

Architecture and Activity-Mediated Refinement of Axonal Projections from a Mosaic of Genetically Identified Retinal Ganglion Cells

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SUMMARY

Our understanding of how mammalian sensory circuits are organized and develop has long been hindered by the lack of genetic markers of neurons with discrete functions. Here, we report a transgenic mouse selectively expressing GFP in a complete mosaic of transient OFF- α retinal ganglion cells (tOFF- α RGCs). This enabled us to relate the mosaic spacing, dendritic anatomy, and electrophysiology of these RGCs to their complete map of projections in the brain. We find that tOFF- α RGCs project exclusively to the superior colliculus (SC) and dorsal lateral geniculate nucleus and are restricted to a specific laminar depth within each of these targets. The axons of tOFF- α RGC are also organized into columns in the SC. Both laminar and columnar specificity develop through axon refinement. Disruption of cholinergic retinal waves prevents the emergence of columnar-but not laminar-specific tOFF- α RGC connections. Our findings reveal that in a genetically identified sensory map, spontaneous activity promotes synaptic specificity by segregating axons arising from RGCs of the same subtype.

INTRODUCTION

The transmission of topographically organized sensory information poses a substantial problem for central nervous system (CNS) development. Sensory axons must travel long distances to reach their targets yet still maintain their local relationships so that the map is preserved. The current model is that molecular guidance cues steer axons through a series of decision points leading to their targets and then spontaneous neural activity and guidance cues refine those projections into an orderly, continuous topographic map (McLaughlin and O'Leary, 2005; Luo and Flanagan, 2007). Within this general model, however, several aspects of the construction of mammalian sensory circuits remain poorly understood. For instance, a hallmark feature of sensory pathways is their parallel organization: different neurons

respond to and convey qualitatively distinct information about a given sensory modality (Callaway, 2005). How axons carrying distinct types of sensory information are organized within continuous topographic maps and how those projections develop is unclear. A major obstacle for studying this aspect of circuit wiring—often called “synaptic specificity” (Benson et al., 2001) is a lack of genetic markers that ensure stable, selective, and complete labeling of functionally identified classes of neurons in mammals. Several basic and important questions related to development of sensory circuits in the mammalian CNS therefore remain unresolved. For example, it is unknown whether emergence of synaptic specificity involves refinement of initially inaccurate projections or whether functionally specific circuits are accurately formed from the outset, as they are in the invertebrate nervous system (Meinertzhagen and Hanson, 1993). A related question is whether neural activity contributes to the emergence of synaptic specificity.

In principle, the connections associated with different types of retinal ganglion cells (RGC) would be ideal for exploring the emergence of synaptic specificity in the mammalian CNS. Conventional dye labeling and electrophysiological recording techniques indicate that there are approximately two dozen anatomically and physiologically distinct subtypes of RGCs (reviewed in Wässle, 2004; Berson, 2008). Each RGC subtype receives qualitatively distinct information about the content of the visual scene by virtue of its laminar-specific dendritic connections with bipolar and amacrine cells in the inner plexiform layer (IPL) (Roska and Werblin, 2001) and in turn, conveys that information to the brain by sending axonal projections to specific retinorecipient nuclei (Berson, 2008). Despite extensive study of the physiology and connectivity associated with different RGCs, the total number of functionally distinct RGC subtypes and the complete wiring diagram corresponding to any one individual RGC subtype still remains unclear (Sun et al., 2002; Kong et al., 2005; Berson, 2008).

In order to study the organization and development of neural circuitry corresponding to distinct RGC subtypes, we screened a library of BAC transgenic mice with GFP expressed under the control of different promoters (Gong et al., 2003). Our goal was to identify mouse lines in which (1) GFP is expressed in a mosaic of RGCs throughout the retina—because mosaic spacing suggests that cell population is comprised of a functionally homogeneous RGC subtype (Wässle, 2004) and (2) there are few

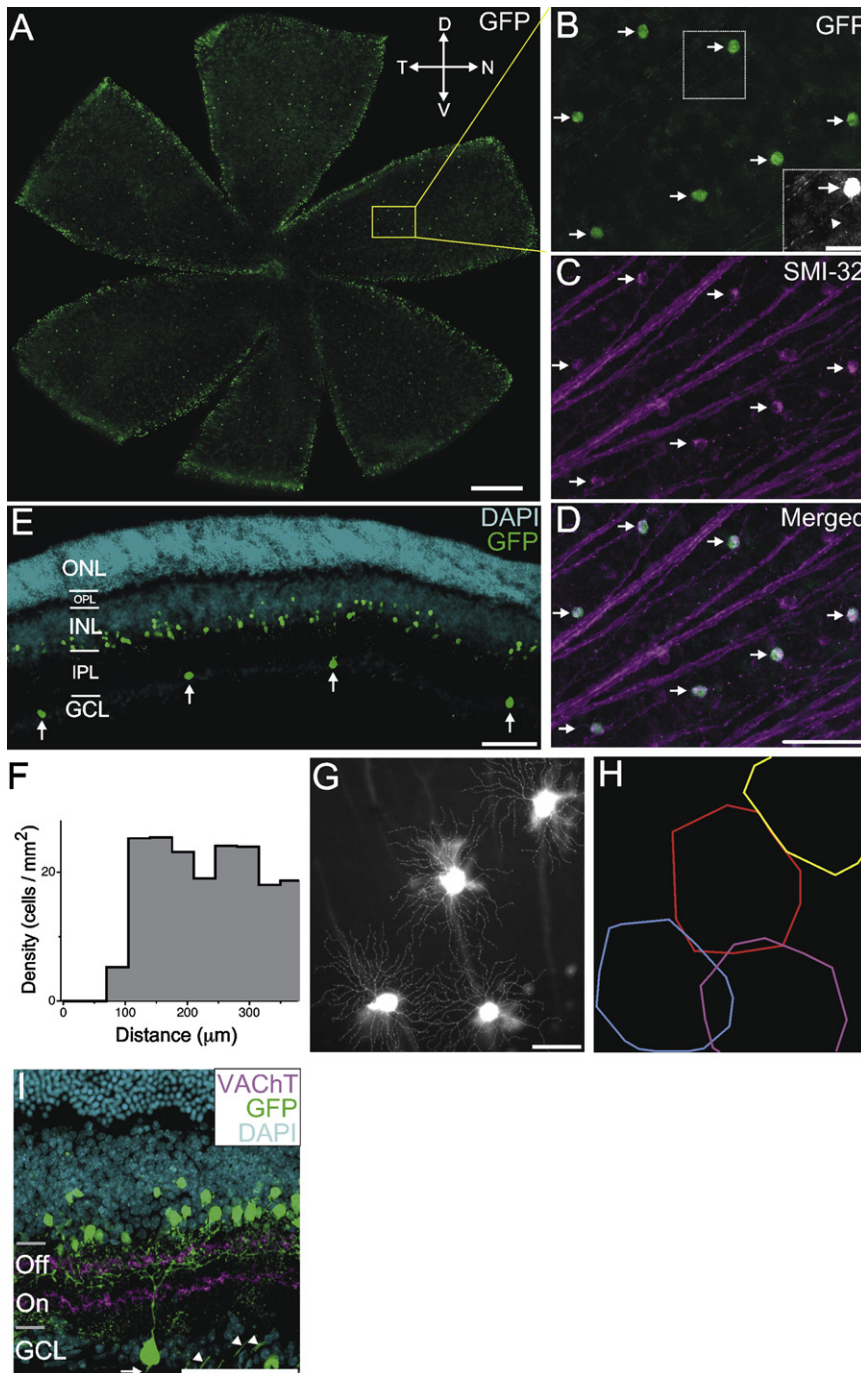


Figure 1. A Mosaic of GFP⁺ OFF- α RGCs in CB2-GFP Mice

(A) Whole-mount CB2-GFP retina, showing GFP⁺ RGCs. Along the relieving cuts, GFP⁺ amacrine cells are also evident (see [E]). D/V/N/T: dorsal, ventral, nasal, temporal axes. Scale is 500 μ m. (B–D) High magnification of the boxed region in (A). (B) GFP⁺ RGCs (arrows). Inset shows a single GFP⁺ RGC (arrow) with its axon (arrowhead). (C and D) Every GFP⁺ RGC is immunopositive for SMI-32 (SMI-32⁺). Scale in (B)–(D) is 125 μ m. Scale in (B) inset is 50 μ m. (E) Vertically-sectioned retina. Blue, DAPI staining indicates cellular layers. GFP⁺ cells are amacrine cells in the inner nuclear layer (INL), and regularly spaced RGCs in the GCL (arrows). Scale is 100 μ m. (F) Density profile of GFP⁺ RGC cells as a function of distance from other GFP⁺ RGCs (Rodieck, 1991). (G) Four neighboring GFP⁺ RGCs filled with Dil to reveal their entire dendritic arbors. (H) Dendritic field boundaries of the same four GFP⁺ cells. (G and H) Scale is 75 μ m. (I) Retinal section stained for DAPI, GFP, and the vesicle-acetylcholine transporter (VAcHT) which labels the middle of the ON and OFF sublaminae in the IPL (horizontal lines denote boundaries of IPL). GFP⁺ RGC dendrites ramify within the OFF sublaminae at the \sim 30%–35% depth of the IPL. Arrow indicates the GFP⁺ RGC's axon. Arrowheads indicate axons from other GFP⁺ RGCs whose somata lie outside the field of view. Scale is 75 μ m.

ulation of regularly spaced cells throughout the ganglion cell layer (GCL) (Figures 1A, 1B, and 1E; mean = 368 ± 14 GFP⁺ RGCs per retina; n = 6 mice). The presence of an axon emanating from each GFP⁺ cell confirmed they were RGCs (Figure 1B, inset). In addition, as expected for RGCs, all the GFP⁺ cells in the RGC layer degenerated in response to optic nerve transection (data not shown). Every GFP⁺ RGC stained with the antibody SMI-32 (Figures 1B–1D), which recognizes a neurofilament antigen enriched in α RGCs (Peichl, 1991). In addition,

or no cells expressing GFP in retinorecipient brain areas, so the organization of axonal projections from the GFP-expressing RGCs can be visualized during development and adulthood.

