of ventral RGC axons to ephrin-B1. Scale bars, in A to F, 500µm. **G.** Quantification of axons fluorescence inside the FC/ephrin-B1 zone. Quantification was performed as described in Material and Methods, abscise value is the ratio of axonal fluorescence inside the zone of interest over the total axonal fluorescence after normalization for the distance, *p value <0,05 & **p value < 0,01 as asserted by ANOVA.

explants were not affected by the source of ephrin-B1 (Figure 25C), while ventral axons showed a clear repulsion to ephrin-B1 (Figure 25D). Then, we performed similar confrontation experiments with explants electroporated with pCAG-EphA4. Although ventral explants overexpressing EphA4 exhibited repulsion to ephrin-B1, similar to control ventral explants (Figure 25F), dorsal explants overexpressing EphA4 were not repelled by ephrin-B1 and continued their growth into the ephrin-B1 border (Figure 25E) confirming that the binding of EphA4 to ephrin-B1 does not promote repulsion by itself.

Overall, these results indicate that EphA4 does not induce repulsion when confronted to ephrin-B1 neither modulates the repulsion mediated by other Eph receptors (Quantification in Figure 25). The lack of repulsion upon contact of EphA4 and ephrin-B1 is compatible with the cell adhesion phenotype induced by EphA4 observed in the retina and reinforces our previous results

suggesting that EphA4 can be involved in mediating adhesive interactions during axon refinement at the visual targets.

How does EphA4/ephrin-B1 signalling mediate cell adhesion?

In order to determine the molecular mechanisms implicated in EphA4/ephrin-B1-induced cell adhesion, we analysed the expression pattern of some proteins well-known to be involved in adhesion, such as L1CAM, N-CAM, E-Cadherin and N-Cadherin, after ectopic introduction of EphA4 in RGCs.

The expression of each of these molecules was determined by immunofluorescence in retinas of P10 mice electroporated with pCAG-EphA4 at E13.5. None of the tested molecules showed any significant variation after EphA4 ectopic expression, neither in localization, nor in staining intensity (Figure 26) compared to control retinas electroporated with pCAG-GFP alone. These results indicate that EphA4-induced cell adhesion is not mediated by recruitment of any of these common cell adhesion molecules or the induction of their transcriptional expression. Further experiments are needed to identify the mechanisms by which EphA4/ephrin-B1 mediated signalling induces cell adhesion.

