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Layer-Specific Developmentally Precise Axon Targeting of Transient Suppressed-by-Contrast Retinal Ganglion Cells

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The mouse retina encodes diverse visual features in the spike trains of >40 retinal ganglion cell (RGC) types. Each RGC type innervates a specific subset of the >50 retinorecipient brain areas. Our catalog of RGC types and feature representations is nearing completion. Yet, we know little about where specific RGC types send their information. Furthermore, the developmental strategies by which RGC axons choose their targets and pattern their terminal arbors remain obscure. Here, we identify a genetic intersection (Cck-Cre and Brn3cCKOAP) that selectively labels transient Suppressed-by-Contrast (tSbC) RGCs, a member of an evolutionarily conserved functionally mysterious RGC subclass. We find that tSbC RGCs selectively innervate the dorsolateral geniculate nucleus (dLGN) and ventrolateral geniculate nucleus (vLGN) of the thalamus, the superior colliculus (SC), and the nucleus of the optic tract (NOT) in mice of either sex. They binocularly innervate dLGN and vLGN but project only contralaterally to SC and NOT. In each target, tSbC RGC axons occupy a specific sublayer, suggesting that they restrict their input to specific circuits. The tSbC RGC axons span the length of the optic tract by birth and remain poised there until they simultaneously innervate their four targets around postnatal day 3. The tSbC RGC axons choose the right targets and establish mature stratification patterns from the outset. This precision is maintained in the absence of Brn3c. Our results provide the first map of SbC inputs to the brain, revealing a narrow target set, unexpected laminar organization, target-specific binocularity, and developmental precision.

**Key words:** axon development; binocular; laminar targeting; retinal projections; thalamus

**Significance Statement**

In recent years, we have learned a lot about the visual features encoded by RGCs, the output neurons of the eye. In contrast, we know little about where RGCs send their information and how RGC axons, which carry this information, target specific brain areas during development. Here, we develop an intersectional strategy to label a unique RGC type, the tSbC RGC, and map its projections. We find that tSbC RGC axons are highly selective. They innervate few retinal targets and restrict their arbors to specific sublayers within these targets. The selective tSbC RGC projection patterns develop synchronously and without trial and error, suggesting molecular determinism and coordination.

**Introduction**

Vision begins in the retina, which transforms the pixel representations of photoreceptors into feature representations of retinal ganglion cells (RGCs), the sole output neurons of the eye (Gollisch and Meister, 2010; Kerschensteiner, 2022). The mouse retina contains >40 RGC types, which send different visual information to the brain (Baden et al., 2016; Bae et al., 2018; Rheume et al., 2018; Tran et al., 2019; Goetz et al., 2022). We have learned a lot about how retinal circuits extract visual features and encode them in spike trains of specific RGC types (Kerschensteiner, 2022). Yet, we know little about where this information is sent.
In total, mouse RGCs innervate >50 brain areas (Morin and Studholme, 2014; Martersteck et al., 2017). However, which brain areas specific RGC types innervate remains, with few exceptions, unknown (Hattar et al., 2006; Huberman et al., 2008, 2009; Kim et al., 2008; Yonehara et al., 2008). Some RGC axons target distinct layers within the dorsolateral geniculate nucleus (dLGN) and superior colliculus (SC), the two largest retinorecipient areas of the mouse brain (Reese, 1988; Huberman et al., 2009; Kim et al., 2010; Hong et al., 2011; Kerschensteiner and Guido, 2017). Our maps of RGC axons in dLGN and SC are incomplete, and the retinal input organization of many other targets remains unexplored. Finally, a subset of RGCs (9/40+ types) innervate ipsilateral and contralateral brain areas to support binocular vision (Dräger and Olsen, 1980; Johnson et al., 2021). Whether these RGC types innervate all or only a subset of their targets binocularly is unclear.