RESULTS

A Transgenic Mouse with a Complete Mosaic of One RGC Subtype Expressing GFP

Calretinin-EGFP mice (CB2-GFP mice) met both criteria of our screen. In the retinas of these mice, GFP is expressed by a pop-

tion, the relatively large (\sim 20–25 μ m) and spherical GFP⁺ somata resembled mouse α RGCs (Pang et al., 2003; Schubert et al., 2005; Volgyi et al., 2005). In CB2-GFP mice, GFP is also expressed by a population of amacrine cells in the inner nuclear layer (INL) (Figures 1A and 1E). Based on their morphology and calretinin expression (Lee et al., 2004) we assume those to be All amacrine, although they may include other amacrine types. In vertical retinal sections (Figure 1E), the GFP⁺ amacrine cells in the INL and the regularly spaced GFP⁺ RGCs (Figure 1E, arrows) were both clearly visible. The somas of GFP⁺ RGCs formed

a highly regular mosaic across the entire retina. This distribution was not random, as cell bodies obeyed a minimum spacing rule, avoiding each other to a distance of approximately 100 μm (Figure 1F). To visualize clearly the dendrites of single cells, we targeted individual GFP⁺ RGCs with electrodes and filled them with Dil ($n = 22$ RGCs from 5 mice). The dendrites of neighboring GFP⁺ RGCs tiled the retina with the amount of overlap typical of mouse OFF- α RGCs (Figure 1G and see Figure S1 available online; Schubert et al., 2005; Volgyi et al., 2005). The Dil fills also revealed that the GFP⁺ RGCs possessed dendritic features characteristic of mouse OFF- α RGCs (Figures 1G, 1H, and S1; Pang et al., 2003; Schubert et al., 2005; Volgyi et al., 2005). We observed that, in every case, the dendrites of the GFP⁺ RGCs were monostratified at the inner 30%–35% depth of the IPL (Figure 1G), within the “OFF” sublaminae (Famiglietti and Kolb, 1976). When the retina was sectioned in the vertical plane and stained for the vesicle acetylcholine transporter (VAcHT) (which delineates ON and OFF sublaminae in the IPL), it was apparent that every GFP⁺ RGC projected its dendrites exclusively into the OFF sublaminae (Figure 1I; >600 GFP⁺ RGCs examined from 12 CB2-GFP mice). Collectively, these observations indicate that a mosaic of OFF- α RGCs selectively express GFP in this mouse line.

We next recorded intracellularly from the GFP⁺ RGCs by visually targeting the cells with a microelectrode under a fluorescence microscope. In response to a flashing visual stimulus, these cells showed transient OFF-type responses, depolarizing and spiking briefly at the offset of a flash (Figure 2A). Visual responses of different GFP⁺ RGCs were very homogeneous, in that the average of each cell’s flash response was highly correlated with the population average (correlation coefficient $r = 0.93 \pm 0.02$, $n = 7$; Figure 2B). Further characterization of these cells using a randomly flickering visual stimulus confirmed the transient OFF-type nature of these cells, which showed a biphasic temporal response that peaked at 105–121 ms relative to the visual stimulus ($n = 3$; Figures 2C and 2D). Taken with our anatomical observations, we conclude that a retinotopic mosaic of transient OFF- α RGCs (tOFF- α RGCs) expresses GFP in this mouse line.

A Highly Organized Map of the tOFF- α RGC Mosaic in the Brain

To determine the connections made by tOFF- α RGCs in the brain we injected cholera toxin beta conjugated to Alexa 594 (CTb-594; red label in Figures 3–8), which labels the axons of all RGCs, into both eyes of CB2-GFP mice and then examined the brain for axons that were both CTb-594⁺ and GFP⁺. Of the approximately two dozen brain regions targeted by RGC axons, tOFF- α RGCs project to only two of these: (1) the superior colliculus (SC), a midbrain structure involved in sensory-motor integration and (2) the dorsal lateral geniculate nucleus (dLGN), a fore-brain structure that filters and relays visual information to primary visual cortex.

The mouse SC receives projections from many different types of RGCs and contains a two-dimensional topographic map of the entire contralateral retina. The dorsal-ventral axis of the retina is mapped along the lateral-medial axis of the SC and the temporal-nasal retinal axis is mapped along the rostral-caudal axis of the SC (Dräger and Hubel, 1976; May, 2005). When the SC is

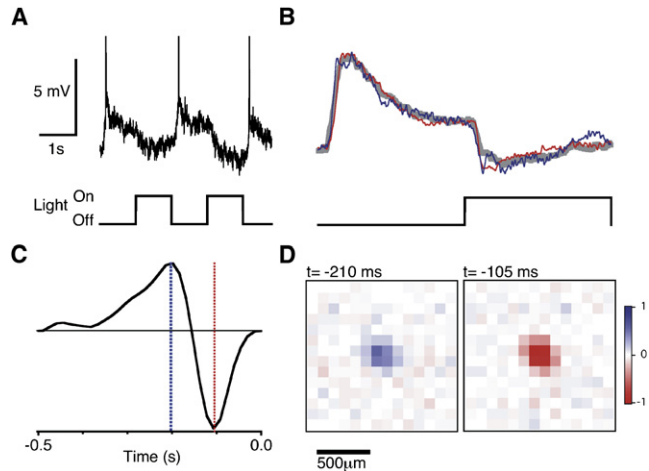


Figure 2. Visual Responses of GFP⁺ tOFF- α RGCs

(A) Membrane potential response of a GFP⁺ tOFF- α RGC in response to a 1 s periodic flash. Action potentials are truncated.

(B) Average subthreshold flash response of seven cells (gray trace), compared to the average flash response of two example cells (colored traces). Spikes were digitally removed prior to averaging.

(C and D) Spatiotemporal receptive field of a GFP⁺ tOFF- α RGC computed from the membrane potential response of the cell to a randomly flickering checkerboard. (C) Temporal filter averaged across the spatial receptive field center. (D) Two spatial slices of the receptive field taken at the times indicated by the colored lines in (C), indicating the biphasic nature of the receptive field center.

viewed in the coronal plane (Figure 3A) the axons of RGCs can be seen entering the SC deep to the pial surface, in the stratum opticum (SO), and their dense terminal arborizations can be observed in the overlying stratum griseum superficiale (SGS) (Figure 3A). As the main retinorecipient division of the SC, the SGS is divided into lower (ISGS) and upper (uSGS) lamina based on the types of afferent input and postsynaptic cells residing along its depth. A limited number of RGC axons also project to the stratum zonale (SZ), a thin layer located just below the pial margin (May, 2005). We observed that the GFP⁺ axons of tOFF- α RGCs terminate in a highly specific and stereotyped manner within the SC. Their axons entered the SC through the SO, along with all other RGC axons, and terminated in the overlying SGS. Within the SGS, however, the GFP⁺ arbors of tOFF- α RGCs were faithfully restricted to the ISGS (Figures 3B and 3C and Figures 3B' and 3C'), avoiding the uSGS and SZ entirely. Viewed in the sagittal plane, laminar-specific targeting of tOFF- α RGC projections to the ISGS was also apparent (Figure 3D). When one eye was removed, all GFP⁺ axons disappeared from the contralateral SC, indicating that the GFP⁺ axons observed in each hemisphere of the SC arise solely from tOFF- α RGCs in the contralateral retina (Figure S2). Retrograde labeling experiments indicated that every GFP⁺ tOFF- α RGC projects to the SC (data not shown). Taken with the fact that the GFP⁺ axons are restricted to the ISGS across the full lateral-medial (Figures 3B and 3C) and rostral-caudal (Figure 3D) extent of the SC, we can conclude that every tOFF- α RGC obeys strict laminar specificity of its axonal projection to this target, regardless of its retinotopic position.