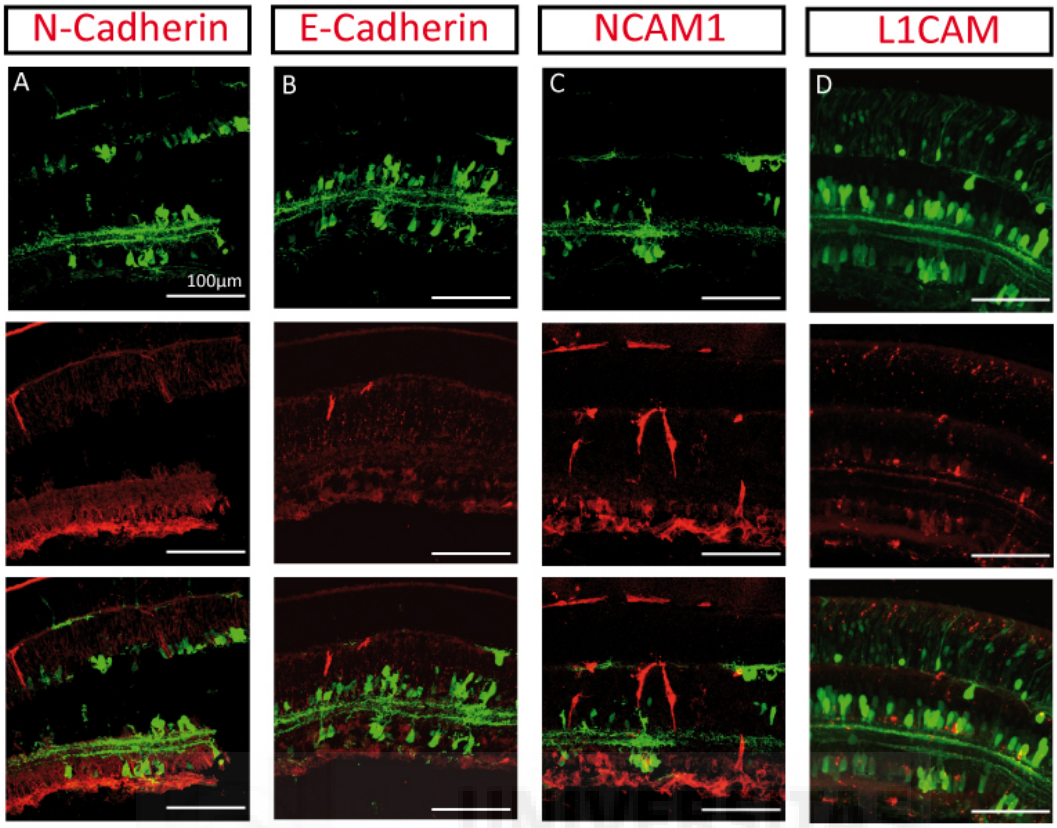

Figure 26: Overexpression of EphA4 does not modify the expression of well-known adhesion molecules-

A-D. Retinal sections from P10 mice, electroporated with pCAG-EphA4 at E13.5 and immunostained against the indicated adhesion molecules. No variation in the expression of these molecules was observed upon ectopic expression of EphA4.

DISCUSSION

In this study, we analysed the role of EphA4 in the development of the visual system. We have demonstrated that in the retina, EphA4 is not expressed in the same spatio-temporal pattern than other EphAs. In fact, the spatio-temporal expression pattern of this receptor seems to be incompatible with a role in the establishment of topography at the visual targets. Instead, we revealed here a previously unknown role for EphA4 in the correct refinement of RGCs axons after topographic mapping and suggested that adhesion is the mechanism underlying this EphA4-dependent refinement process.

Eph and ephrins in the visual system

At the beginning of the 90s, several groups demonstrated the existence of a high temporal to low nasal gradient (T>N) of Eph receptors in the vertebrate retina. The lack of reliable antibodies for those receptors pushed researchers to analyse the corresponding mRNA levels by radioactive (Brown et al., 2000; Diaz et al., 2003; Reber et al., 2004), fluorescent (Barbieri et al., 2001) or more classic colorimetric *in situ* hybridization (Feldheim et al., 1998; Feldheim, 2004; Carreres et al., 2011a). Spatiotemporal localization of these molecules was also asserted by LacZ transgenic mice (Feldheim, 2004) and mRNA levels compared by RT-PCR (Cowan et al., 2005; Carreres et al., 2011a). Thanks to all these approaches, it is extensively accepted today that EphA3, which is not expressed by murine RGC, is expressed in a T>N gradient in the chick retina, while EphA5 and EphA6 follow a similar T>N gradient in the mouse.

The rest of the EphA receptors are not expressed either in chick or mouse RGCs, except EphA4. The expression pattern of EphA4 in the retina is quite different from other Eph receptors (Cheng et al., 1995; Holash and Pasquale, 1995; Connor et al., 1998; Hornberger et al., 1999). Only four studies have previously described the expression pattern of EphA4 in the retina: Two of these studies claimed that *EphA4 mRNA* is present in RGCs around P0 without any notable gradient (Feldheim et al., 1998; Reber et al., 2004). The other two papers stated that during early embryonic development, *EphA4 mRNA* is only present in the optic disc (Petros et al., 2006; Agrawal et al., 2014). Our spatiotemporal expression pattern analysis confirms these previous studies and reveals that *EphA4 mRNA* expression starts just before E18, it is maintained during postnatal development and slowly fades away at P10 to completely disappear by P20. We also carefully analysed both D-V and M-L axis of retinal sections and found no evidence of differential expression in those axis at any stages.

