HHS Public Access

Author manuscript

Dev Neurobiol. Author manuscript; available in PMC 2016 January 16.

Published in final edited form as:

Dev Neurobiol. 2015 December ; 75(12): 1385–1401. doi:10.1002/dneu.22291.

Transient ipsilateral retinal ganglion cell projections to the brain: Extent, targeting and disappearance

Célia A. Soares[1,2,3] and **Carol A. Mason**[1,*]

Célia A. Soares: cam4@columbia.edu; Carol A. Mason: a47479@alunos.uminho.pt

[1]Departments of Pathology and Cell Biology, Neuroscience, and Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, USA

[3]ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal

Abstract

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

Keywords

dorsocentral retina; *in utero* electroporation; optic tract; lateral geniculate nucleus; superior colliculus

INTRODUCTION

The development of neuronal circuits is a dynamic process involving the formation of projections that do not persist in mature circuits (Luo and O'Leary, 2005). The elimination

*Corresponding authors: Carol A. Mason and Celia A. Soares, Mail address: Department of Pathology and Cell Biology, College of Physicians and Surgeons Columbia University 14-509 P&S Bldg., 630 W. 168th St., New York, NY 10032, Phone 212-305-2105 (office), -5882 (lab), FAX-5498.

[2]Present address : Life and Health Science Research Institute, School of Health Sciences, University of Minho, Braga, Portugal.

of transient projections ranges from small-scale events such as local pruning of axonal branches and synaptic boutons, as in the vertebrate neuromuscular junction (Tapia et al., 2012), to large-scale elimination of major axon projections or their collaterals (Stanfield and O'Leary, 1985; Luo and O'Leary, 2005) and wholesale elimination of neurons themselves, as in programmed cell death (Francisco-Morcillo et al., 2014).

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

Anterograde labeling with DiI has been widely used to label axonal projections in fixed developing nervous tissue (Colello and Guillery, 1990; Marcus and Mason, 1995), and can be used to chart the time course of axonal projections. However, this approach provides snapshots of the status of developing cells and is not prospective, whereby cells could be labeled at early stages then the time course of projections followed. In order to chart the early DC ipsilateral RGC projection at later developmental stages, we used *in utero* electroporation of a GFP plasmid at E12.5. This strategy allows prospective analysis of the number, projection, and disappearance of this cohort of RGC axons.

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

METHODS

Animals

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

Tissue fixation

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

DiI Labeling and Quantification

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

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

(Petros et al., 2009; Erskine et al., 2011; Escalante et al., 2013) and expressed as a percentage. The ipsilateral ratio was calculated at each distance at E13.5 and E15.5, and the values of the 3 segments averaged for each case.

In utero electroporation

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

Tissue processing of electroporated embryos and pups

Embryos and pups were sacrificed as described above. The left retina of each embryo or pup was dissected, immunostained for GFP (rabbit polyclonal anti-GFP, 1:1000, Invitrogen), flattened as a whole mount, and confirmed for successful electroporation. Retinal whole mounts were imaged and ImageJ was used to calculate the GFP^+ retinal area and pixel intensity of GFP^+ cells. Only the cases in which the GFP^+ area comprised more than 5% of the total retinal area were used for further analysis. Whole heads or brains were vibratome sectioned frontally at 100μm and immunostained for GFP.

Immunohistochemistry

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

Image processing and quantification

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

Quantification of electroporated RGC axons in the optic tract

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

Axon reconstruction in the superior colliculus

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

Statistical analyses

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

RESULTS

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

DiI labeling of the transient ipsilateral RGC projection from central retina

To determine how far the contralateral and ipsilateral axons of the DC RGCs project in the early period of RGC axon growth, we labeled RGC axons by placing DiI on the optic nerve head of one retina in E13.5 and E14.5 embryos and measured the extent of the ipsilateral and contralateral projection from the DC retina in brain sections. After E15.5, however, since the retinal projection includes axons from the central retina (and not only from the DC retina as in previous ages) as well as the permanent ipsilateral RGCs from VT retina (Colello and Guillery, 1990), we labeled only the DC retina at E15.5 to selectively visualize the early ipsilateral component in Fig. 1A–D, G. As previously reported (Marcus and Mason, 1995), we found that the ipsilateral axons are the first to reach the OT by E12.75 (data not shown). The extent of the retinal axons was measured along the length of the OT from the ventral midline to the distal most axonal tips. Since the DC ipsilateral axons are the first retinal cohort to reach the OT, and assuming that ipsilateral and contralateral axons grow at the same rate, we predicted that the DC retinal ipsilateral axons would extend farther than the contralateral axons at early ages. However, surprisingly, we found that the central retinal ipsilateral axons extend to a similar distance as the contralateral axons in the OT at E13.5, but by E14.5 and E15.5, the contralateral cohort projects past the ipsilateral cohort (Fig. 1C, G). There was no statistically significant difference in extent of axon growth between these two populations at E13.5 (ipsilateral: 1616 μm ± 149, contralateral: 1513 μm ± 153, Mann Whitney p= 0.54, n= 7 embryos) (Fig. 1D). At E14.5 and E15.5 there is a trend for the contralateral axons to project farther than the DC ipsilateral axons (E14.5, ipsilateral: 1783μm ±124, contralateral: 2325μm ±85, n=4; E15.5, ipsilateral: 1443μm ±170, contralateral: 3076μm ±108, n=5. At E14.5 and E15.5, the difference between the extent of growth of ipsilateral and the contralateral axons from DC retina was statistically significant (Mann Whitney E14.5, p= 0.029; E15.5, p= 0.0079). It is important to note that whereas the contralateral axons continue to extend more caudally, the distal extent of the DC ipsilateral axon projection remains relatively constant, with the majority reaching only below the future dLGN, and remaining at this relative distance from E13.5 to E15.5 (p= 0.47). The extent of contralateral axon growth, on the other hand, increases over this period, (p= 0.0014).

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

Quantification of DiI fluorescence intensity enables inference of the relative abundance of axons in a tract, but cannot provide information on the actual number of axons. The percentage of ipsilateral axons relative to contralateral at E14.5 in the OT was previously estimated to be 8% by DiI anterograde labeling (Erskine et al., 2011), but this value was not

known for ages E13.5 and 15.5. Therefore, we next estimated the percentage of ipsilateral axons in the proximal OT at these ages, in each case labeling the entire RGC projection by placing a crystal on the optic nerve head and inferring values from pixel intensity of DiI labeling (Erskine et al., 2011) (Fig. H–L). Measures were taken at three areas along the OT: 500, 750, and 1000 μm from the ventral midline in frontal sections. First, we determined whether there was a statistically significant difference in the ipsilateral ratio (iROI/ (iROI + cROI)) for the brains examined at E13.5 within these three areas (n = 6 embryos; E15.5, n=7 embryos). As there was no difference in the ipsilateral ratio in the three OT segments (E13.5, p= 0.12; E15.5, p=0.13), the ipsilateral ratios from the three areas were averaged, and one mean value per case was considered. In whole eye-labeled preparations at E13.5, the first age at which RGC axons are seen within the OT, the ipsilateral RGC axons were estimated to comprise 20.07% of the total RGC labeled axon population in the OT (± 1.09, n=6) (Fig. 1H–J). By E15.5, there was a dramatic decrease in the overall proportion of ipsilateral to contralateral axons in the OT to 5.32% (± 0.88, n=7), even with the addition to the ipsilateral projection in the OT of the permanent ipsilateral axons from VT retina (Fig. 1H, K–L).