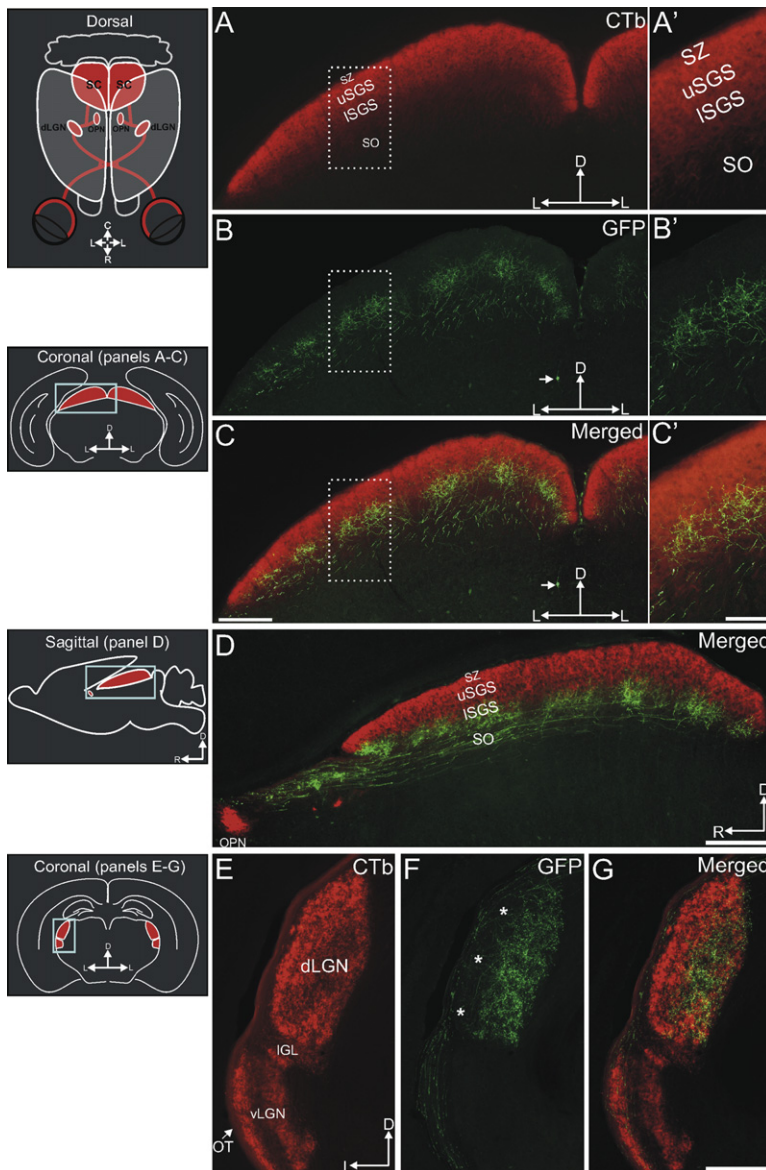


Figure 3. Axonal Projections of GFP⁺ tOFF- α RGCs

(Left margin) Schematics of RGC projections in the mouse. (Top left) Dorsal view. The retinas and optic pathways are in red. Superior colliculus (SC), dorsal lateral geniculate nucleus (dLGN), and olivary pretectal nucleus (OPN). Here and in (A–G) R: rostral, C: caudal, L: lateral, M: medial. Schematics of coronal and sagittal view of the brain at the level of the SC (middle panels) and dLGN (bottom panel).

(A–D) Laminar- and columnar-specific tOFF- α RGC axonal projections to the SC. (A) CTb-594 labeling (red) of all RGC axons. CTb-594 weakly labels RGC axon shafts but densely labels RGC axon terminals and thereby completely labels all retinorecipient nuclei. SO, stratum opticum; ISGS, lower stratum griseum superficiale; uSGS, upper stratum griseum superficiale; SZ, stratum zonale. (A') High-magnification view of box in (A). (B) GFP⁺ tOFF- α RGC axons in the SC. The axons enter the SC through the SO, and selectively arborize in the ISGS. The GFP⁺ arbors are also organized into columns. Small arrow indicates a GFP⁺ cell in the deeper SC; such cells do not contribute to the GFP⁺ axons in the retinorecipient SC (see Figure S2). (B') High-magnification view of box in (B). (C) Merged view of (A) and (B). (C') High-magnification view of box in (C). (A–C) Scale is 250 μ m. Coronal plane is shown; D: dorsal, L: lateral. Scale in (A')–(C') is 125 μ m. (D) Sagittal view of CTb-594 and GFP⁺ tOFF- α RGC axons in the SC. Scale is 250 μ m.

(E–G) Retino-dLGN projections viewed in the coronal plane. (E) CTb-594 labeled RGC axons fill the entire dLGN, IGL, and vLGN. OT, optic tract. (F) GFP⁺ tOFF- α RGC axons bypass the vLGN and IGL, turn medially into the dLGN, and project through the lateral third of the dLGN (asterisks) to selectively arborize in the inner dLGN, forming a “layer.” (G) Merged view of (E) and (F). Scale is 250 μ m.

Another salient feature of the tOFF- α RGC projections to the SC was that, within the ISGS, their arbors were regularly spaced to form patches of innervated and noninnervated territory (Figures 3A–3D). Based on their regular increased density of projections within the topographic map, we refer to these structures as columns. Retinocollicular projections are known to be organized into columns of alternating right-eye and left-eye RGC input in cats (May, 2005). The GFP⁺ columns observed in the SC of CB2-GFP mice do not relate to eye specificity, however, because all the GFP⁺ axons in the SC originate from the contralateral retina (Figure S2).

We counted \sim 90 GFP⁺ columns per SC hemisphere (88–96 columns; $n = 6$ mice). Given that an entire mosaic of \sim 370 tOFF- α RGCs expresses GFP and projects to the contralateral SC, we could not determine how many tOFF- α RGCs project to each column. In the caudal SC, however, RGC axons were

sparse enough that we could observe cases where individual GFP⁺ axons bifurcated to terminate in two neighboring, separate columns (Figure S3). Because the degree of retinocollicular convergence varies according to position within the retinotopic map (Dräger and Hubel, 1976; Berson, 1988), it is unclear if such bifurcation is a general rule for all tOFF- α RGCs or is limited to the caudal SC. We observed regularly spaced GFP⁺ columns across all axes of the SC (Figures 3A–3D), indicating that there is a highly organized columnar map corresponding to the mosaic of tOFF- α RGCs within this target.

Unlike the dLGN of carnivores and primates (Callaway, 2005), the rodent dLGN lacks overt laminar cytoarchitecture corresponding to eye-specific inputs or to functionally specialized visual processing streams (Reese, 1988). We were therefore surprised to observe strict laminar specificity of tOFF- α RGC projections to the dLGN. GFP⁺ axons projected through the lateral third of the dLGN to selectively terminate within the inner/medial portion of the nucleus, where they form a well-demarcated “layer” (Figures 3E–3G). Eye removal experiments indicated that the GFP⁺ axons projecting to the dLGN arose exclusively from the contralateral retina (Figure S2). Given that every GFP⁺ RGC projects to the SC, the layer-specific tOFF- α RGC projections to the dLGN must be the collaterals of retinocollicular axons. Together, the presence of laminar- and columnar-specific

projections to the SC and the laminar-specific projections to dLGN indicates there is a highly precise brain map associated with the tOFF- α RGC mosaic.

Emergence of Laminar and Columnar Specificity Involves Axonal Refinement

How do the highly specific patterns of axonal connections made by tOFF- α RGCs arise during development? This could occur by a mechanism in which each RGC axon is guided directly and precisely to its proper layer and column(s), as has been described for retinal projections in the fly and in the chick (Meinertzhagen and Hanson, 1993; Inoue and Sanes, 1997). Alternatively, tOFF- α RGC axons could at first project broadly within their targets, and subsequently refine to achieve laminar and columnar specificity, as is the case for retinotopic and eye-specific mapping in mammals (McLaughlin and O'Leary, 2005; Torborg and Feller, 2005). Because tOFF- α RGC projections to the SC exhibit both laminar and columnar specificity (Figure 3) and because the SC develops relatively early (Edwards et al., 1986), we examined the development of these features in the retinocollicular pathway.

Studies in mice and hamsters have shown that RGC axons reach the SC slightly before birth and initially overshoot their correct retinotopic termination zone. Between postnatal day 0 (P0) and \sim P4, RGC axons retract within the SO, establishing a coarse retinotopic map (McLaughlin and O'Leary, 2005). Subsequently, RGC axons elaborate terminal arbors in the overlying SGS (Sachs et al., 1986). We observed that on P4, the axons of tOFF- α RGCs do not exhibit their mature pattern of laminar or columnar specificity (Figures 4A–4C'). While tOFF- α RGC axons tended to arborize in the ISGS and rudimentary columns were evident (Figures 4A–4C), it was readily apparent that many GFP⁺ tOFF- α RGC axons extend across the entire retinorecipient depth of the SC (Figures 4A–4C'), a pattern never observed in adulthood (Figure 3). Indeed, on P4, many GFP⁺ axons projected all the way up to the pial margin of the SC (arrows in Figures 4B' and 4C'). On P10, by contrast, laminar and columnar specificity of tOFF- α RGC retinocollicular projections resembled the adult pattern (Figures 4D–4F'). Quantitative analysis confirmed that a significantly greater number of GFP⁺ axons project to the uSGS and SZ (the upper half of the retinorecipient SC; 0%–50% depth from the pial margin) in P4 mice as compared to P10 or adult mice (Figure 4G). There was also significantly less columnar specificity of tOFF- α RGC retinocollicular projections in P4 mice compared to P10 or adult mice (Figure 4H).