The role of EphA4 in visual development

Along the last two decades, several groups have tried to address the role of EphA4 in chick and mice retina (Hornberger et al., 1999; Walkenhorst et al., 2000; Bevins et al., 2011). A milestone study in the field proposed that EphA4 is necessary for the rostro-caudal (R-C) mapping of nasal axons to the tectum (Walkenhorst et al., 2000). This study was based on *in vitro* experiments performed with chick retinas, and described the existence of a functional gradient of EphA4. According to the authors, although the protein is expressed uniformly across the retina, its phosphorylation state makes it only functional in the temporal part of the retina (Connor et al., 1998; Hornberger et al., 1999). However, this functional gradient has only been identified in the

chick retina and the existence of such functional gradient in mouse has since been challenged (Reber et al., 2004).

In addition, it has been assumed for a long time that all EphAs in the retina, including EphA4, are mostly interchangeable (Bevins et al., 2011). However, although it is possible that these proteins may replace each other when they are forced to, like in our overexpression experiment (see brackets in Figure 18C); the expression pattern of EphA4 together with our functional results, clearly reveal that endogenous EphA4 does not play the same role as EphA5 or -A6 in the mouse retina. Around E18, when expression of EphA4 starts in the GCL, most of the RGCs have already grown their axons into the SC. After P10, when EphA4 is down-regulated, the topographic mapping of those axons is finished since days ago. EphA5 and -A6 have been amply demonstrated as essential for establishing retinal topography in the SC and they are expressed from E13 to early postnatal days (Feldheim, 2004; Carreres et al., 2011a). Therefore, it is difficult to imagine a mechanism by which EphA4, with a different spatiotemporal pattern, plays the same topographic role. On the contrary, EphA4 expression pattern is rather compatible with a role in refinement and/or pruning of RGCs axons because these processes occur after gross topography is established and better coincide with the temporal expression of EphA4.

Our functional approaches have clearly demonstrated that EphA4 does not play the same function than other EphA receptors. By electroporation of EphA6, we did observe that most axons were shifted to the rostral part of the SC. However, our results also show that overexpression of EphA4 is not sufficient to shift the axons to the rostral part of the SC at the same extension than EphA6 (Empty arrowhead in Figure 18C). This is in agreement with our loss-of-function experiments showing that visual axons from EphA4KO mice were able to reach their correct RC position in the SC (Figure 17B) and points that EphA4 is not involved in ephrin-A5 repulsion-mediated topography. A

result also confirmed by electroporation of Cre-recombinase in EphA4cKO mice, where no defects in axon guidance were found along the visual pathway (Figure 15F). Taken together these results clearly show that EphA4 is not responsible for the R-C topographic organization of retinal axons in the SC. In fact, the phenotype of EphA3/EphA4 transgenic mice indirectly supports our hypothesis. Transgenic mice expressing ectopic EphA3 in RGCs show an increased repulsive response of axons in the SC. When those mice were crossed with EphA4$^{+/-}$ mice, an even stronger repulsion was observed (Reber et al., 2004). If EphA4 mediates repulsion, having less EphA4 protein should result in decreased repulsion. The phenotype observed in EphA3KI;EphA4$^{+/-}$ mice may be however explained by a reduced adhesion, facilitating the EphA3/ephrin-A5 mediated repulsion of the axons.

As we have already discussed, EphA4 expression pattern in the retina is not compatible with establishing map topography. However, it may be compatible with an implication in the fine refinement of the axons in the SC. Our experiments suggest that this is in fact the case and we propose that EphA4, by regulating adhesion, might be responsible for the limitation of the exploratory behaviour in the SC once the axons have reached the correct terminal zones. EphA4KO axons arborize over greater areas than WT axons in the SC (Figure 17C), which is similar to the observations made in mutant mice lacking ephrin-B1, where axons in the SC expand over larger but topographically correct TZ (Thakar et al., 2011). According to our results and knowing that ephrin-B1 is expressed in the SC in a M>L gradient (Hindges et al., 2002), the phenotype of ventral axons should be stronger than the dorsal axons in the EphA4KO mice. However, this is an issue that remains to be analysed in future experiments.

Does EphA4/ephrin-B signalling mediates cell adhesion?