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

Thus, ipsilateral axons from the DC retina are the first to enter the OT at E12.5, and the contralateral RGC axons reach the OT at E13.5, confirming previous reports (Marcus and Mason, 1995). Even though the ipsilateral axons are the first to arrive in the OT, after E13.5 they are overtaken in their extent by the contralateral axons. These two populations of DC retina RGC axons are well segregated at E13.5 in their position in the OT, with the ipsilateral axons medially and the contralateral laterally. At early stages of development (E13.5–14.5) the DC ipsilateral RGC cohort comprises a greater proportion of the RGC axons within the OT relative to contralateral axons (Fig. 1H–J) and by E15.5 the total ipsilateral projection (including the DC transient ipsilateral projection (Fig. 1G) as well as the first axons to extend from the permanent VT ipsilateral cohort (Fig. 1L)) decreases relative to the contralateral RGC axons (Fig. 1H).

Prospective labeling of RGCs by early electroporation of GFP

Prospective labeling, i.e., “fate mapping”, of axon projections is difficult to perform with DiI, especially at postnatal ages when DiI is less effective as an axonal marker. To trace the projection of RGC axons from the DC retina prospectively, we electroporated membrane-bound green fluorescent protein (GFP) into embryonic retinas *in utero* at E12.5, allowing RGC labeling *in vivo* and visualization of RGC axons at selected later stages of development, into the postnatal period. E12.5 is the earliest age at which it is technically feasible to label the retina without labeling the brain, since before that age, the subretinal space is connected to the brain ventricles. The injection and electroporation of a GFP

plasmid into the subretinal space at E12.5 predominantly targets RGC precursors in the central retina (Garcia-Frigola et al., 2007; Petros et al., 2009) (Fig. 2A–B). In our experiments, the central retina including both the dorsal and ventral central regions was consistently labeled with GFP. Thus, *in utero* electroporation of GFP at E12.5 labels a cohort of RGCs different from those labeled with DiI as described above. While DiI labels most of the RGCs projecting to the brain at the time of DiI application, electroporation of GFP in the subretinal space at E12.5 labels progenitor cells that will differentiate into RGCs a day or two later, and allowing prospective examination of RGC axon projection at later ages.

Embryos were collected at E14.5, 15.5, 16.5, P0, and P4. To determine whether the GFP signal from a standard injected concentration of GFP plasmid, deteriorated through time after electroporation, pixel intensity in the GFP^+ area in the retina was measured in retinal whole mounts at the time points listed above. The pixel intensity was similar across the ages examined, with no statistical difference (Min: 0, Max: 255, Mean: 40.3 ± 2.26, p= 0.078, n = 30). This argues against the possibility that the observed decrease in ipsilateral RGC axons over time is a consequence of loss of GFP expression. Moreover, the contralateral projection is strongly labeled with the membrane-bound GFP in the most distal point of their extent, implicating that the GFP signal remains robust from E12.5 until P4, and that this prospective labeling technique is valid and useful for developmental studies.

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

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

Next we determined how far the ipsilateral RGC axons electroporated at E12.5 project from E14.5-P4, and whether they invade the SC and/or dLGN. We quantified the number of axons in 500μm sectors along the OT from the ventral midline to their most dorsal extent at 4000 μm in frontal sections (Fig. 4A, B). A greater number of central retinal ipsilateral axons is in the proximal optic tract at E15.5 and E16.5 than at later ages (Fig. 4C, G, H), but their number is still markedly lower than the contralateral RGC axons from the same cohort of electroporated RGCs (Fig. 4E), and few of these axons extend beyond 3000μm distal to the midline. In Fig. 4D, at E16.5, a central retinal ipsilateral axon with a growth cone, and

therefore presumably still extending, is seen in the OT (Fig. 4D, arrow). At E17.5 and thereafter the number of axons decreases but the remaining axons extend farther than at earlier ages (Fig. 4G, H). There was no evidence of axonal degeneration, i.e., large axonal swellings disconnected from neurites.

Thus, the DC retinal RGCs, electroporated at E12.5 with GFP reach the OT after E14.5. The central retinal ipsilateral RGC axons from this group of cells project along the OT primarily between E15.5 and E16.5. Subsequently, their number decreases abruptly. At the time of decline of central retinal ipsilateral RGC axon number, E16.5 to E17.5, and prior to this time, most axons do not seem to have projected to the SC.

Target entry of RGC axons electroporated at E12.5

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

The SC is the first target in the brain to receive retinal projections, with the earliest axons entering at E15.5 (Godement et al., 1984). In mouse, from E16.5 to P0 RGC axons extend in the SC, overshooting their topographically appropriate target (McLaughlin and O'Leary, 2005). Only at P2 do they start to form branches in the topographically appropriate area in the SC. At P4, RGCs begin to prune branches projecting outside the final target area. This refinement process is established by P10 (Feldheim and O'Leary, 2010). In the cohort of RGCs electroporated at E12.5, the first axons reach the rostral SC at E16.5 and invade the SC at E17.5 (Fig. 6). At E17.5 the RGC axons project to the most superficial dorsal area of the SC but do not invade the inner-most area of the SC, in agreement with previous reports (Godement et al., 1984). The central retinal ipsilateral RGC axons electroporated at E12.5 project to the rostral-most area of the SC (Fig. 6A, A′), while the contralateral axons project more caudally (Fig. 6A–C). By P0, the contralateral RGC axons occupy most of the SC, and invade deeper layers of the SC than at E17.5 (Fig. 7A).

In order to better visualize the rostral-caudal distribution of the RGC axons in the SC, a schematic reconstruction of the SC was created, showing the area occupied by the central

retinal contralateral axons in green, and individual central retinal ipsilateral RGC axons as red tracings (Fig. 7E–J). At P0, the few remaining ipsilateral RGC axons from the central retina project more caudally in the SC compared with E17.5, but do not project as far caudally as the contralateral axons in the same cohort of electroporated RGCs (Fig. 7B, E–G). Only by P4 do the central retinal ipsilateral RGC axons reach the caudal SC. At the same age, it is possible to notice a slight decrease in the contralateral RGC axon territory, likely reflecting pruning of the axons outside of the appropriate topographic area (Fig. 7H–J). Both central retinal contralateral and ipsilateral axons do not show complex branches at P0, when compared with axons at P4. At P4, the contralateral RGC axons form complex arbors in a specific area in the SC (Fig. 7C). A few ipsilateral axons formed a branched arbor but others were much more simple (Fig. 7D, H–J).