It is unlikely that the lack of laminar and columnar specificity observed in P4 CB2-GFP mice is related to immaturity of the SC layers or to increased numbers of RGCs expressing GFP at this age because development of the retinorecipient SC layers and RGC apoptosis are completed by P4 (Edwards et al., 1986; Farah and Easter, 2005). Indeed, we found that both the thickness of the retinorecipient SC (Figure 4I) and the number of GFP⁺ RGCs per retina were indistinguishable between P4, P10 and adult CB2-GFP mice (Figure 4J). Also, as in the adult mouse (Figure S2), the GFP⁺ axons observed in the SC of developing CB2-GFP mice arise solely from tOFF- α RGCs in the contralateral retina and not from other sources (Figure S4). We therefore conclude that the develop-

ment of laminar- and columnar-specific retinocollicular projections involves axonal retraction and refinement. Thus, in addition to any potential mechanisms that direct tOFF- α RGC axons to their correct laminar and columnar positions, there must also be mechanisms in place to remove the incorrect projections made by these RGCs during early postnatal development.

Cholinergic Spontaneous Retinal Activity Regulates Emergence of Columnar Specificity

Laminar and columnar specificity of tOFF- α RGC projections to the SC emerges between P4 and P10, which is prior to the onset of vision (Demas et al., 2003). During this period, however, "waves" of neural activity sweep across the retina, inducing correlated firing of neighboring RGCs (Wong, 1999). Given that P4 to P10 coincides with the presence of cholinergic-mediated retinal waves (Bansal et al., 2000; McLaughlin et al., 2003), we tested the role of that activity on the emergence of laminar- and columnar-specific tOFF- α RGC projections to the SC and the dLGN.

In the first set of experiments, we mated CB2-GFP mice to mice lacking the β 2-subunit of the nicotinic acetylcholine receptor (β 2nAChR^{-/-}) (Picciotto et al., 1995; Xu et al., 1999) to generate CB2-GFP; β 2nAChR^{-/-} mice. β 2nAChR^{-/-} mice exhibit significantly altered patterns of RGC bursting during the first 8 days of postnatal life, after which spontaneous and visually evoked RGC activity returns to normal (Bansal et al., 2000; McLaughlin et al., 2003). At P4/5, the degree of laminar- and columnar-specificity in the SC was qualitatively and quantitatively indistinguishable between CB2-GFP; β 2nAChR^{+/+} (WT) mice, CB2-GFP; β 2nAChR^{+/-} mice, and CB2-GFP; β 2nAChR^{-/-} mice (Figure 5). On P10, however, CB2-GFP; β 2nAChR^{-/-} mice exhibited a severely abnormal pattern of tOFF- α RGC projections to the SC (Figures 6A–6F). Whereas overall target selection and laminar specificity were normal in the SC (Figures 6A–6E) and in the dLGN (Figure 7), there was an obvious reduction in columnar specificity among tOFF- α RGC axons in the SC of CB2-GFP; β 2nAChR^{-/-} mice (Figures 6C, 6D, and 6F).

Remarkably, even though spontaneous retinal activity in β 2nAChR^{-/-} mice returns to normal by P8 (Bansal et al., 2000; McLaughlin et al., 2003; Torborg and Feller, 2005), columnar specificity remained severely abnormal in adult CB2-GFP; β 2nAChR^{-/-} mice (Figures 6F and 6I). This shows there is a critical period for columnar segregation of tOFF- α RGC projections that is mediated by β 2nAChR-mediated spontaneous retinal activity during the first postnatal week. Importantly, the number of GFP⁺ tOFF- α RGCs and the retinorecipient thickness of the SC were both normal in CB2-GFP; β 2nAChR^{-/-} mice (Figures 6G and 6H). We thus attribute the permanent loss of columnar specificity in CB2-GFP; β 2nAChR^{-/-} mice to a lack of β 2nAChR-mediated RGC activity and not to altered RGC survival or indirect effects of the mutation on SC cells.

β 2nAChR^{-/-} mice lack β 2nAChRs throughout the brain, including in the SC where they are normally expressed (Picciotto et al., 1995; Xu et al., 1999). So even though several aspects of RGC axon targeting are normal in CB2-GFP; β 2nAChR^{-/-} mice (Figures 6 and 7), the above experiments do not unequivocally determine whether cholinergic spontaneous retinal activity instructs columnar-specific refinement of tOFF- α RGC projections

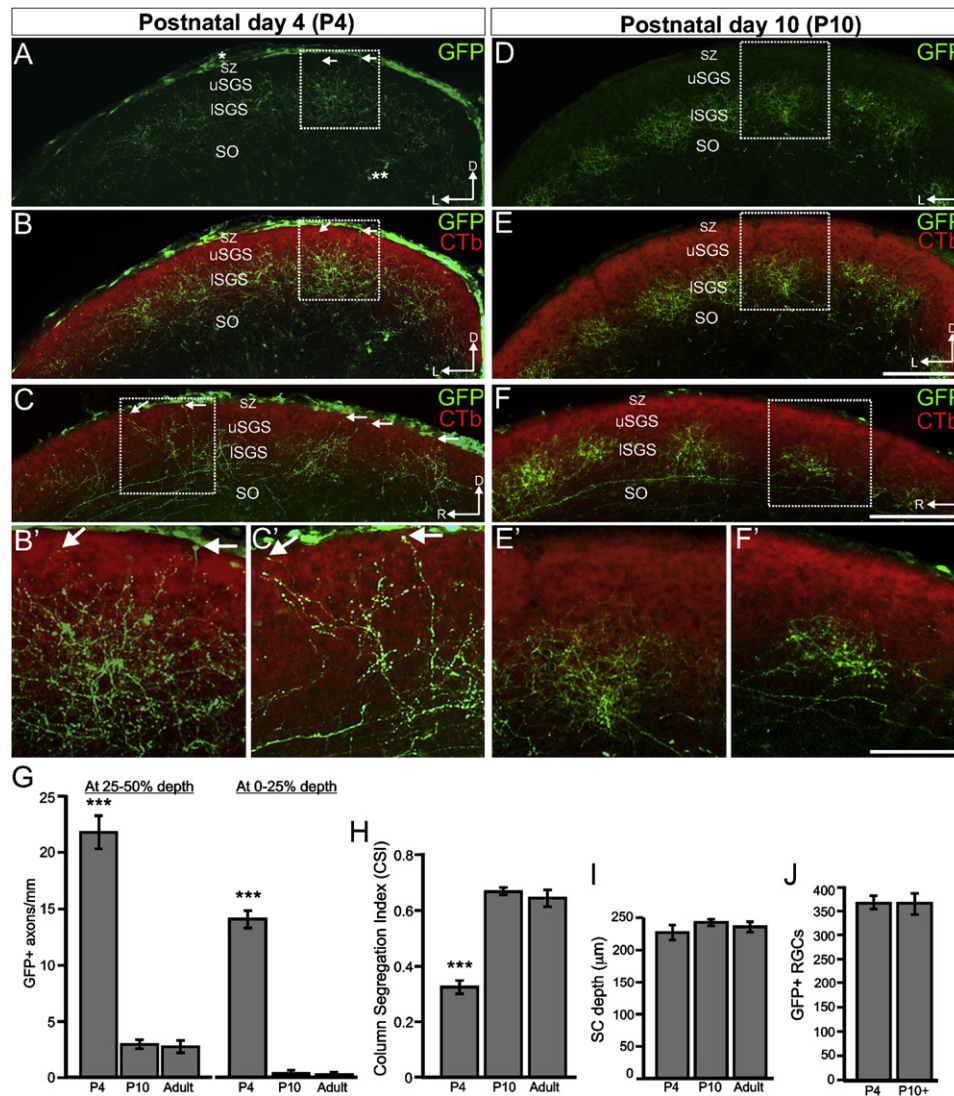


Figure 4. Development of Laminar- and Columnar-Specific tOFF- α RGC Projections Involves Axonal Refinement

(A–C) Targeting of tOFF- α RGC axons to the SC is imprecise early in development. (A and B) Coronal view of the SC in a P4 CB2-GFP mouse. tOFF- α RGC axons exhibit rudimentary columnar and laminar specificity, but unlike in adult CB2-GFP mice (Figure 3), many GFP⁺ axons project into the uSGS and SZ, and up to the pial margin (arrows). (B') Higher-magnification view of the boxed region in (A) and (B). Arrows indicate GFP⁺ axons projecting to the uppermost SC. (C and C') Sagittal view of the SC on P4. GFP⁺ axons are distributed across the retinorecipient depth of the SC. Arrows indicate GFP⁺ axons projecting to the pial margin (arrows). Asterisks in (A) indicate GFP⁺ glia at the pial margin and a few GFP⁺ neurons in the deeper, non-retinorecipient SC (see Figure 3). GFP expression in these glia disappears by \sim P8. Neither the GFP⁺ glia nor GFP⁺ SC neurons contribute to the GFP⁺ axons in the young or adult SC (Figures S2 and S3).

(D–F') Targeting of tOFF- α RGC axons to the SC is adult-like by P10; GFP⁺ axons are restricted to the ISGS and segregated into distinct columns. (A–F) Scale is 250 μ m. (B', C', E', and F') Scale is 125 μ m.

(G) Quantification of laminar specificity as a function of age (see Experimental Procedures). The number of GFP⁺ axons/mm projecting to the 25%–50% (upper third) and 0%–25% (upper quarter) of the SC depth is significantly greater on P4 compared to P10 or adult ($p < 0.0001$). P10 versus adult were not significantly different (25%–50% depth, $p = 0.74$; 0%–25% depth, $p = 0.23$; t test, \pm SEM) ($n = 4$ adult mice, $n = 6$ P10 mice, $n = 7$ P4 mice).