In general, EphA/ephrin-A signalling has been shown to promote repulsion. However, the response mediated by EphB/ephrin-B signalling is more ambiguous. In mice, the medial part of the SC expresses high concentration of ephrin-Bs during development. And yet the medial SC is the final target of axons coming from the ventral retina, expressing high concentration of EphBs. On the contrary, the lateral SC (low ephrin-Bs) is the target of axons coming from the dorsal retinal (low EphBs)(Thakar et al., 2011). However, this idea has been challenged by experiments conducted with retinal explants *in vitro* ((Walkenhorst et al., 2000) and Figure 25), but also *in vivo* in the cortex (Villar-Cerviño et al., 2013) and in the optic chiasm (Williams et al., 2003). Some authors have proposed that repulsion can be turned into attraction depending on relative concentrations of ligand and receptor (Lemke and Reber, 2005). However, the demonstration of such a process in systems where EphB/ephrin-B is involved has not yet been provided. Knowing that EphA4 can bind to both ephrin-A and ephrin-B ligands (Gale et al., 1996), we wondered whether EphA4 could work in synergy with retinal EphBs to switch repulsion into attraction. Our *in vitro* experiments involving electroporated explants gave us the possibility to test this hypothesis (Figure 25). However, the results of these experiments clearly reveal that EphA4/ephrin-B signalling does not influence EphB/ephrin-B signalling and together with the rest of our functional experiments, this favours the hypothesis that EphA4/ephrin-B1 signalling directly mediates adhesion instead of influencing other axonal responses.

EphA4 is a tyrosine kinase receptor and as such, it has been shown to have ligand independent kinase activity, as it is the case for the PDGF/VEGF related receptor (PVR) (Inaki et al., 2012), the oncogene protein RET (Runeberg-Roos et al., 2007), but also with some EphAs and –Bs

(Mellitzer et al., 1999; Tanaka et al., 2003). For this reason, when we electroporated EphA4 at a high concentration, we believe EphA4 is constitutively activated. This would also be in accordance with previous results from our lab where overexpression of EphA6 and a constitutively active mutant form of EphA6, gave identical phenotypes after electroporation (Carreres et al., 2011a). Hence, the ectopic patchy phenotype observed in all cell types through the retina after electroporation of EphA4 is likely ligand independent. This could also explain why there are no phenotypic differences between EphA4 expressing axons presented to ephrin-B1 or FC in culture (Figure 25C vs 25E and 25D vs 25F). Nevertheless these results led us to discard the idea of EphA4 as an attractive/repulsive switch but reinforced the notion that EphA4 does not mediate repulsion but cell adhesion.

Biochemical studies, where dissociation constants between Eph and ephrins were calculated, showed that ephrin-B2 and -B3 are more likely to interact with EphA4 than with ephrin-B1. *In vivo* however, post-translational glycosylation on ephrin-B1 N139Q residue has been shown to modulate ephrin-B1 binding properties to EphA4 (Arvanitis et al., 2013). In the developing cortex, EphA4/ephrin-B1 interaction has been reported (North et al., 2009), also in platelets, where binding of EphA4/ephrin-B1 is responsible for an adhesion phenotype *in vivo* (Prevost et al., 2002; 2004; 2005) shows that, EphA4/ephrin-B1 binding occurs endogenously , despite the *in vitro* observations.

From a phylogenetic perspective, EphA4 closest relative is EphA5 (Drescher, 2002). The N-terminal ligand-binding domain is the less conserved domain among the Eph receptor family, as it is responsible for the affinity of each member to the ligand. In contrast, most of the cytosolic domains are highly conserved. Here we have shown that these domains are necessary for EphA4 forward

signalling as electroporation of Epha4ΔC did not induce ectopic cell patches in the retina (Figure 22C and 22D). Electroporation of Epha4ΔSAM demonstrated that the SAM was not implicated in the adhesion phenotype, while electroporation of Epha4ΔKDSAM proved that the Kinase domain was necessary (Figure 23) pushing further our understanding of EphA4 forward signalling. The specific substrate of EphA4 phosphorylation specifically involved in adhesion remains to be determined. However, EphBs have been shown to phosphorylate the lysine residue 1229 of L1CAM, leading to increased neurites outgrowth and a modulation of adhesion (Dai et al., 2012). This observation suggests that L1CAM may be a potential partner for EphA4 during adhesive interactions, an issue that needs to be further analysed.