Retinal projections to the brain serve as a model for studying the development of long-range neural projections and supported the discovery of conserved growth programs and molecular cues that guide RGC axons toward their targets (Erskine and Herrera, 2007; Moore and Goldberg, 2011; Mason and Slavi, 2020; Williams et al., 2020). Yet, how RGC axons invade specific targets and organize their arbors within, and whether this process involves trial and error or proceeds orderly, is unknown (Kim et al., 2010; Osterhout et al., 2011, 2014, 2015; Su et al., 2021). Individual RGCs innervate multiple targets through axon collaterals to ensure complete visual field representation in each retinorecipient area (Fernandez et al., 2016). Whether collaterals innervate targets simultaneously or sequentially is unclear (Osterhout et al., 2014). Brn3 POU domain transcription factors are part of the gene regulatory network that controls RGC specification and terminal identity (Mu and Klein, 2004; Badea et al., 2009). Brn3a, Brn3b, and Brn3c are expressed in distinct but overlapping sets of RGCs (Xiang et al., 1995; Badea and Nanthas, 2011; Parmhans et al., 2018, 2021). RGC axon targeting is severely affected in Brn3b KO mice, but the contributions of Brn3a and Brn3c to this process remain to be tested (Badea et al., 2009, 2012).

Most RGCs increase their firing rates to light increments (i.e., positive contrast), light decrements (i.e., negative contrast), or both. However, Levick (1967) and Rodieck (1967) discovered RGCs in cats and rabbits, respectively, that fire at high rates in the absence of stimuli and are silenced by positive and negative contrast steps. Suppressed-by-Contrast (SbC) RGCs have since been identified in nonhuman primates and mice, indicating they are a conserved output of the mammalian retina (de Monasterio, 1978; Jacoby et al., 2015; Tien et al., 2015). The contributions of SbC signals to vision remain mysterious, in part because the projection patterns of SbC RGCs are unknown (Masland and Martin, 2007).

Here, we discover a genetic intersection that selectively labels transient SbC (tSbC) RGCs in mice. We use this intersection to map the tSbC RGC projections to the brain and study their development.
Materials and Methods

Experimental animals. We used the following mouse lines, alone or in combination: Cck-Cre (Taniguchi et al., 2011), Brn3cCKOAP (Badea et al., 2012), and Ai9 (Madsen et al., 2010). All mice were crossed onto a C57Bl6/J background for at least five generations before inclusion in this study. We used Cck-Cre Brn3cCKOAP/CKOAP mice to test the contributions of Brn3c to the development of tSbC RGC projections. Although we did not independently confirm Brn3c deletion, we are confident that tSbC RGCs in these mice lack Brn3c because (1) alkaline phosphatase (AP) expression reports successful recombination and Brn3c excision, and (2) the recombined Brn3cCKOAP allele has previously been used successfully as a null allele (Badea et al., 2012; Shi et al., 2013). Throughout our study, we used mice of both sexes, and because we observed no differences, combined their data.

Adeno-associated virus. A Brainbow adeno-associated virus (AAV; AAV-EF1a-BbTagBY, catalog #45185-AAV9, Addgene) was injected (250 nl) into the vitreous of newborn Cck-Cre Brn3cCKOAP/CKOAP mice as described previously (Cai et al., 2013; Soto et al., 2013).

Tissue preparation and histochemistry. Tissues were either collected fresh or after transcardial perfusion. For fresh tissue collection, animals were deeply anesthetized with CO2, decapitated, and their retinas and brains collected. Retinas and brains were fixed by immersion in 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature and overnight at 4°C, respectively. For transcardial perfusions, animals were injected with ketamine (80 mg/kg) and xylazine (8 mg/kg) and perfused with ice-cold PBS followed by 4% paraformaldehyde (Sigma-Aldrich). Fixed brain and retina vibratome slices and fixed retinal whole mounts were washed twice for 20 min in PBS at room temperature. Before AP staining, brain sections and retinas in PBS were heated in a water bath at 65–70°C for 90 min to inactivate endogenous AP. AP staining was then developed in the heat-inactivated tissue in AP buffer (0.1 mM Tris, 0.1 mM NaCl, 50 mM MgCl2, pH to 9.5) with 3.4 μl/ml of NBT/BCIP overnight at room temperature with gentle agitation (Badea et al., 2003). After staining, the tissue was washed three times for 20 min in PBS with 0.1% Tween 20 and fixed overnight in PBS with 4% PFA at 4°C. To improve imaging, the tissue was dehydrated through an ethanol series (50, 75, 85, 95, and 100%, 100 proof, for 20 min, then 100%, 200 proof, overnight) and cleared with 2:1 Benzyl benzoate (BB)/Benzyl alcohol (BA). The tissue was then mounted in 2:1 BB/BA between glass slides and coverslips before the NBT/BCIP precipitate was dissolved in BB/BA. We used a rabbit polyclonal anti-Brn3c antibody (Xiang et al., 1995) to stain Cck-Cre Ai9 retinas and a murine anti-secreted embryonic alkaline phosphatase (SEAP) antibody 0.03 mg/ml (VIB Core Protein) to stain Cck-Cre Brn3cCKOAP/CKOAP retinas.