(H) Columnar segregation index (see Experimental Procedures). There is significantly less columnar specificity on P4, as compared to P10 or adult ($p < 0.0001$), whereas columnar specificity is indistinguishable between P10 and adult ($p = 0.53$) ($n = 3$ mice per age).

(I) The depth (retinorecipient thickness) of the SC is not different between P4, P10, or adult CB2-GFP mice (P4 versus P10, $p = 0.3$; P4 versus adult, $p = 0.48$; t test; $n = 5$ P4 mice, $n = 4$ P10 mice, $n = 4$ adult mice).

(J) The number of GFP⁺ RGCs per retina is not different on P4 compared to P10 and adult (P10+) ($p = 0.96$; t test; \pm SEM; $n = 5$ P4 mice, $n = 4$ P10 mice, and $n = 4$ adult mice).

or whether there is a more general requirement for β 2nAChRs for columns to develop. To address this, we injected epibatidine (epi), a cholinergic agonist that perturbs spontaneous RGC

bursting in the early postnatal retina (Penn et al., 1998; Cang et al., 2005) into one eye, and we injected saline into the other eye of CB2-GFP; β 2nAChR^{+/+} (WT) mice every 48 hr from P4 to

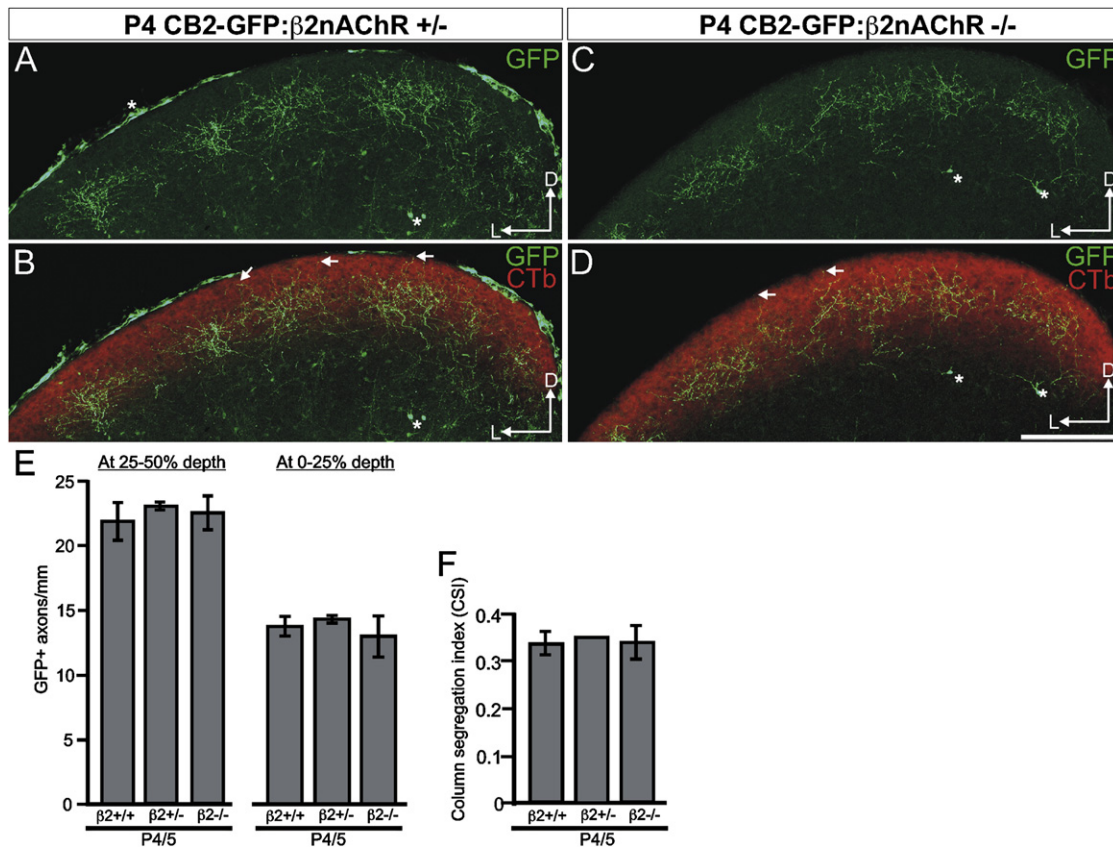


Figure 5. Genetic Removal of $\beta 2nAChR$ s Does Not Alter the Initial Targeting of tOFF- α RGC Axons

(A–D) On P4, targeting of tOFF- α RGC axons to the SC is normal in CB2-GFP: $\beta 2nAChR^{+/+}$ mice and CB2-GFP: $\beta 2nAChR^{-/-}$ mice. Coronal view of the SC in a P4 CB2-GFP: $\beta 2nAChR^{+/+}$ mouse (A and B) and a P4 CB2-GFP: $\beta 2nAChR^{-/-}$ mouse (C and D). As in WT P4 mice (Figure 4) laminar and columnar specificity are not mature at this age. Arrows indicate GFP⁺ axons that project up near the pial margin. Scale is 250 μ m.

(E) Quantification of laminar specificity for each genotype on P4/5; laminar specificity is indistinguishable between WT and $\beta 2nAChR^{+/+}$ mice (25%–50% depth, $p = 0.74$; 0%–25% depth, $p = 0.54$), between $\beta 2nAChR^{+/+}$ and $\beta 2nAChR^{-/-}$ mice (25%–50% depth, $p = 0.82$; 0%–25% depth, $p = 0.45$), and between WT and $\beta 2nAChR^{-/-}$ mice (25%–50% depth, $p = 0.73$; 0%–25% depth, $p = 0.60$), indicating that all genotypes start out with similar patterns of axon targeting on P4/5. (F) Quantification of columnar specificity across genotypes on P4/5 (WT versus $\beta 2nAChR^{+/+}$, $p = 0.67$; $\beta 2nAChR^{+/+}$ versus $\beta 2nAChR^{-/-}$, $p = 0.61$; WT versus $\beta 2nAChR^{-/-}$, $p = 0.97$).

Error bars are \pm SEM.

P10. We then examined the pattern of tOFF- α RGCs projections to the SC on P10. Since all the GFP⁺ tOFF- α RGCs in each eye project to the contralateral SC (Figures S2 and S4), we could directly compare the retinocollicular projections from the epi-treated retina to the retinocollicular projections from the saline-treated retina, in the two SC hemispheres of the same mouse. In the SC hemisphere contralateral to the saline-injected eye, tOFF- α RGC axons were restricted normally to the ISGS and organized into distinct columns (Figures 8A and 8B). In the SC hemisphere contralateral to the epi-treated eye, laminar specificity was normal but columnar specificity was perturbed (Figures 8A and 8B). The defects in columnar specificity induced by epi treatment were nearly as severe as those observed in CB2-GFP: $\beta 2nAChR^{-/-}$ mice (Figure 8C). We therefore conclude that $\beta 2nAChR$ -mediated spontaneous activity that occurs in the retina from P4–P10 mediates columnar-specific refinement of tOFF- α RGC projections to the SC.

DISCUSSION

Elucidation of the Map of Projections from a Mosaic of RGCs

We provide the first description of a complete mosaic of RGCs (tOFF- α RGCs) selectively labeled with GFP. This enabled us to relate the mosaic spacing, dendritic anatomy, and electrophysiology of these RGCs with their complete map of connections in the brain. Mouse tOFF- α RGCs are thought to be homologous to OFF-brisk-transient-Y RGCs in cats and monkeys (Pang et al., 2003; Schubert et al., 2005; Volgyi et al., 2005; Berson, 2008). However, the complete pattern of projections arising from tOFF- α /Y RGCs has not previously been elucidated. We find here that tOFF- α RGCs are remarkably specific with respect to which brain nuclei they project to, and the organization of their projections within the retinotopic maps of those nuclei. In the mouse, tOFF- α RGCs project