EphA4 mechanisms of action in other systems and processes

EphA4 has been implicated in differentiation and proliferation in several tissues and animal models (Irie et al., 2009; Torii et al., 2009; Stiffel et al., 2014; Gerstmann et al., 2014; Chen et al., 2015). In the retina, if EphA4 would be involved in proliferation or differentiation a difference in the type and/or number of labelled neurons after EphA4 electroporation and/or in the EphA4KO mice should be detected. However, we do not observe such phenomenon (Figure 15E, 16, 19G and 19H). In addition, an increase in proliferation would not explain how the electroporated cells stay tightly packed together in patches as we observe in EphA4 electroporated retinas, as the tangential migration is known to push some cell types away from their progenitors during INM (Fekete et al., 1994). Therefore, in the retina, we do not support the idea that EphA4 plays a role in proliferation or differentiation.

EphA4 has also been proposed to be important in cell sorting during early development of zebrafish embryos (Cooke et al., 2005). In this study, two intermingled populations of rhombomere neurons, one expressing ephrin-B2 and the other expressing EphA4, have been shown to migrate away from each other upon transactivation. This mechanism is dependent on two distinct transactivation of EphA4: 1/ EphA4/ephrin-B2 activation mediates repulsion of ephrin-B2 expressing cells through reverse signalling. 2/ Another ligand mediates adhesion of EphA4 expressing cells through forward signalling. The authors do not describe the ligand responsible for this second activation of EphA4. However, we believe that ephrin-B1 is a strong candidate to fill this position, since it has been shown to be expressed at the correct time and location (De Vellis and Carpenter, 1999). Another compelling evidence for EphA4/ephrin-B1 involvement in adhesion can be found in the immune system. It has been shown that transactivation of EphA4/ephrin-B1 in platelets leads to L1CAM mediated adhesion (Prevost et al., 2002). Since both EphA4 and ephrin-B1 are expressed in the same cells, determination of forward and reverse signalling involvement was not possible in that study, however the authors discovered that EphA4 mediated adhesion might also be supported by integrin AlphaIIbBeta3 and plasma glycoprotein fibrinogen (Prevost et al., 2005). Similar adhesion mechanisms could explain the patches observed after overexpression of EphA4 in the retina. The formation of ectopic patches by adhesion can be explained by the endogenous tangential dispersion suffered by RGC and amacrine cells during development (Reese et al., 1995): The INM of progenitors during retinal formation is pushing new differentiated cells in random directions tangentially to the plane of the retina, resulting in homogenous dispersion of RGCs in WT. In the case of an EphA4-electroporated induced adhesion, targeted cells, if randomly pushed in contact with other adherent cells, would not be able to disperse properly, resulting in aggregates of electroporated cells, surrounded by non-electroporated cell.

In the otic system, EphA4 is known to be necessary for correct axonal fasciculation (Coate et al., 2012). Axon fasciculation needs cell adhesion to occur. We did not observe defasciculation of retinal axons in EphA4KO mice (Empty arrowhead in Figure 15F). It is interesting to notice that L1CAMKO mice do not show fasciculation defects either (Demyanenko and Maness, 2003), showing that fasciculation and reduced adhesion are not necessarily linked. This might explain why we have found adhesion of axon terminals at the SC after ectopic electroporation of EphA4 suggesting that although this receptor is not important to maintain axon fasciculation along the visual pathway, it may induce cell adhesion at the axon terminals. EphA4 has also been implicated in adhesion during regrowth experiments after optic nerve crush (Joly et al., 2014) or spinal cord dorsal hemi-section (Goldshmit et al., 2004). In these studies, EphA4KO axons show a decreased adhesion but improved regeneration. These results are compatible with our observation that axons lacking EphA4 arborize over a greater area once they have reached their correct TZs in the SC (Figure 17B).