Thus, the central retinal ipsilateral and contralateral RGC axons electroporated in the retina at E12.5 show quite different behaviors projecting to and within their targets. Although the contralateral and ipsilateral axons extend past the dLGN by E17.5, neither population enters the dLGN until after E17.5. The few ipsilateral axons that do enter have only simple branches through P4, while the contralateral axons form full arbors by that time. In the SC, the ipsilateral RGC axons project into this target later (P0) than the contralateral RGC axons, and although they branch, they do not reach the same complexity at P4 as the contralateral axons.

DISCUSSION

Using DiI labeling and *in utero* electroporation of GFP, we have followed the progression and waning of the transient ipsilateral retinal ganglion cell (RGC) axons projecting from the dorsocentral (DC) and central retina in the early period of RGC axon growth. As seen by DiI labeling, the ipsilateral RGC axons projecting from the DC retina at E13.5 comprise a greater proportion of axons extending in the OT compared with the contralateral axons at that time and compared with the RGC axons forming the permanent ipsilateral projection from the VT retina. Subsequently there is a precipitous drop in the proportion of ipsilateral to contralateral RGC axons that originate in the DC retina. RGC precursors that are electroporated in the central retina at E12.5, enter the OT at E14.5 and that can be followed until P4, display similar timing of elimination. The ipsilateral RGC axons observed after both labeling paradigms the DC/central retina seem to disappear through a process independent of interactions with their targets since so few of the ipsilateral RGC axons observed in this study ever reach targets in the brain beyond the dorsal OT just rostroventral to the LGN. While the mechanism of the disappearance of the early ipsilateral RGC axons is not understood, these data provide a more detailed picture of the ephemeral ipsilateral RGC projection than in previous studies.

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

The disappearance of ipsilateral RGC axons from the DC retina has been acknowledged, but previous studies analyzed this projection in mice with whole eye anterograde labeling with HRP (Godement et al., 1987), retrograde labeling from the OT with DiI and examined only

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

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

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

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

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

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

Electroporation *in utero* of a GFP plasmid at E12.5 targeted the central retina and allowed visualization of the retinal projections from the central retina to both sides of the brain. We described two differences in target innervation between the projections of the early ipsilateral and contralateral central retinal RGCs. First, while central retinal contralateral RGC axons project to the dLGN after P0 and make complex arbors by P4, the ipsilateral RGC axons project only short branches to the dLGN that branch minimally, if at all. A previous study that labeled RGCs in the peripheral retina by electroporation at postnatal ages found no difference in the features of dLGN innervation by the contralateral and ipsilateral axons, especially their morphology, although they noted that arborization of RGC axons in the dLGN at P4 lagged behind the arborization in the SC by almost one week (Dhande et al., 2011). However, in the Dhande study the permanent ipsilateral RGCs from the VT retina were visualized, whereas in our study the transient ipsilateral RGCs in DC/central retina were labeled. Second, there is a delay in the progression of the central retinal ipsilateral RGC axons in the OT from E15.5 to E17.5 and in the rostral-caudal axis of the SC at P0, compared with the extension of the contralateral axons electroporated in the same cohort. Nonetheless, the timing of branching in the SC seems similar in both contra- and ipsilateral populations. The strategy of innervation of RGC axons to their targets seems to change throughout development (Osterhout et al., 2014). Early-born RGC axons that project as early as E15.5 innervate multiple targets and subsequently retract the projections from inappropriate targets. However, later-born RGC axons accurately project to their appropriate and final targets. Various RGC subtypes have different birth dates, molecular markers, projections and functional roles (Hong et al., 2011; McNeill et al., 2011; Osterhout et al., 2011; Osterhout et al., 2014; Triplett et al., 2014). To date, molecular markers for the transient ipsilateral RGCs from DC and central retina are lacking and thus it is not clear whether the transient RGCs observed in the present study have a distinct molecular profile or belong to a RGC subtype. Judging from the time of their projection, the transient ipsilateral RGCs labeled from the DC retina with DiI and the central RGCs electroporated with GFP at E12.5 could belong to the first group of early-born, early-projecting, non-imaging forming RGCs expressing cadherin 3 and cadherin 4 that project from the DC retina at E14.5 (Osterhout et al., 2014).

Mechanisms of elimination of transient axonal projections

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

The elimination of axonal projections in inappropriate targets has been attributed to the absence of appropriate trophic factors in the target (Lom and Cohen-Cory, 1999; Yamaguchi and Miura, 2015), or the absence of appropriate receptors or trophic factors in the growing neurons themselves (Cohen-Cory et al., 2010; Harvey et al., 2012). In support of these hypotheses, growing RGC axons have an intrinsic supply of neurotrophic factors supporting growth toward targets but when axons reach their target they become dependent on target-derived neurotrophic factors (Spalding et al., 2004; Marshak et al., 2007). The ipsilateral RGC axons that we electroporated at E12.5 in the central retina grow more slowly than the contralateral RGC axons, and the slower progression could reflect a reduced intrinsic supply of neurotrophic factors before reaching the target. In addition, we did not observe the majority of early-projecting ipsilateral RGC axons reaching their first retinal target, the SC, or entering the dLGN, implying that the DC and central retinal ipsilateral axons do not perceive target-derived neurotrophic factors that would ensure their progression toward and entry to the target and/or survival.

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

Summary and Conclusions

Anterograde DiI labeling and in utero electroporation of GFP have provided new details on the transient ipsilateral projection from the retina distal to the optic chiasm and visual targets in the brain. DiI provides a snapshot of the early stages of RGC axon growth from the DC retina and GFP electroporation at E12, a prospective chronicle of the extent, targeting and disappearance of the transient ipsilateral RGC projection from the central retina. Both approaches have shown for the first time that the majority of the transient ipsilateral RGC axons do not innervate targets, and provides precise spatiotemporal information on their

disappearance. This study will provide a basis for further analysis of this transient projection by fate mapping, and investigation of the mechanisms underlying its elimination.

Acknowledgments

We thank Qing Wang, Taka Kuwajima and Florencia Marcucci for training in *in vivo* electroporation, Dinardo Rodriguez and the BRAINYAC program for participating in the DiI experiments, Mika Melikyan for mouse breeding assistance, and members of the Mason lab, Joana Palha and Riva Marcus for discussion and comments on the manuscript. Supported by NIH R01 EY012736 and P30 EY019007 (CAM); and the Portuguese Foundation for Science and Technology fellowship SFRH/BD/74926/2010, the Luso-American Development Foundation, and the MD-PhD Program, University of Minho (CAS).