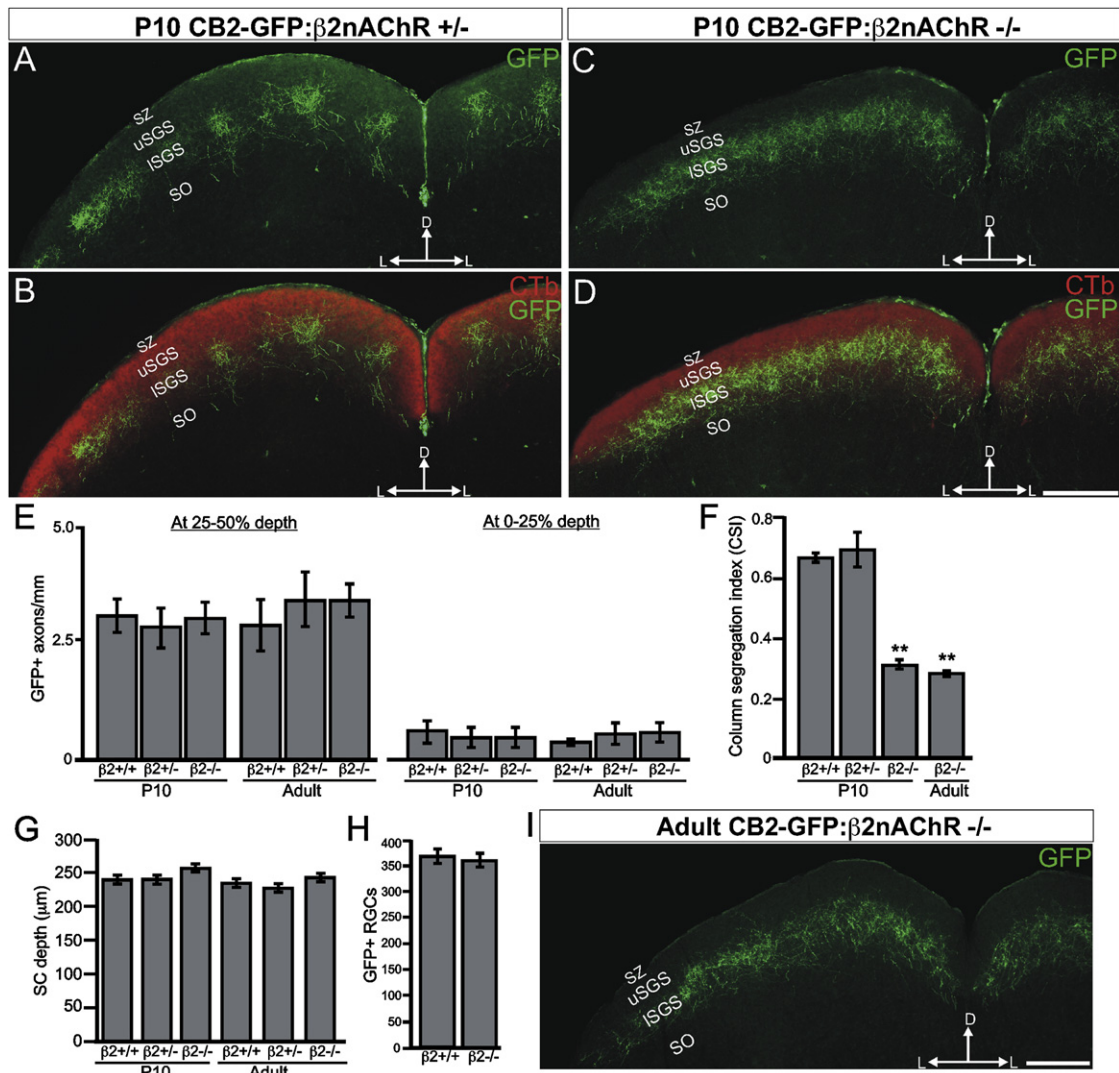


Figure 6. Cholinergic Spontaneous Activity Mediates Columnar- but Not Laminar-Specific Refinement of tOFF- α RGC Projections to the SC (A and B) Coronal view of tOFF- α RGC projections to the SC in a P10 CB2-GFP; $\beta 2nAChR^{+/-}$ mouse. Laminar specificity and columnar specificity are similar to WT P10 mice (see Figure 4).

(C and D) Coronal view of tOFF- α RGC projections to the SC in a P10 CB2-GFP; $\beta 2nAChR^{-/-}$ mouse. Laminar specificity is normal for this age but columnar specificity is severely perturbed. (A–D) Scale is 250 μm .

(E) Quantification of laminar specificity according to genotype. No significant differences were present between WT, $\beta 2nAChR^{+/-}$, or $\beta 2nAChR^{-/-}$ CB2-GFP mice on P10 ($p = 0.3-0.7$; $\pm SEM$; $n = 3$ mice per condition).

(F) Quantification of columnar specificity in the P10 and adult SC, according to genotype. At P10 and at adulthood, CB2-GFP; $\beta 2nAChR^{-/-}$ mice exhibit significantly less columnar specificity than $\beta 2nAChR^{+/-}$ mice or WT ($\beta 2nAChR^{+/+}$) mice (** $p < 0.001$; $n = 3$ mice per genotype; $\pm SEM$; Student's t test).

(G) The thickness of the retinorecipient SC depth is not different between WT, $\beta 2nAChR^{+/-}$ or $\beta 2nAChR^{-/-}$ mice at P10 or in adulthood ($p = 0.2-0.5$, t test, $n = 3$ mice per genotype).

(H) The number of GFP+ RGCs per retina is not different between WT and $\beta 2nAChR^{-/-}$ CB2-GFP mice ($p = 0.75$; t test; $\pm SEM$; $n = 4$ mice per genotype).

(I) GFP+ tOFF- α RGCs projections to the SC of an adult CB2-GFP; $\beta 2nAChR^{-/-}$ mouse. Columnar specificity is severely reduced (quantified in [F]). Scale is 250 μm .

only to the dLGN and the SC, and within both these targets, they form precisely targeted laminar-specific connections. Laminar-specificity of retinocollicular projections from functionally distinct RGCs has been observed previously by recording from and filling individual RGC axons in the optic tract of cats, monkeys, and hamsters (Bowling and Michael, 1980; Berson, 1988; Mooney and Rhoades, 1990; Tamamaki et al., 1995)

and was suggested from retrograde filling experiments in mice (Hofbauer and Dräger, 1985). Those studies agree with our findings that α/Y RGCs project to the ISGS. But because previous studies relied on filling of randomly-targeted individual RGC axons, our results are the first to delineate the pathway corresponding to a complete mosaic of one subtype of RGCs.

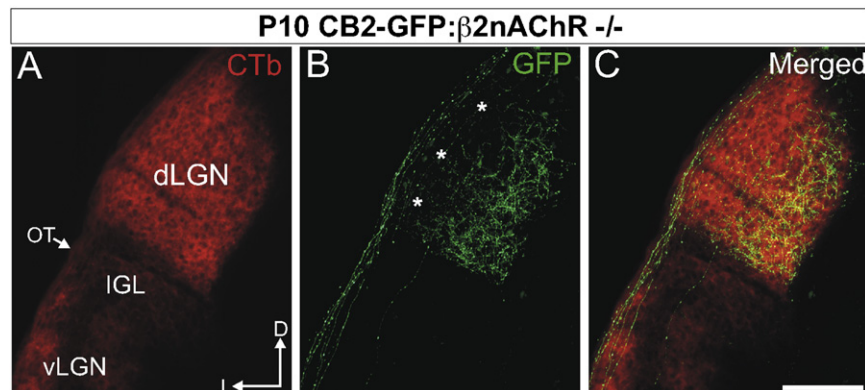


Figure 7. Disruption of Cholinergic Spontaneous Retinal Activity Does Not Prevent Laminar-Specific Targeting of tOFF- α RGC Axons to the dLGN

(A–C) Retino-dLGN projections in a P10 CB2-GFP; β 2nAChR $^{-/-}$ mouse. (A) CTb-594 (red) labeled RGC retino-dLGN projections. (B) GFP $^{+}$ tOFF- α RGC axons avoid the outer third (asterisks) of the dLGN and selectively terminate within the inner portion of the dLGN, forming a distinct layer just as in WT CB2-GFP mice (see Figure 3). Note also that the GFP $^{+}$ tOFF- α RGC axons avoid the vLGN and IGL, just as in WT CB2-GFP mice. (C) Merged view of (A) and (B). Scale is 150 μ m.

Whereas laminar organization of retinogeniculate projections from functionally distinct RGCs is a hallmark feature of the carnivore and primate visual pathway (Callaway, 2005), this feature was thought to be absent in rodents (Reese, 1988). We find, however, that tOFF- α RGCs project to stereotyped lamina in the dLGN of the mouse, revealing an unexpectedly high degree of synaptic specificity in the rodent visual system. Our discovery that the axons from the mosaic of tOFF- α RGCs form a columnar map in the SC reveals the precision with which single RGC subtypes arrange their projections within a topographic map. These results and those in recent reports (Yonehara et al., 2008; Kim et al., 2008) highlight the usefulness of genetic methods to identify functionally distinct RGC subtypes and to delineate their projections within multiple brain targets, even when those targets consist of compact neuropil with no obvious cytoarchitectural divisions.

Emergence of Synaptic Specificity in the Mammalian CNS Involves Axonal Refinement

Our results show that mammalian RGC projections achieve laminar and columnar specificity by first transitioning through a period of imprecision (Figure 4). The emergence of synaptic specificity in the mammalian visual system thus differs from that of the invertebrate CNS where laminar and columnar specificity of photoreceptor projections emerge by directed growth of appropriately targeted synaptic connections (Meinertzhagen and Hanson, 1993; Clandinin and Zipursky, 2002). It is unlikely that the imprecision we observed simply reflects tOFF- α RGCs axons contacting the proper target SC cells that have not yet migrated to their final positions, because cell migration and lamination in the SC is completed by P5 (Edwards et al., 1986). Also, the SC follows an inside-out gradient of neurogenesis (Edwards et al., 1986), meaning that ISGS neurons develop before the uSGS. Therefore, tOFF- α RGCs project through and beyond their correct target lamina before retracting to form laminar-specific connections in the ISGS.