We describe here a new role for EphA4 in visual system development. These observations open a new set of questions that need to be addressed to have a complete understanding of EphA4 mechanisms of action. For instance: 1/ Which are the mechanisms allowing EphA4 to respond specifically to ephrin-B1 but not ephrin-B2 in this context? 2/ What CAMs are involved with EphA4 during axon refinement? 3/ How does EphA4-mediated refinement interplay with previously known mechanisms involved in this process such as spontaneous activity? Therefore, this study shed some light on the role of EphA4 in the retina, but also raises new questions, hopefully driving a renewal of interest for the role of EphA4 in this and other systems and processes.

CONCLUSIONS

- EphA4 is highly expressed in the developing mouse retina. During embryonic stages (from E14 to P0) is expressed in the optic disc and then, at postnatal stages (from birth to at least P14) is homogenously expressed in the RGC layer.

- EphA4 is not necessary for the formation or maintenance of retinal cytoarchitecture

- EphA4 is essential for RGC axon terminal refinement at the termination zone in the superior colliculus, but it is dispensable to determine map topography.

- EphA4 forward signalling mediates cell adhesion in a manner that depends on the kinase function of this receptor.

- Activation of EphA4 by ephrin-B1, but not ephrin-B2, mediates adhesion through a yet undetermined cell adhesion molecule.

CONCLUSIONES

- EphA4 esta expresado en la retina del ratón durante el desarrollo. Durante los estadios embrionarios (desde E14 hasta P0) se expresa en el disco óptico, después, en estadios postnatales (desde P0 hasta al menos P14) se expresa homogéneamente en la capa de las RGC.

- EphA4 no es necesario para la formación o el mantenimiento de la cito arquitectura de la retina.

- EphA4 es esencial para el refinamiento de los terminales axónicos de las RGC en la zona adecuada del colículo superior, pero no es necesario para el establecimiento de los mapas topográficos visuales.

- La señalización "forward" de EphA4 provoca adhesión celular, y es dependiente de la función quinasa del receptor.

- La activación de EphA4 por ephrin-B1, pero no por ephrin-B2, resulta en adhesión al través de una molécula de adhesión celular, aun no determinada.

ANNEX

Here we compile a review of literature about the different Eph/ephrin members present in the mice retina, at all stages, and with their respective expression patterns according to each author.

Protein	Detection	Age	Expression	Authors	Date	Journal
Ephrin-As	EphA2-FC	E10	N>T	Marcus RC, et al.	1996	Developmental Biology
Ephrin-Bs	EphB2-FC	E10	D>V	Marcus RC, et al.	1996	Developmental Biology
EphA4	IHC	E11	absent	Greferath U, et al.	2002	Mechanisms of Development
EphA3	cISH	E12	absent	Agrawal P, et al.	2014	Developmental Dynamics
EphA4	cISH	E12	OD	Agrawal P, et al.	2014	Developmental Dynamics
EphA4	cISH	E12	OD	Petros TJ, et al.	2006	MCN
EphA7	cISH	E12	absent	Agrawal P, et al.	2014	Developmental Dynamics
EphA1	cISH	E13	absent	Agrawal P, et al.	2014	Developmental Dynamics
EphA2	cISH	E13	present	Agrawal P, et al.	2014	Developmental Dynamics
EphA3	cISH	E13	absent	Agrawal P, et al.	2014	Developmental Dynamics
EphA4	cISH	E13	OD	Agrawal P, et al.	2014	Developmental Dynamics
EphA4	IHC	E13	present	Greferath U, et al.	2002	Mechanisms of Development
EphA5	cISH	E13	T>N	Carreres MI, et al.	2011	Jneuro
EphA6	cISH	E13	T>N	Carreres MI, et al.	2011	Jneuro
EphA7	cISH	E13	absent	Agrawal P, et al.	2014	Developmental Dynamics
EphA4	cISH	E14	OD	Petros TJ, et al.	2006	MCN
EphA4	IHC	E14	OD	Petros TJ, et al.	2006	MCN
EphA5	cISH	E14	T>N	Carreres MI, et al.	2011	Jneuro
EphA6	cISH	E14	T>N	Carreres MI, et al.	2011	Jneuro
EphA6	rISH	E14	T>N	Diaz E, et al.	2003	PNAS
EphA6	rISH	E14	D=V	Diaz E, et al.	2003	PNAS
EphB1	cISH	E14	D>V	Erskine L, et al	2011	Neuron
Ephrin-A5	rISH	E14	N>T	Marcus RC, et al.	1996	Developmental Biology
Ephrin-As	EphA5-AP	E14	P>C	Son AI, et al.	2014	IOVS