References

Campbell DS, Okamoto H. Local caspase activation interacts with Slit-Robo signaling to restrict axonal arborization. J Cell Biol. 2013; 203:657–672. [PubMed: 24385488]

Chan SO, Chung KY, Taylor JS. The effects of early prenatal monocular enucleation on the routing of uncrossed retinofugal axons and the cellular environment at the chiasm of mouse embryos. Eur J Neurosci. 1999; 11:3225–3235. [PubMed: 10510186]

Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol. 2010; 70:271–288. [PubMed: 20186709]

Colello RJ, Guillery RW. The early development of retinal ganglion cells with uncrossed axons in the mouse: retinal position and axonal course. Development. 1990; 108:515–523. [PubMed: 2340812]

Cowan WM, Fawcett JW, O'Leary DD, Stanfield BB. Regressive events in neurogenesis. Science. 1984; 225:1258–1265. [PubMed: 6474175]

Dhande OS, Hua EW, Guh E, Yeh J, Bhatt S, Zhang Y, Ruthazer ES, Feller MB, Crair MC. Development of single retinofugal axon arbors in normal and beta2 knock-out mice. J Neurosci. 2011; 31:3384–3399. [PubMed: 21368050]

Drager UC. Birth dates of retinal ganglion cells giving rise to the crossed and uncrossed optic projections in the mouse. Proc R Soc Lond B Biol Sci. 1985; 224:57–77. [PubMed: 2581263]

Erskine L, Reijntjes S, Pratt T, Denti L, Schwarz Q, Vieira JM, Alakakone B, Shewan D, Ruhrberg C. VEGF signaling through neuropilin 1 guides commissural axon crossing at the optic chiasm. Neuron. 2011; 70:951–965. [PubMed: 21658587]

Escalante A, Murillo B, Morenilla-Palao C, Klar A, Herrera E. Zic2-dependent axon midline avoidance controls the formation of major ipsilateral tracts in the CNS. Neuron. 2013; 80:1392–1406. [PubMed: 24360543]

Feldheim DA, O'Leary DD. Visual map development: bidirectional signaling, bifunctional guidance molecules, and competition. Cold Spring Harb Perspect Biol. 2010; 2:a001768. [PubMed: 20880989]

Francisco-Morcillo J, Bejarano-Escobar R, Rodriguez-Leon J, Navascues J, Martin-Partido G. Ontogenetic Cell Death and Phagocytosis in the Visual System of Vertebrates. Dev Dyn. 2014

Garcia-Frigola C, Carreres MI, Vegar C, Herrera E. Gene delivery into mouse retinal ganglion cells by in utero electroporation. BMC Dev Biol. 2007; 7:103. [PubMed: 17875204]

Godement P, Saillour P, Imbert M. The ipsilateral optic pathway to the dorsal lateral geniculate nucleus and superior colliculus in mice with prenatal or postnatal loss of one eye. J Comp Neurol. 1980; 190:611–626. [PubMed: 7400384]

Godement P, Salaun J, Imbert M. Prenatal and postnatal development of retinogeniculate and retinocollicular projections in the mouse. J Comp Neurol. 1984; 230:552–575. [PubMed: 6520251]

Godement P, Vanselow J, Thanos S, Bonhoeffer F. A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. Development. 1987; 101:697–713. [PubMed: 2460302]

Guillery RW, Mason CA, Taylor JS. Developmental determinants at the mammalian optic chiasm. J Neurosci. 1995; 15:4727–4737. [PubMed: 7623106]

Harvey AR, Ooi JW, Rodger J. Neurotrophic factors and the regeneration of adult retinal ganglion cell axons. Int Rev Neurobiol. 2012; 106:1–33. [PubMed: 23211458]

Hong YK, Kim IJ, Sanes JR. Stereotyped axonal arbors of retinal ganglion cell subsets in the mouse superior colliculus. J Comp Neurol. 2011; 519:1691–1711. [PubMed: 21452242]

Imai T, Sakano H. Axon-axon interactions in neuronal circuit assembly: lessons from olfactory map formation. Eur J Neurosci. 2011; 34:1647–1654. [PubMed: 22103421]

Lom B, Cohen-Cory S. Brain-derived neurotrophic factor differentially regulates retinal ganglion cell dendritic and axonal arborization in vivo. J Neurosci. 1999; 19:9928–9938. [PubMed: 10559401]

Luo L, O'Leary DD. Axon retraction and degeneration in development and disease. Annu Rev Neurosci. 2005; 28:127–156. [PubMed: 16022592]

Marcus RC, Mason CA. The first retinal axon growth in the mouse optic chiasm: axon patterning and the cellular environment. J Neurosci. 1995; 15:6389–6402. [PubMed: 7472403]

Marshak S, Nikolakopoulou AM, Dirks R, Martens GJ, Cohen-Cory S. Cell-autonomous TrkB signaling in presynaptic retinal ganglion cells mediates axon arbor growth and synapse maturation during the establishment of retinotectal synaptic connectivity. J Neurosci. 2007; 27:2444–2456. [PubMed: 17344382]

Matsuda T, Cepko CL. Controlled expression of transgenes introduced by in vivo electroporation. Proc Natl Acad Sci U S A. 2007; 104:1027–1032. [PubMed: 17209010]

McLaughlin T, O'Leary DD. Molecular gradients and development of retinotopic maps. Annu Rev Neurosci. 2005; 28:327–355. [PubMed: 16022599]

McNeill DS, Sheely CJ, Ecker JL, Badea TC, Morhardt D, Guido W, Hattar S. Development of melanopsin-based irradiance detecting circuitry. Neural Dev. 2011; 6:8. [PubMed: 21418557]

Osterhout JA, El-Danaf RN, Nguyen PL, Huberman AD. Birthdate and outgrowth timing predict cellular mechanisms of axon target matching in the developing visual pathway. Cell Rep. 2014; 8:1006–1017. [PubMed: 25088424]

Osterhout JA, Josten N, Yamada J, Pan F, Wu SW, Nguyen PL, Panagiotakos G, Inoue YU, Egusa SF, Volgyi B, Inoue T, Bloomfield SA, Barres BA, Berson DM, Feldheim DA, Huberman AD. Cadherin-6 mediates axon-target matching in a non-image-forming visual circuit. Neuron. 2011; 71:632–639. [PubMed: 21867880]

Pak W, Hindges R, Lim YS, Pfaff SL, O'Leary DD. Magnitude of binocular vision controlled by islet-2 repression of a genetic program that specifies laterality of retinal axon pathfinding. Cell. 2004; 119:567–578. [PubMed: 15537545]

Petros TJ, Rebsam A, Mason CA. Retinal axon growth at the optic chiasm: to cross or not to cross. Annu Rev Neurosci. 2008; 31:295–315. [PubMed: 18558857]

Petros TJ, Rebsam A, Mason CA. In utero and ex vivo electroporation for gene expression in mouse retinal ganglion cells. J Vis Exp. 2009

Petros TJ, Shrestha BR, Mason C. Specificity and sufficiency of EphB1 in driving the ipsilateral retinal projection. J Neurosci. 2009; 29:3463–3474. [PubMed: 19295152]

Pfeiffenberger C, Cutforth T, Woods G, Yamada J, Renteria RC, Copenhagen DR, Flanagan JG, Feldheim DA. Ephrin-As and neural activity are required for eye-specific patterning during retinogeniculate mapping. Nat Neurosci. 2005; 8:1022–1027. [PubMed: 16025107]

Pittman AJ, Law MY, Chien CB. Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points. Development. 2008; 135:2865–2871. [PubMed: 18653554]

Plump AS, Erskine L, Sabatier C, Brose K, Epstein CJ, Goodman CS, Mason CA, Tessier-Lavigne M. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron. 2002; 33:219–232. [PubMed: 11804570]