Imaging and analysis. We brightfield imaged the cleared tissue with 4× and 10× objectives on an Olympus BX51 microscope and a 2× objective on a Leica M205 FCA stereo microscope. We used an Olympus FV1000 laser-scanning confocal microscope with a 20× objective to analyze Brn3c-staining in Cck-Cre Ai9 mice and a 60× objective to image AAV-Brainbow-positive cells and SEAP staining in Cck-Cre Brn3cCKOAP/CKOAP mice. The dendritic territories, the ascending and descending branches, and the stratification profile were analyzed in image stacks with a voxel size of 0.207 μm (x/y/z-axis) to 0.5 μm (z-axis).

Density recovery profiles (DRPs) of labeled RGCs were calculated following the definitions of Rodieck (1991) using scripts written in MATLAB. RGC dendrite and axon stratification profiles were analyzed in Fiji (Schindelin et al., 2012). To determine whether labeled RGCs are regionally enriched or distributed evenly across the retina, we measured their density in four quadrants of each retina and defined an asymmetry index as follows:

\[
\text{Asymmetry index} = \frac{D_{\text{max}} - D_{\text{min}}}{D_{\text{max}} + D_{\text{min}}},
\]

where \(D_{\text{max}}\) is the density of cells in the most populated quadrant, and \(D_{\text{min}}\) is the density of cells in the least populated quadrant. Territories of ON and OFF dendrites were measured as the areas of the smallest convex polygons to encompass the respective arbors in maximum intensity projections. The distribution of ascending and descending branches was calculated as a function of the distance from the soma. The dendritic stratification profile was calculated as the mean fluorescent values of the tSbC RGCs as a function of distance normalized from the inner nuclear layer to the ganglion cell layer. Data were analyzed using Fiji and custom scripts written in MATLAB.

Results

An intersectional genetic strategy selectively labels tSbC RGCs.

We previously characterized tSbC RGCs in Cck-Cre transgenic mice injected with AAVs or crossed to a fluorescent reporter...
strain (Ai9, tdTomato; Tien et al., 2015). In our previous characterization, we referred to these cells simply as SbC RGCs (Tien et al., 2015) because of their light responses, resemblance to SbC RGCs in rabbits (Levick, 1967; Sivyer et al., 2010), and because they were the only RGC type with SbC responses known in mice. Since then, two additional mouse SbC RGC types have been identified (Jacoby et al., 2015; Jacoby and Schwartz, 2018; Wienbar and Schwartz, 2022). These cells differ morphologically and functionally from the cell type we identified. One is suppressed for longer periods after contrast steps [i.e., sustained SBC (sSbC) RGC; Jacoby et al., 2015], and one fires action potentials in bursts [i.e., bursty SBC (bSbC) RGC; Wienbar and Schwartz, 2022]. Following a change in nomenclature proposed by Jacoby and Schwartz (2018), we now refer to the cell type we originally identified as tSbC RGCs.