Ephrin-As	EphA2-FC	E14	N>T	Marcus RC, et al.	1996	Developmental Biology
Ephrin-B2	rISH	E14	D>V	Marcus RC, et al.	1996	Developmental Biology
Ephrin-Bs	EphB2-FC	E14	D>V	Marcus RC, et al.	1996	Developmental Biology
EphA4	IHC	E15	present	Greferath U, et al.	2002	Mechanisms of Development
EphA4	cISH	E15	OD	Petros TJ, et al.	2006	MCN
EphA5	RTPCR	E15	present	Carreres MI, et al.	2011	Jneuro
EphA5	RTPCR	E15	present	Cowan CW, et al.	2005	Neuron
EphA6	RTPCR	E15	present	Carreres MI, et al.	2011	Jneuro
EphA6	RTPCR	E15	present	Cowan CW, et al.	2005	Neuron
EphAs	Ephrin-A4-FC	E15	T>N	Marcus RC, et al.	1996	Developmental Biology
EphB1	RTPCR	E15	present	Cowan CW, et al.	2005	Neuron
EphB1	RTPCR	E15	present	Garcia-frigola C, et al.	2008	Development
EphB1	rISH	E15	VT>rest	Pak W, et al	2004	Cell
EphBs	Ephrin-B1-FC	E15	V>D	Marcus RC, et al.	1996	Developmental Biology
Ephrin-A2	RTPCR	E15	present	Cowan CW, et al.	2005	Neuron
Ephrin-A5	RTPCR	E15	present	Carreres MI, et al.	2011	Jneuro
Ephrin-A5	RTPCR	E15	present	Cowan CW, et al.	2005	Neuron
Ephrin-As	EphA5-FC	E15	N>T	Marcus RC, et al.	1996	Developmental Biology
Ephrin-B2	RTPCR	E15	present	Cowan CW, et al.	2005	Neuron
Ephrin-Bs	EphB2-FC	E15	D>V	Marcus RC, et al.	1996	Developmental Biology
EphA5	cISH	E16	T>N	Carreres MI, et al.	2011	Jneuro
EphA6	cISH	E16	T>N	Carreres MI, et al.	2011	Jneuro
EphB1	cISH	E16	VT	Herrera E, et al.	2004	Development
EphB2	cISH	E16	V>D	Barbieri AM, et al.	2002	Development
Ephrin-B2	fISH	E16	D>V	Barbieri AM, et al.	2002	Development
EphA4	IHC	E17	present	Greferath U, et al.	2002	Mechanisms of Development
EphA5	cISH	E17	T>N	Carreres MI, et al.	2011	Jneuro
EphA6	cISH	E17	T>N	Carreres MI, et al.	2011	Jneuro