Pont-Lezica L, Beumer W, Colasse S, Drexhage H, Versnel M, Bessis A. Microglia shape corpus callosum axon tract fasciculation: functional impact of prenatal inflammation. Eur J Neurosci. 2014; 39:1551–1557. [PubMed: 24593277]

Raper J, Mason C. Cellular strategies of axonal pathfinding. Cold Spring Harb Perspect Biol. 2010; 2:a001933. [PubMed: 20591992]

Simon DJ, Weimer RM, McLaughlin T, Kallop D, Stanger K, Yang J, O'Leary DD, Hannoush RN, Tessier-Lavigne M. A caspase cascade regulating developmental axon degeneration. J Neurosci. 2012; 32:17540–17553. [PubMed: 23223278]

Spalding KL, Rush RA, Harvey AR. Target-derived and locally derived neurotrophins support retinal ganglion cell survival in the neonatal rat retina. J Neurobiol. 2004; 60:319–327. [PubMed: 15281070]

Stanfield BB, O'Leary DD. The transient corticospinal projection from the occipital cortex during the postnatal development of the rat. J Comp Neurol. 1985; 238:236–248. [PubMed: 4044913]

Tapia JC, Wylie JD, Kasthuri N, Hayworth KJ, Schalek R, Berger DR, Guatimosim C, Seung HS, Lichtman JW. Pervasive synaptic branch removal in the mammalian neuromuscular system at birth. Neuron. 2012; 74:816–829. [PubMed: 22681687]

Triplett JW, Wei W, Gonzalez C, Sweeney NT, Huberman AD, Feller MB, Feldheim DA. Dendritic and axonal targeting patterns of a genetically-specified class of retinal ganglion cells that participate in image-forming circuits. Neural Dev. 2014; 9:2. [PubMed: 24495295]

Walsh C, Guillery RW. Age-related fiber order in the optic tract of the ferret. J Neurosci. 1985; 5:3061–3069. [PubMed: 3840527]

Williams SE, Mann F, Erskine L, Sakurai T, Wei S, Rossi DJ, Gale NW, Holt CE, Mason CA, Henkemeyer M. Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. Neuron. 2003; 39:919–935. [PubMed: 12971893]

Yamaguchi Y, Miura M. Programmed Cell Death in Neurodevelopment. Dev Cell. 2015; 32:478–490. [PubMed: 25710534]

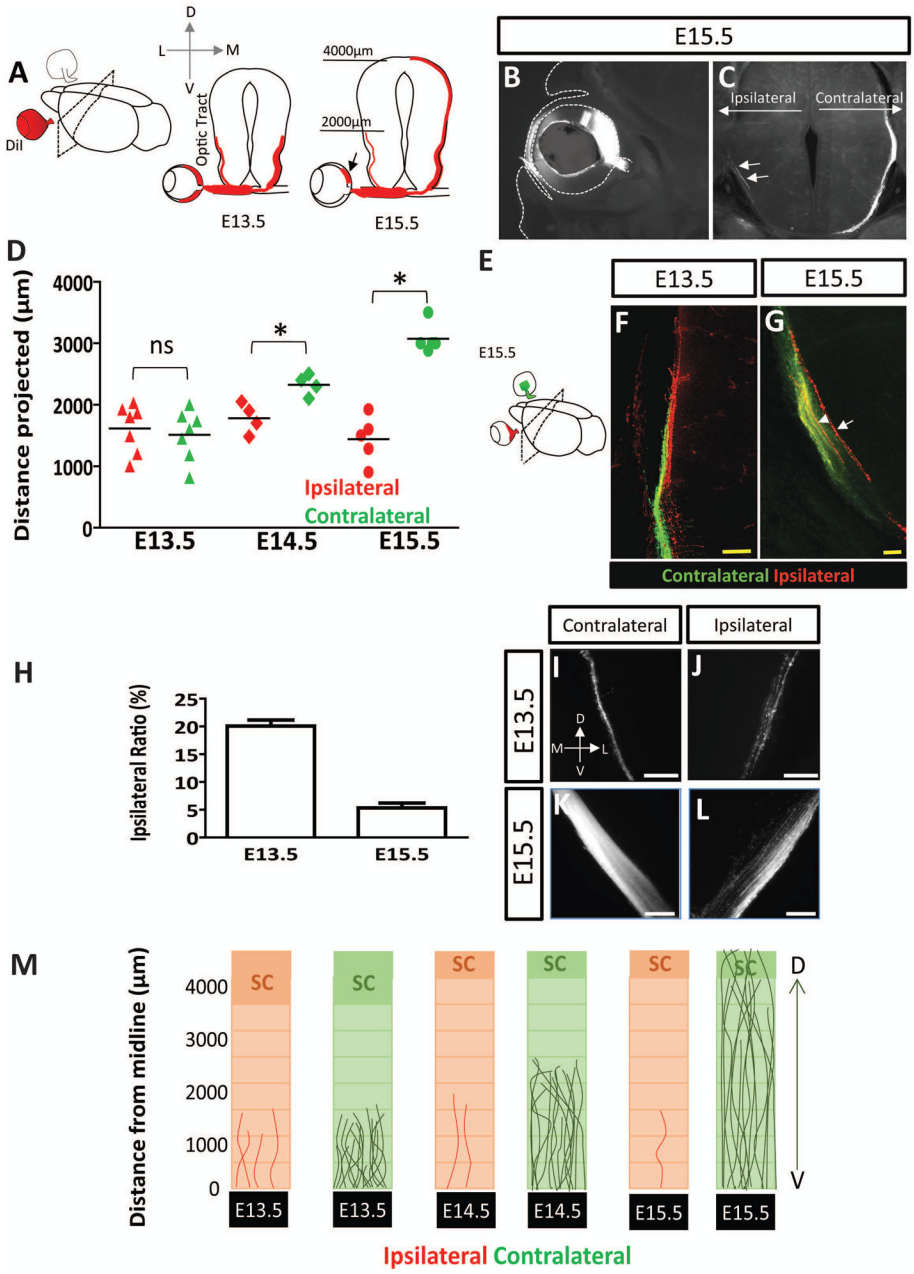

Figure 1. Retinal ganglion cell axon projections in the first stage of extension – DiI labeling