The bistratified dendrites of tSbC RGCs target synaptic laminae outside the inner plexiform layer (IPL) ChAT bands (i.e., the plexus of ON and OFF starburst amacrine cells) and are connected by numerous ascending and descending processes (Tien et al., 2015). An RGC type matching this morphologic description was reported in Brn3cCKOAP/+ mice crossed to a ubiquitously expressed sparsely induced Cre line (R26CreERT mice; Badea and Nathans, 2011). Both Cck-Cre Ai9 and R26CreERT Brn3cCKOAP/+ label multiple RGC types. To examine their...
To analyze how tSbC RGCs establish target- and layer-specific projections, we first needed to test whether Cck-Cre Brn3cCKOAP+ mice directly innervate a subset of retinorecipient areas. To map the projections of tSbC RGCs, we cut coronal sections of Cck-Cre Brn3cCKOAP+ mouse brains (P30) and stained them for alkaline phosphatase. Neurons in the brain did not express alkaline phosphatase. All brain labeling was eliminated by binocular enucleation (data not shown), indicating that it reflects the expression of alkaline phosphatase in tSbC RGC axons.

We comprehensively surveyed the retinorecipient areas of Cck-Cre Brn3cCKOAP+ mouse brains (P30) and found labeling in only four (of >50; Morin and Studholme, 2014; Martersteck et al., 2017). In the LGN complex, the dLGN and ventrolateral geniculate nucleus (vLGN) were labeled, whereas the intergeniculate leaflet was clear (Fig. 2B). Labeling in dLGN encompassed all but the most medial aspect, and in vLGN, labeling was restricted to the most temporal layer (Fig. 2B). In SC, labeling was strongest in the upper layer of the retinorecipient superficial SC (sSC; Fig. 2C). Finally, we observed narrowly stratified labeling in the nucleus of the optic tract (NOT; Fig. 2D). Thus, tSbC RGC axons innervate a small subset of retinorecipient areas (4/50+) and target specific layers within each of these areas.

### Developmentally Precise tSbC RGC Axon Targeting

We recently discovered that only nine of the >40 mouse RGC types innervate ipsilateral and contralateral brain areas to support binocular vision (Johnson et al., 2021). The remaining types innervate only contralateral targets. tSbC RGCs are part of the ipsilaterally projecting set (Johnson et al., 2021). To test whether tSbC RGCs innervate all their targets binocularly, we examined alkaline phosphatase labeling in the brains of monocularly enucleated Cck-Cre Brn3cCKOAP+ mice. We enucleated mice at P25 and analyzed RGC projection patterns at P30 to allow for Wallerian degeneration of RGC axons from the removed eye (Knöferle et al., 2010). All four targets received contralateral tSbC RGC input (Fig. 3A–C), but only the dLGN and vLGN received input from ipsilateral tSbC RGCs (Fig. 3A). Thus, tSbC RGCs binocularly innervate only a subset of their targets, the first example of target-specific RGC binocularity outside the special case of the retinohypothalamic tract (Magnin et al., 1989; Fernandez et al., 2016).

### Cck-Cre Brn3cCKOAP+ mice label tSbC RGCs throughout postnatal development

To analyze how tSbC RGCs establish target- and layer-specific axonal projections, we first needed to test whether Cck-Cre Brn3cCKOAP+ mice selectively label these cells during development. We prepared retinal whole mounts from P0, P5, P10, P15, and P20 mice to address this question. At P0, some alkaline-phosphatase-expressing postmitotic RGCs have not yet retracted their apical processes and completed migration to the ganglion cell layer (Saigo et al., 2016; Fig. 4A); at all other
Development of the tSbC RGC projections

We started to study the development of tSbC RGC projections by tracking their innervation of the LGN complex from P0 to P20. At P0 and P1, labeling was restricted to the optic tract with few branches or axon terminals visible in the dLGN or vLGN (Fig. 5A–C). However, by P3, axonal arborizations occupied all but the medial aspect of dLGN and a lateral band of vLGN (Fig. 5A–C). These patterns were maintained to maturity (Fig. 5A–C). We observed no aberrant tSbC RGCs axons in the intergeniculate leaflet at any stage of postnatal development.