EphB1	rISH	E17	VT>rest	Pak W, et al	2004	Cell
EphB1	LacZ	E17	VT>rest	Thakar et al.	2012	Nat Comms
EphB2	LacZ	E17	V>D	Thakar et al.	2012	Nat Comms
Ephrin-A5	cISH	E17	N>T	Carreres MI, et al.	2011	Jneuro
Ephrin-B2	LacZ	E17	D>V	Thakar et al.	2012	Nat Comms
EphA4	IHC	E18	OD	Petros TJ, et al.	2006	MCN
EphA5	cISH	E18	T>N	Carreres MI, et al.	2011	Jneuro
EphA6	cISH	E18	T>N	Carreres MI, et al.	2011	Jneuro
EphA3	cISH	P0	absent	Brown A, et al.	2000	Cell
EphA3	rISH	P0	absent	Brown A, et al.	2000	Cell
EphA3	cISH	P0	T>N	Feldheim DA, et al.	1998	Neuron
EphA4	cISH	P0	T=N	Feldheim DA, et al.	1998	Neuron
EphA5	rISH	P0	T>N	Brown A, et al.	2000	Cell
EphA5	cISH	P0	T>N	Feldheim DA, et al.	1998	Neuron
EphA5	cISH	P0	T>N	Feldheim DA, et al.	2004	Jneuro
EphA5	LacZ	P0	T>N	Feldheim DA, et al.	2004	Jneuro
EphA6	rISH	P0	T>N	Brown A, et al.	2000	Cell
EphA6	cISH	P0	T>N	Feldheim DA, et al.	2004	Jneuro
EphAs	Ephrin-A5-AP	P0	T>N	Feldheim DA, et al.	1998	Neuron
EphB2	rISH	P0	V>D	Hindges R, et al.	2002	Neuron
EphB3	rISH	P0	V>D	Hindges R, et al.	2002	Neuron
Ephrin-A2	cISH	P0	N=T	Pfeiffenberger C, et al.	2006	Jneuro
Ephrin-A3	WB	P0	absent	Fang Y, et al.	2013	Stem Cell
Ephrin-A3	cISH	P0	N=T	Pfeiffenberger C, et al.	2006	Jneuro
Ephrin-A5	RTPCR	P0	present	Suetterlin P, et al.	2014	Neuron
Ephrin-B1	rISH	P0	D>V	Hindges R, et al.	2002	Neuron
Ephrin-B2	rISH	P0	D>V	Hindges R, et al.	2002	Neuron
EphA4	rISH	P1	T=N	Reber M, et al.	2004	Nature

EphA5	rISH	P1	T>N	Reber M, et al.	2004	Nature
EphA6	rISH	P1	T>N	Reber M, et al.	2004	Nature
EphA6	rISH	P1	T>N	Reber M, et al.	2004	Nature
EphAs	rISH	P1	T>N	Reber M, et al.	2004	Nature
EphB1	LacZ	P1	VT>rest	Thakar et al.	2012	Nat Comms
EphB2	LacZ	P1	V>D	Thakar et al.	2012	Nat Comms
Ephrin-A5	cISH	P1	N>T	Suetterlin P, et al.	2014	Neuron
Ephrin-B2	LacZ	P1	D>V	Thakar et al.	2012	Nat Comms
EphB2	rISH	P4	V>D	Hindges R, et al.	2002	Neuron
EphB3	rISH	P4	V>D	Hindges R, et al.	2002	Neuron
Ephrin-A3	WB	P4	present	Fang Y, et al.	2013	Stem Cell
Ephrin-A5	cISH	P4	N>T	Suetterlin P, et al.	2014	Neuron
Ephrin-A5	Ephrin-A5-GFP	P5	N>T	Yoo S, et al.	2011	EMBOJ
EphA4	WB	P6	present	Cowan CW, et al.	2005	Neuron
EphB1	LacZ	P8	VT>rest	Thakar et al.	2012	Nat Comms
EphB2	LacZ	P8	V>D	Thakar et al.	2012	Nat Comms
Ephrin-A5	cISH	P8	N>T	Suetterlin P, et al.	2014	Neuron
EphrinB2	LacZ	P8	D>V	Thakar et al.	2012	Nat Comms
Ephrin-A3	WB	P10	present	Fang Y, et al.	2013	Stem Cell
EphA4	IHC	adult	CillBody	Fang Y, et al.	2013	Stem Cell
EphA4	RTPCR	adult	CillBody	Fang Y, et al.	2013	Stem Cell
EphA4	IHC	adult	Muller	Joly S, et al.	2014	EJN
EphA4	IHC	adult	present	Ruilin Zhu et al.	2014	Chin Med J
EphA7	RTPCR	adult	CillBody	Fang Y, et al.	2013	Stem Cell
Ephrin-A3	WB	adult	present	Fang Y, et al.	2013	Stem Cell
Ephrin-A3	IHC	adult	RGC	Joly S, et al.	2014	EJN
Ephrin-A3	IHC	adult	INL	Joly S, et al.	2014	EJN

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