(**A, B**) DiI was applied to the retina of fixed mouse embryos to visualize retinal ganglion cell (RGC) projections to the brain, either to the whole retina at E13.5 and E14.5 (left) or only to the dorsocentral (DC) retina at E15.5 (right, and B). (**C**) DiI labeling in the ipsilateral and contralateral optic tracts at E15.5 when only the DC is labeled. (**D**) The distance that RGC axons extend from the ventral midline to the dorsal thalamus over time. While the extent to which the contralateral RGC axons project varies from E13.5 to E15.5 ($p= 0.0014$), the extent of the ipsilateral RGC projection does not ($p= 0.47$). Each mark = a single brain, horizontal bar = mean, $p< 0.05$. (**E–G**) DiI or DiA was applied in fixed embryos, to the entire retina at E13.5, when only RGCs from DC retina extend axons, and to the DC retina

at E15.5, and the brain sectioned frontally. (**F**) At E13.5, DC ipsilateral axons occupy a more medial position in the OT compared with contralateral RGCs. (**G**) At 15.5, most DC ipsilateral RGC axons continue to occupy the medial-most position in the OT (arrow) but a few ipsilateral axons are positioned in the lateral OT mingled with contralateral axons (arrowhead). (**H**) DiI crystals were applied to the whole retina at E13.5 and E15.5, and the embryos were sectioned frontally. The proportion of ipsilateral to contralateral RGC axons was estimated from pixel intensity (PI) of DiI in the Region of Interest (ROI) within the OT: PI ipsilateral ROI / (PI ipsilateral ROI + PI contralateral ROI) × 100, expressed as a percentage. The ipsilateral RGCs within the OT at E13.5 represent 20.07% (±1.09), n=6, of the total projection in both OTs. At E15.5 the percentage of ipsilateral RGCs decreases to 5.32% (±0.88), n=7. p= 0.0012. Data represents mean ± SEM. (**I–L**) Representative frontal sections through the contra and ipsilateral OT after DiI labeling of the optic nerve head in fixed brains at E13.5 and E15.5, performed as in A. Note that at E15.5 (K, L), the entire retinal projection was labeled, and thus includes the transient ipsilateral axons from central retina and the permanent ipsilateral RGCs from ventrotemporal retina. (**M**) Schematic representation of the extent and relative number of retinal projections from the midline to the optic tract (OT) and superior colliculus (SC). At E13.5 when the first axons project to the OT, the ipsilateral and contralateral projections project to the same distance. After E14.5 the contralateral projection projects farther than the ipsilateral axons. *p< 0.05, ** p< 0.01. SC: superior colliculus, OT: Optic tract, ROI: region of interest. D/L/M/V: dorsal, lateral, medial, and ventral. All scale bars= 100μm.

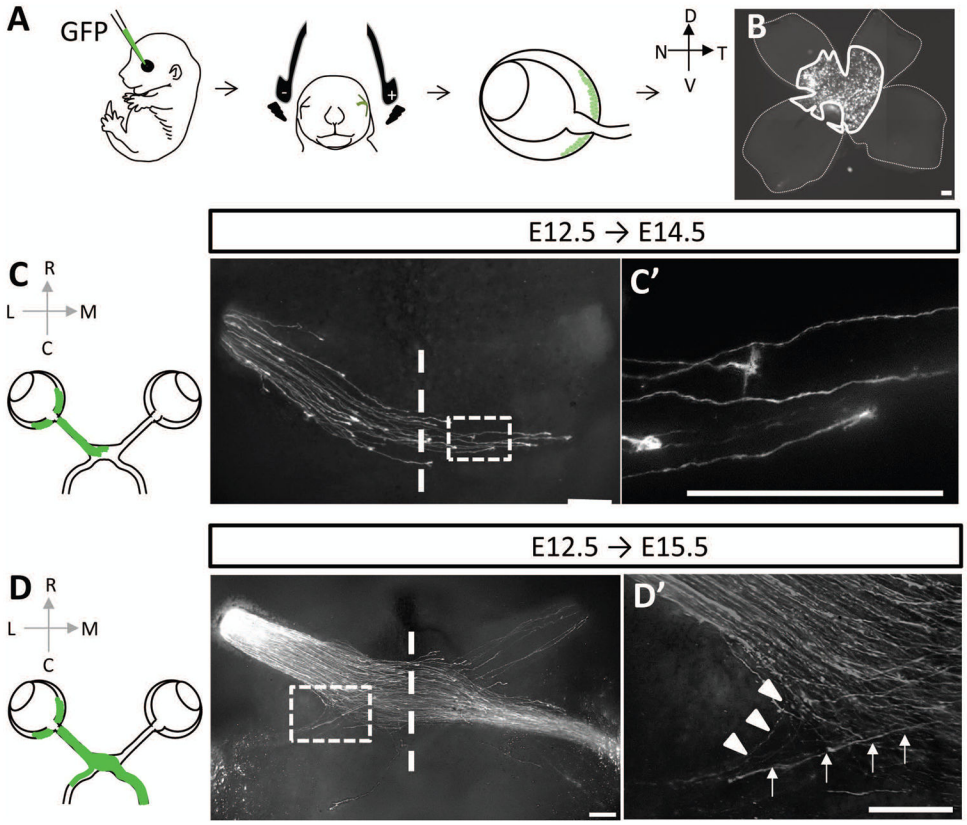

Figure 2. Electroporation of GFP into the central retina at E12.5 labels RGCs that cross the midline at E14.5

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

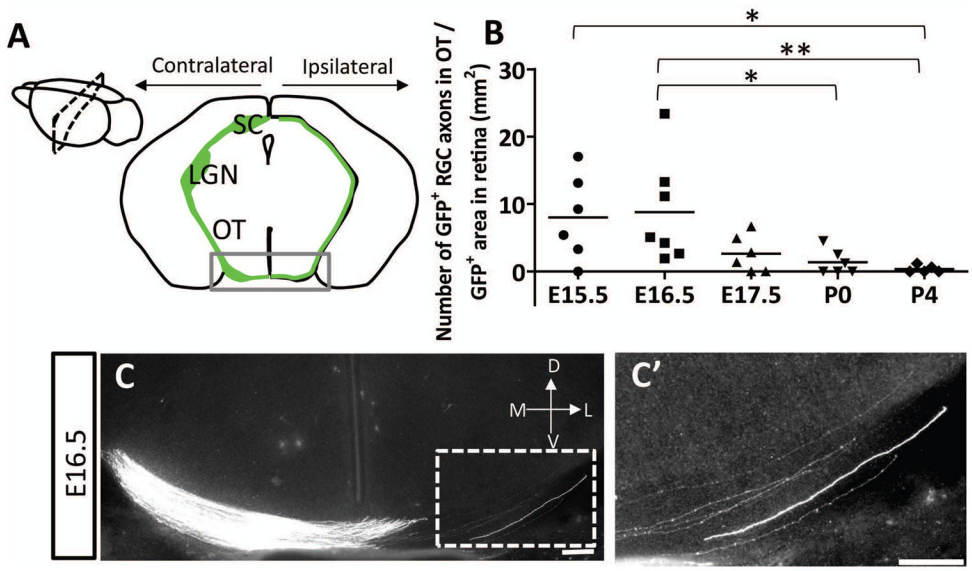

Figure 3. In the cohort of RGCs electroporated with GFP at E12.5, the number of DC ipsilateral axons decreases after E16.5