We next turned to the SC. Like LGN, tSbC RGC axons had few branches in sSC at P0 and P1 but densely innervated the uppermost layers of sSC by P3, which they continued to occupy at maturity (Fig. 6A–C). We found that neurons in the deeper layers SC (dSC) expressed alkaline phosphatase during early postnatal development (P0 and P5). We confirmed that the sSC labeling reflects tSbC RGC axons entering from the optic tract in sagittal sections (Fig. 7) and through binocular enucleations in developing mice (Fig. 8).

Finally, although tSbC RGC axons were present in the optic tract at P0, we observed few branches in the NOT (Fig. 9A–C). By P3, tSbC RGC axon terminals targeted a narrow band in NOT and maintained this position to maturity (Fig. 9A–C). At no point in our developmental time series did we find tSbC RGCs axons in other pretectal areas or any retinoreceptor targets other than dLGN, vLGN, SC, and NOT (Fig. 8). Thus, tSbC RGC axons choose their targets without developmental errors, invade them simultaneously between P1 and P3, and form laminar arborization patterns that are precise from the outset.

tSbC RGC mosaics and axonal projections develop independently of Brn3

Brn3 transcription factors contribute to RGC differentiation and terminal identity (Mu and Klein, 2004; Badea et al., 2009). Brn3a, Brn3b, and Brn3c are expressed in multiple RGC types, and their contributions to RGC differentiation and axon targeting have not been studied with cell-type resolution. We generated Cck-Cre Brn3cCKOAP/CKOAP mice to explore the influence of Brn3c on tSbC RGCs (Badea et al., 2012). We observed no differences in the density of tSbC RGCs (Fig. 9A,B; Cck-Cre Brn3cCKOAP/CKOAP, 205 ± 5 cells/mm², n = 5 retinas; Cck-Cre Brn3cCKOAP+, 215 ± 15 cells/mm², n = 10, p = 1, Mann–Whitney U test), their mosaic distributions (Fig. 9A,B; exclusion zones, Cck-Cre Brn3cCKOAP/CKOAP, 31.7 ± 1.2 μm, n = 5 retinas; Cck-Cre Brn3cCKOAP+, 29.8 ± 1.3 μm, n = 10, p = 0.21, Mann–Whitney U test), or axonal targeting (Fig. 9C–H), between P20 Cck-Cre Brn3cCKOAP/CKOAP and Cck-Cre Brn3cCKOAP+ mice. We do not know when in development, Brn3c is removed in these mice, but we know that Cre-dependent alkaline phosphatase expression is detectable at least from P0 onward. Thus, the postnatal development of tSbC RGCs and their axonal projections appears to be independent of Brn3c.

Discussion

Here, we discover a transgenic intersection (Cck-Cre and Brn3cCKOAP) that marks a single RGC type in the mouse retina,
the tSbC RGC. Immunohistochemistry (Fig. 1) and single-cell RNA sequencing support the notion that tSbC RGCs coexpress Cck and Brn3c (Tran et al., 2019; Goetz et al., 2022). We use Cck-Cre Brn3cCKOAP mice to map tSbC RGC projections and study their development.

The tSbC RGCs selectively innervate four of the >50 retinorecipient brain areas, dLGN, vLGN, SC, and NOT (Fig. 2). The dLGN and SC are the main image-forming retinal targets (Morin and Studholme, 2014; Martersteck et al., 2017). Both pass information to visual cortex (among other places), dLGN directly to primary visual cortex (V1), and SC via the lateral posterior nucleus of the thalamus to postthalamic cortex (Kerschensteiner and Guido, 2017; Beltramo and Scanziani, 2019; Bennett et al., 2019). SbC responses have been recorded in dLGN, SC, and V1 (Niel and Stryker, 2008; Piscopo et al., 2013; Ito et al., 2017), suggesting that some dLGN and SC neurons receive exclusive or predominant input from tSbC RGCs to relay their signals to visual cortex and other second-order targets. In addition, high-resolution imaging of RGC axon responses suggests that SbC inputs converge with conventional retinal signals onto some dLGN neurons (Liang et al., 2018). The combination of SbC and conventional signals could serve as a contrast gain control mechanism in which SbC signals boost responses to low-contrast stimuli while avoiding response saturation to high-contrast stimuli.