Spontaneous Retinal Activity Segregates Axons Arising from RGCs of the Same Subtype

Cholinergic retinal waves are thought to segregate RGC axons from the two eyes and glutamatergic waves are thought to segregate axons from RGCs serving different functions (Torborg and Feller, 2005; Kerschensteiner and Wong, 2008). Here, we pro-

vide evidence that cholinergic spontaneous retinal activity also segregates axons arising from RGCs in the same eye and of the same subtype. In previous studies, the consequences of altering spontaneous activity were assessed either by (1) randomly labeling individual RGC axons (Sretavan et al., 1988), (2) labeling all the RGC axons arising each eye (Penn et al., 1998; Huberman et al., 2002; Torborg and Feller, 2005), or (3) by labeling groups (~500) of axons arising from retinotopically close but functionally diverse RGCs (Grubb et al., 2003; McLaughlin et al., 2003; Chandrasekaran et al., 2005). Thus, it was unclear precisely which features of axon targeting and refinement were impacted by activity. Indeed, some studies have argued that activity is merely permissive for axon growth and targeting (Crowley and Katz, 2000; Huberman et al., 2003). Our observation that retinocollicular projections are similar between β 2nAChR $^{-/-}$ mice and control mice before refinement on P4 (Figure 5) and that laminar specificity emerges despite manipulations that severely perturb columnar specificity (Figures 6–8) argues against a merely permissive influence of spontaneous retinal activity on axon growth. The data presented here thus identify cholinergic retinal waves as a key influence on the emergence of synaptic specificity by clustering groups of axons arising from RGCs of the same subtype.

Our finding that cholinergic spontaneous retinal activity is not necessary to refine tOFF- α RGCs projections into specific lamina within their targets differs from eye-specific laminar refinement, which clearly requires cholinergic transmission (Penn et al., 1998; Rossi et al., 2001; Muir-Robinson et al., 2002; Huberman et al., 2002; Torborg and Feller, 2005). It is worth noting that glutamatergic waves appear in β 2nAChR $^{-/-}$ mice by P8 (Bansal et al., 2000) whereas we evaluated laminar specificity on P10 because it is the earliest age when laminar and columnar refinement are adult-like. It is therefore possible that glutamatergic waves rescue laminar specificity of tOFF- α RGC projections between P8 and P10. Nevertheless, allowing an extended period of survival past P10 does not rescue columnar specificity (Figure 6), indicating a lasting, critical role for cholinergic spontaneous retinal activity in columnar but not laminar refinement.

The fact that laminar specificity can emerge in the absence of cholinergic retinal activity is consistent with previous studies on the role of activity in lamina-specific targeting of retinal projections in the fly and chick (Inoue and Sanes, 1997; Hiesinger et al., 2006). We also observed normal laminar-specific targeting

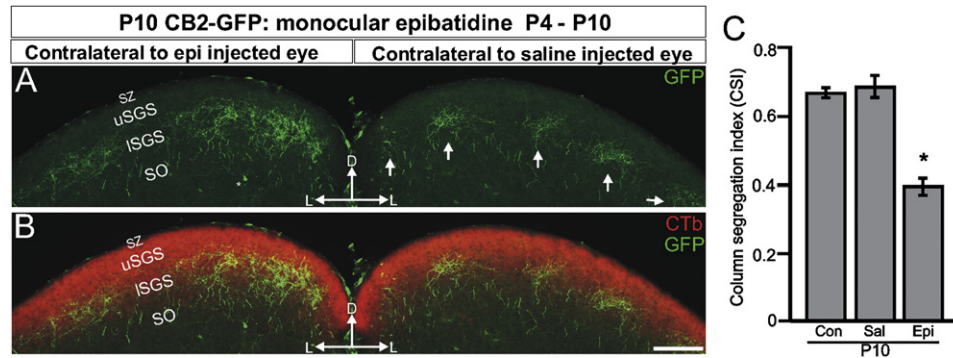


Figure 8. Columnar-Specific Refinement Is Mediated by Cholinergic Spontaneous Activity in the Retina

(A and B) RGC projections to the SC of a P10 WT CB2-GFP mouse that received epibatidine (epi) injections into the right eye to disrupt spontaneous retinal activity and saline (control) injections into the left eye, from P4–P10. Normal laminar-specific targeting of GFP⁺ tOFF- α RGC axons to the ISGS is evident in both SC hemispheres. Columnar segregation is normal in the saline hemisphere but is perturbed in the SC hemisphere contralateral to the epi-injected eye. Scale is 250 μ m. (C) Quantification of columnar specificity in the SC hemisphere receiving input from control, saline- or epi-injected retina (epi versus saline ** $p < 0.001$; saline versus control $p = 0.67$; \pm SEM; t test; $n = 2$ mice).

of tOFF- α RGCs dendrites to the OFF sublamina of the IPL of CB2-GFP; $\beta 2nAChR^{-/-}$ mice (data not shown), which was expected based on previous results (Bansal et al., 2000). Although we do not rule out the possibility that noncholinergic forms of retinal spontaneous activity could mediate laminar-specific targeting of tOFF- α RGC axons or dendrites, we speculate that RGCs select their laminar-specific connections primarily on the basis of cell-type specific expression of adhesion molecules, as is the case in the fly and chick visual system (Inoue and Sanes, 1997; Clandinin and Zipursky, 2002; Chen and Clandinin, 2008; Nern et al., 2008; Yamagata and Sanes, 2008). In CB2-GFP mice, tOFF- α RGCs can be identified by virtue of their GFP expression early in development (Figure S5), making these mice potentially useful for studying development of RGC morphology, mosaic spacing and tiling, as well as for identifying candidate genes that control lamina-specific targeting of tOFF- α RGC axons and dendrites.

Activity-Dependent Segregation of tOFF- α RGC Projections into Columns: Relevance to Models of Ocular Dominance Column Development

Our findings that emergence of columnar-specific projections from tOFF- α RGCs involves axonal refinement and requires spontaneous retinal activity are consistent with the classic model of cortical ocular dominance column (ODC) formation (LeVay et al., 1978; Stryker and Harris, 1986; Huberman et al., 2006) and with experiments on ODCs in the tectum of three-eyed frogs (Reh and Constantine-Paton, 1985). Our results contrast, however, with the fly visual system, where columnar targeting of photoreceptor axons is precise from the outset and does not require synaptic transmission (Hiesinger et al., 2006). This raises the question of whether, in mammals, the segregation of visual projections into columns reflects generic mechanisms of activity-dependent axon sorting that occur during development (Adams and Horton, 2003) or whether columns serve a particular function related to the types of neural information they carry.

Along these lines, we considered what aspects of retinocollicular circuitry tOFF- α RGC columns might correspond to and why

they require cholinergic spontaneous retinal activity in order to develop. Based on the mapping of retinal coordinates onto the SC in the mouse (Dräger and Hubel, 1976), we determined that the size of each GFP⁺ column in the SC corresponds to about 240 to 300 μ m across the retina. Cholinergic waves correlate the firing of RGCs whose cell bodies are located within 0 to 300 μ m of each other (McLaughlin et al., 2003). Given the average spacing of tOFF- α RGCs, cholinergic waves should correlate nearby groups of 3 to 4 GFP⁺ RGCs. These measurements support the idea that the columns described here arise from the axon terminals of neighboring GFP⁺ RGCs that experience correlated activity in the retina. In this way, the columns we describe can be thought of as anchoring axons arising from closely positioned RGCs of the same subtype, through Hebbian mechanisms.

Why does columnar specificity fail to develop in $\beta 2nAChR^{-/-}$ mice? In these mutants, correlated RGC firing is significantly lower than normal for RGCs situated up to 300 μ m apart (McLaughlin et al., 2003). This should reduce Hebbian strengthening of axonal inputs from groups of GFP⁺ RGCs that project to the same SC cells and prevent weakening and elimination of RGC axons misprojecting to intercolumn zones or to incorrect columns. This model is consistent with our observation that qualitatively and quantitatively, some columnar architecture is present in both WT and $\beta 2nAChR^{-/-}$ mice on P4/5 (Figure 5) and that columnar specificity is severely reduced but not entirely absent in P10 and adult $\beta 2nAChR^{-/-}$ mice (Figure 6). This model is also consistent with studies that perturbed retinal waves or blocked NMDA receptors in the SC during development and observed increased receptive field sizes of SC neurons (Chandrasekaran et al., 2005; Huang and Pallas, 2001).

One consideration is that mouse tOFF- α RGCs are coupled to one another through connexin36 gap junctions (Schubert et al., 2005). Genetic removal of connexin36 abolishes tOFF- α RGC coupling (Schubert et al., 2005), and yet the same manipulation does not perturb early spontaneous retinal activity or eye-specific refinement in the dLGN (Torborg and Feller, 2005). Thus, it is unlikely that genetic removal of $\beta 2nAChRs$ or epibatidine

prevents columnar segregation of tOFF- α RGC axons by reducing gap junction coupling of these RGCs. We thus conclude that altered correlated RGC firing (Bansal et al., 2000; McLaughlin et al., 2003; Cang et al., 2005; Torborg and Feller, 2005) is the factor primarily responsible for the defective columnar refinement of tOFF- α RGC projections in CB2-GFP: β 2nAChR^{-/-} and epibatidine-treated mice.