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

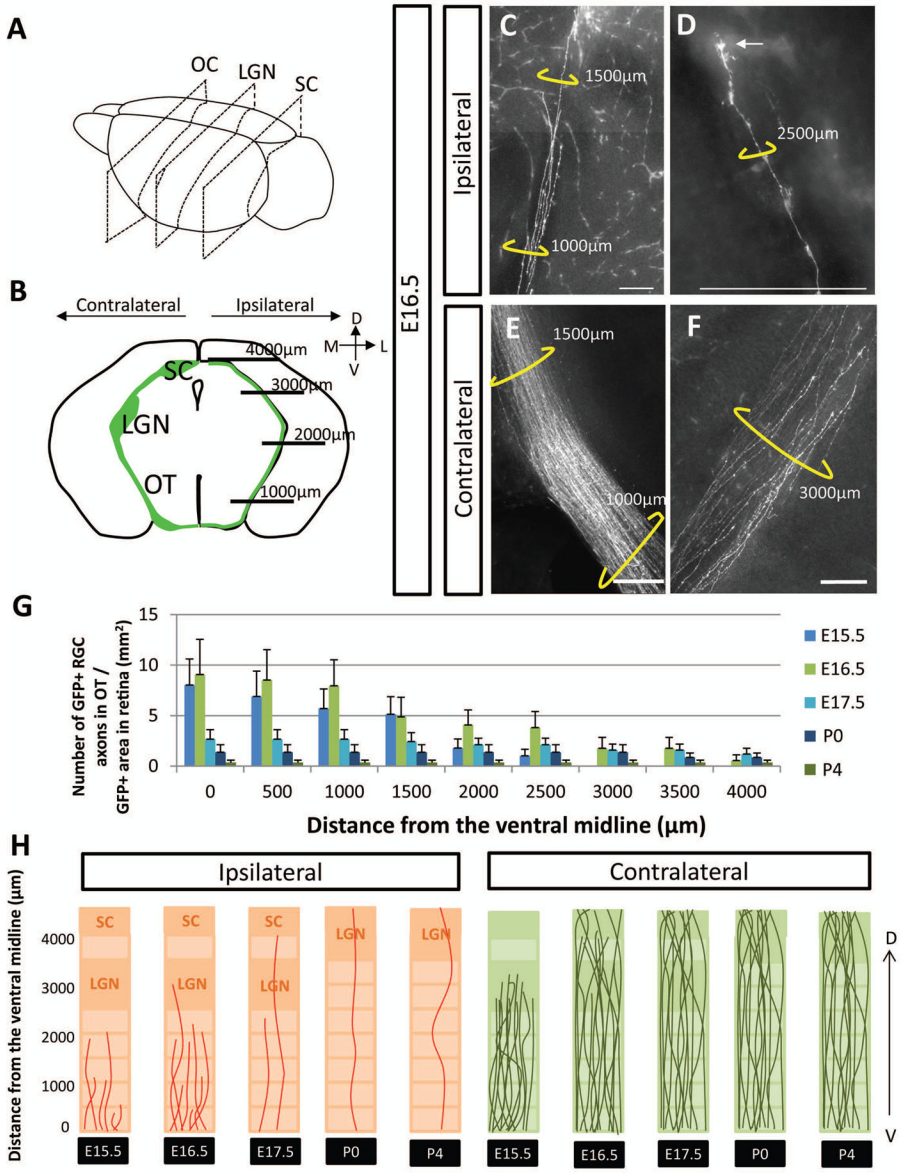

Figure 4. In the cohort of RGCs electroporated with GFP at E12.5, only a few central ipsilateral axons project distal to the optic tract after E16.5

(**A**) Schema of brain indicating sampling of frontal sections through the optic chiasm (OC), lateral geniculate nucleus (LGN) and a rostral section through the superior colliculus (SC). (**B**) Schema of the RGC axon projection in the brain in frontal view, indicating the distance measured from the OC midline through the optic tract (OT) to dLGN and SC targets. (**C–F**) The optic tract at different distances from the ventral midline, at E16.5. (**C**) Few GFP$^+$ axons from RGCs from the central retina are in the proximal ipsilateral OT at E16.5 compared with the contralateral projection (C, D vs E, F). (**D**) In the same cases as in C., the few ipsilateral axons that extend along the OT have growth cones. (**E**) At E16.5, many GFP$^+$ RGC axons project contralaterally. (**F**) The contralateral RGC axons project further along the OT than the ipsilateral RGC axons (**G**) The number of central retina ipsilateral RGC

axons was quantified in contiguous 500μm sectors beginning from the ventral midline to the SC. A greater number of ipsilateral axons from the central retina are in the proximal optic tract at E15.5 and E16.5 than at later ages, but few axons extend beyond 3000 μm from the midline. After E16.5 only a few ipsilateral axons extend farther. (**H**) Scheme of the contralateral and ipsilateral axons labeled by electroporation at E12.5 in the central retina, by number and length, from E15.5 to P4. The darker shaded bars represent the LGN and the SC. The contralateral axons from the central retina extend toward and reach targets compared with ipsilateral axons from the central retina at the same developmental stage. dLGN: dorsal lateral geniculate nucleus, OT: optic tract, SC: superior colliculus. D/L/M/V: dorsal, lateral, medial, and ventral. All scale bars= 100μm.

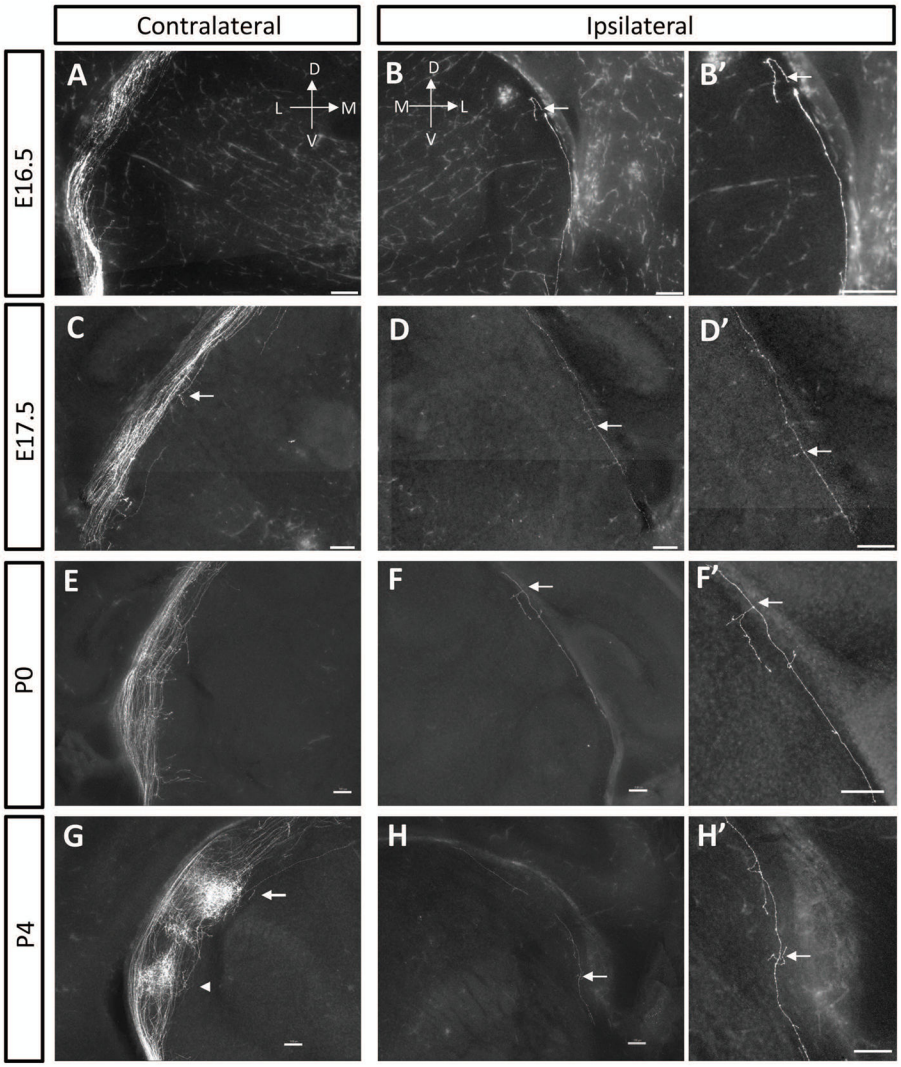

Figure 5. Few ipsilateral RGCs electroporated at E12.5 in the central retina project to the dorsal lateral geniculate nucleus