The NOT is part of the accessory optic system, which controls gaze-stabilizing eye movements via the optokinetic reflex arc (Simpson, 1984). The NOT, in particular, mediates ipsiversive horizontal eye movements (Kato et al., 1986; Yukishin et al., 2000; Macé et al., 2018). NOT neuron light responses have, to our knowledge, not been characterized in mice. In rabbits, they are unfailingly direction selective (Collewijn, 1975). NOT is strongly innervated by ON direction-selective RGCs in rabbits and mice (Pu and Amthor, 1990; Dhande et al., 2013). Thus, it seems likely that tSbC RGCs converge with ON direction-selective RGCs onto NOT neurons, possibly for contrast gain control. It seems less likely that tSbC RGC signals are maintained as a separate information stream in this target. Why tSbC RGCs do not innervate the medial terminal nucleus—the nucleus of the accessory optic system mediating gaze-stabilizing vertical eye movements—is unclear.

The vLGN has recently been shown to mediate influences of light on mood and learning (Huang et al., 2019, 2021). The vLGN also regulates defensive responses to visual threats (Fratzl et al., 2021; Salay and Huberman, 2021), but this regulation appears to be independent of its light responses and retinal input (Fratzl et al., 2021). The vLGN is subdivided into a retinorecipient external (vLGNe) and a nonretinorecipient internal (vLGNi) layer. A recent study identified transcriptionally distinct cell types arranged in four sublaminae within vLGNe (Sabbagh et al., 2020). Our labeling distribution suggests that tSbC RGCs provide input to the outer two layers of vLGN (Figs. 2, 5).

The laminar targeting of axons in the other retinorecipient areas suggests that tSbC RGCs provide input to specific circuits in these targets as well. It will be interesting to see whether and where tSbC RGCs axons overlap with axons of other SbC RGC types (Jacob and Schwartz, 2018; Wienbar and Schwartz, 2022). Our discovery of a genetic intersection to target tSbC RGCs should allow us in the future to selectively perturb these cells to clarify their contributions to visual processing and behaviors mediated by the targets identified here.

A previous study found that early-born RGC axons grow along the optic tract prenatally and transiently innervate more targets than they ultimately maintain (Osterhout et al., 2014). In contrast, late-born RGC axons grow along the optic tract postnatally and make few, if any, targeting errors (Osterhout et al., 2014). We find that tSbC RGC axons span the length of the optic tract at P0, indicating that they grow prenatally. They remain poised there until around P3, when they simultaneously and without error innervate their four targets (Figs. 5–9), revealing further cell-type-specific diversity in the developmental strategies of retinofugal projections.

Some axons of RGCs refine their terminal arbors slowly during postnatal development to attain specific laminar positions in their target areas (Kim et al., 2010; Hong et al., 2014; Osterhout et al., 2014). We find that tSbC RGC axons establish mature lamination patterns shortly after invading their targets (Figs. 5–9). Brn3 transcription factors are important nodes of the gene regulatory networks that govern RGC differentiation and terminal identity, and Brn3c has been suggested to shape axon projection patterns (Wang et al., 2002; Mu and Klein, 2004; Badea et al., 2009). Using cell-type-specific Brn3c deletion and axon mapping, we find no difference in the patterns of tSbC RGC axons developing with and without Brn3c (Fig. 10). The molecular mechanisms that drive tSbC RGC axon growth keep tSbC RGC axons poised in the optic tract, make them simultaneously invade their targets, and guide them to the correct laminar position, thus, remain unknown. The developmentally stable and selective genetic intersection we identify for tSbC RGC targeting should facilitate future efforts to uncover these mechanisms.

Finally, we find that tSbC RGCs innervate the ipsilateral vLGN and dLGN but only the contralateral SC and NOT (Fig. 3). This could indicate that only a subset of tSbC RGCs innervates SC and NOT or that some tSbC RGC axons bifurcate at the optic chiasm to innervate targets on both sides of the brain. Both options raise interesting developmental and molecular questions and could be distinguished using single-cell labeling approaches in future studies (Hong et al., 2011; Fernandez et al., 2016; Herrera et al., 2019; Mason and Slavi, 2020).

References