At this time, we do not know what the columns arising from tOFF- α RGCs contribute to visual processing or behavior. Interestingly, others have described columns of somatosensory input to the deeper nonretinorecipient SC layers that are in register with the retinotopic map (Dräger and Hubel, 1976). Whether somatosensory columns align with tOFF- α RGC columns is unclear, but it is tempting to speculate about their possible relationship in multimodal sensory integration. Ultimately, tools that allow manipulation of neural activity in particular RGC subtypes will allow us to determine their contribution to visual perception and behavior. In the meantime, our findings show that transgenic mice with genetically identified subtypes of retinal neurons expressing GFP can serve as useful tools for elucidating previously uncharted structure-function relationships in the visual system and for studying the mechanisms underlying development of synaptic specificity in the mammalian CNS.

EXPERIMENTAL PROCEDURES

CB2-GFP Mice

Mice were obtained from the Mouse Regional Resource Center (detailed information about CB2-GFP mice, also called Calb2 or calretinin-EGFP, is available at: <http://www.mmrc.org/strains/283/0283.html>). Mice were generated on Swiss-webster background and were crossed onto C57/Bl6 background for multiple generations in our laboratory. In the text, postnatal day 0 corresponds to the day of birth. GFP expression in the retina of CB2-GFP was reflective of mRNA in situ for calretinin in the retina, but there was less GFP in the brain than would be expected on the basis of calretinin mRNA expression (data not shown). This reflects positional effects of the CB2-GFP transgene insertion (Gong et al., 2003).

CB2-GFP: β 2nAChR^{-/-} Mice

These mice were generated by breeding β 2nAChR^{-/-} mice (Xu et al., 1999; obtained from D. Feldheim, UC Santa Cruz) to CB2-GFP mice. The CB2-GFP: β 2nAChR^{-/-} offspring were backcrossed to β 2nAChR^{-/-} mice on a C57/Bl6 background, and the mixed background CB2-GFP: β 2nAChR^{-/-} offspring were then used to breed and expand the line. Genotyping was carried out as previously described (Xu et al., 1999).

Retinal Histology

Mice were perfused with saline followed by 4% paraformaldehyde (PFA), the eyes removed and postfixed 4 hr in 4% PFA at 4°C, then the retina was extracted. For whole mount, relieving cuts were made to allow the retina to lay flat. Retinas were incubated in blocking solution (10% goat serum, 0.25% Triton-X in PBS) for 2 hr, then transferred to primary antibodies (1:1000 rabbit anti-GFP from Molecular Probes; 1:5000 mouse SMI-32, Sternberger monoclonals) for 48 hr at 4°C, washed in PBS (5 × 1 hr), transferred to secondary antibody (1:1000 goat anti-rabbit Alexa 488, 1:1000 goat anti-mouse Alexa 594; Molecular probes) for 2 hr, washed in PBS (5 × 1 hr), mounted, and coverslipped with Vectashield (Vectorlabs). Retinal sections were as follows: the retina was immersed in 30% sucrose, then cryosectioned at 12 μ m. Block = 1 hr, primary incubation = 24 hr at 4°C, secondary incubation = 1 hr. VACHT staining (1:1000 goat anti-VACHT, Chemicon), required the use of donkey serum rather than goat serum.

Dil Labeling of Single RGCs

Methods were as described previously (Kim and Jeon, 2006). Whole eyes were fixed for 8 hr in 4% PFA, the retina extracted and stored in PBS. GFP⁺ cells were filled iontophoretically using an Axoprobe-1A amplifier and the lipophilic dye, Dil. Electrodes had resistances of about 50 MOhm and were about 0.5 μ m in size. A positive current of between 5 and 20 nA was applied for 20–30 min to fill each cell.

Quantification of GFP⁺ RGCs

The number of GFP⁺ RGCs per retina was counted from 5× or 10× mounted images of whole-mount stained retinas. t tests were applied for statistical comparisons here and unless otherwise noted, for all quantitative measurements listed below. SEMs are shown.

Quantification of Mosaic Regularity

The density recovery profile (Rodieck, 1991) describes the average density of cells at a given distance from the soma, and was calculated as

$$\rho(s) = \frac{\sum_i n_i(s)}{\sum_i A_i(s)} \quad (1)$$

where $n_i(s)$ is a histogram of the number of cells in an image at distance s from cell i , and $A_i(s)$ is the area contained in that image at distance s from cell i . $\rho(s)$ was calculated from all GFP⁺ RGCs in 27 images of central and peripheral retina from 6 different mice.

Electrophysiology

The dark-adapted mouse retina was isolated under an infrared microscope into oxygenated Ringer's solution (124 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₂, and 22.2 mM glucose), and the pH was maintained at 7.4 by bubbling of 95% O₂, 5% CO₂ at 36°C. A flattened retina-eyecup preparation (Hu et al., 2000) was placed with ganglion cells facing upwards in a superfusion chamber on the stage of an upright fluorescence microscope and held in place with a transparent dialysis membrane containing several ~200 μ m holes. For intracellular recordings, sharp microelectrodes were filled with 2 M potassium acetate, having a final impedance of 250–350 M Ω . Fluorescent retinal ganglion cells were detected at ×40 magnification by excitation with a blue LED. To minimize effects of adaptation and photodamage, single fluorescent images were taken at a very dim excitation intensity (~10 mW/m²), within the range of intensities used for visual stimulation, and exposed for 10–20 s. To facilitate direct access to the ganglion cell soma by the intracellular electrode, the inner limiting membrane was microdissected using a patch pipette. After a fluorescent cell was identified, it was impaled under oblique infrared illumination.

Stimulation and analysis was as follows: light stimuli were projected from a computer video monitor through a custom-made lens system and focused from above onto the photoreceptor layer through a ×10 water immersion objective a mean photopic intensity of ~8 mW/m². Flashes (0.5 Hz) were presented using the maximum and minimum intensities of the monitor. The spatiotemporal receptive field of all neurons was measured by the standard method of reverse correlation of the membrane potential to a flickering checkboard stimulus (110 μ m regions changing every 30 ms; Baccus et al., 2008).

To compute correlation coefficients between the average flash responses of neurons, spikes were first removed by setting a threshold for the derivative of the membrane potential, and then the average was computed from 10–15 flashes. The correlation coefficient r between two average flash responses $x(t)$ and $y(t)$ was calculated as

$$r = \frac{\int_0^T (x(t) - \langle x \rangle)(y(t) - \langle y \rangle) dt}{\sqrt{\int_0^T (x(t) - \langle x \rangle)^2 dt} \sqrt{\int_0^T (y(t) - \langle y \rangle)^2 dt}} \quad (2)$$

where T is the duration of the recording, and $\langle \dots \rangle$ denotes the time average.

Brain Histology

Mice were perfused, brains removed, postfixed for 8 hr in 4% PFA, immersed in 30% sucrose, and sectioned at 30 μ m on a freezing microtome. GFP staining was carried out as above except that primary antibody incubation was overnight at 4°C, and secondary incubation was 90 min at RT.

CTb-594 Labeling of RGC Axons

Two to five microliters of CTb-594 (0.5% diluted in saline; Molecular Probes) was injected into the vitreous of each eye (2 μ l/eye on P4/5, 3.5 μ l/eye at P10; 5 μ l/eye in adults). The mouse was perfused 24 hr later (ages in text refer to age of animal at perfusion). CTb-594 was visualized without any immunostaining.

Epibatidine Treatment

One microliter of 1 mM epi was injected into the vitreous of one eye every 48 hr on P4, P6, P8 (Rossi et al., 2001). An equivalent volume of saline vehicle was injected into the opposite eye and both eyes were labeled with CTb-594 on P9, and brain tissue harvested on P10.

Quantification of Retinorecipient SC Depth

The distance from the base of the SO to the pial surface of the SC was measured from CTb-594 labeling at four locations across each SC section. Measures were taken from 2–4 SC sections per animal and averaged before statistical comparison.

Quantification of Laminar Specificity

The depth of the retinorecipient SC was divided into quarters along its depth such that the pial margin was 0% and the base of the SO was 100%. Zero to fifty percent depth corresponds well to the combined thickness of the uSGS and SZ. One and one-half to two millimeter line scans were made across the rostral-caudal (sagittal sections) and medial lateral (coronal sections) SC and the number of GFP⁺ axons intersected per mm was calculated in the 0%–25% and 25%–50% depths. Two to four sections per animal were analyzed to generate an average per animal. The density of GFP labeling in the 50%–75% and 75%–100% depths of the SC (the ISGS and SO) was too high to count individual axons. We therefore quantified the degree of mistargeting to the 0%–50% depth (the uSGS and SZ).

Quantification of Columnar Specificity

To measure the degree of columnar specificity in the SC, we calculated a column segregation index (CSI) (Crowley and Katz, 2000) according to

$$CSI = 1 - \frac{m - b}{c - b} \quad (3)$$

where c is the image intensity in the center of a fluorescent patch, m is the intensity at the midpoint between patches, and b is the intensity of the background tissue, measured in square regions 100 μ m on a side. CSI ranged between 0 and 1 and was averaged over all adjacent patch/interpatch pairs in 2–4 SC sections per animal. Sample sizes reflect the number of animals. For no age or condition was the CSI zero, indicating that some columnar structure was present even on P4 and after activity manipulations. The near-complete lack of columnar segregation in some CB2-GFP: β 2nAChR^{-/-} mice made it difficult to identify interpatch zones in those animals. We therefore made line scans across the extent of the ISGS and took the troughs as interpatch zones.

SUPPLEMENTAL DATA

The Supplemental Data include five figures and can be found with this article online at <http://www.neuron.org/cgi/content/full/59/3/425/DC1/>.

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