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

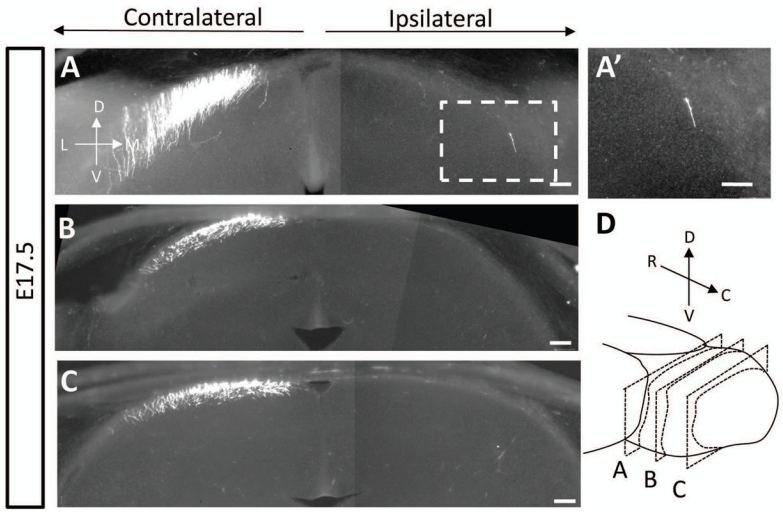

Figure 6. Few RGCs electroporated at E12.5 in the central retina project to the superior colliculus at E17.5

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

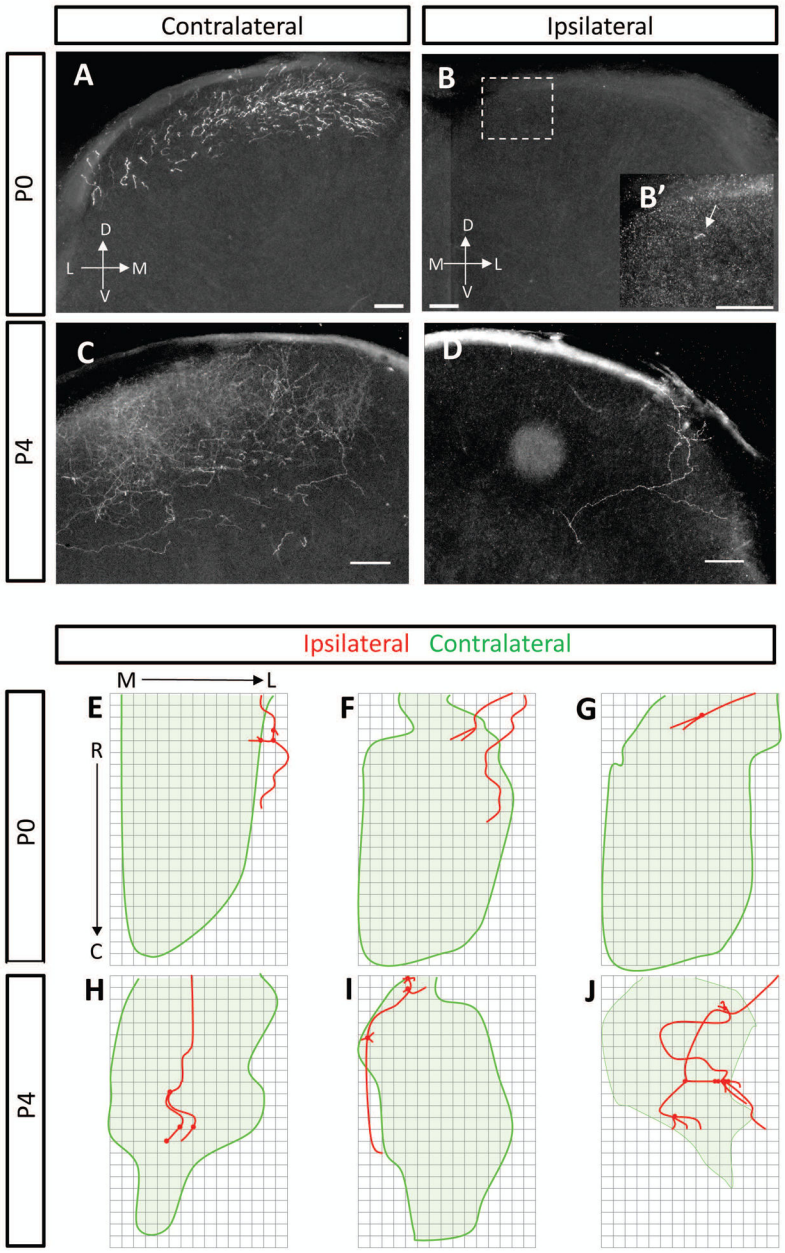

Figure 7. Some ipsilateral RGCs electroporated at E12.5 in the central retina project to and arborize in the superior colliculus at P0 and P4

(**A–D**) Frontal section of the superior colliculus (SC) at P0 and P4 after electroporation of GFP into central retina at E12.5. (**A–B**) At P0, the contralateral RGCs electroporated in central retina project to most of the SC (pale green shaded area). At the same age, only a few central retina ipsilateral axons, with simple morphology, are seen (B and B′). (**C–D**) At P4, many more contralateral axons projecting into the SC are branched when compared with RGC axons P0. The few ipsilateral axons projecting into the SC are also more branched than at P0. (**E–J**) Reconstruction of projections to the SC of RGCs electroporated at E12.5 in the central retina. Each square represents a 100 × 100μm area in the SC. The green shading

represents the area occupied by the electroporated contralateral RGCs. The red tracing represents individual ipsilateral RGC axons in the same cases in which the contralateral projections were estimated. At P4, ipsilateral axons project more caudally in the SC and have more branches when compared with P0. Note that the electroporated central ipsilateral RGC axons project to the lateral SC unlike the permanent ipsilateral RGCs from ventrotemporal retina, which project medially (not shown). C/D/L/M/R/V: caudal, dorsal, lateral, medial, rostral, and ventral. All scale bars= 100μm.