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Role of Adenylate Cyclase 1 in Retinofugal Map Development

Onkar S. Dhande^{1,2}, Shivani Bhatt¹, Anastacia Anishchenko³, Justin Elstrott³, Takuji Iwasato⁴, Eric C. Swindell⁵, Hong-Ping Xu¹, Milan Jamrich⁵, Shigeyoshi Itohara⁴, Marla B. Feller³, and Michael C. Crair^{1,*}

¹Department of Neurobiology, Yale University, New Haven, Connecticut 06510

²Program in Developmental Biology, Baylor College of Medicine, Houston, Texas 77030

³Department of Molecular and Cell Biology and Helen Willis Neuroscience Institute, University of California at Berkeley, Berkeley, California 94720

⁴Laboratory for Behavioral Genetics, RIKEN Brain Science Institute (BSI), Saitama 351-0198, Japan

⁵Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

Abstract

The development of topographic maps of the sensory periphery is sensitive to the disruption of adenylate cyclase 1 (AC1) signaling. AC1 catalyzes the production of cAMP in a Ca^{2+} /calmodulin-dependent manner, and AC1 mutant mice (AC1^{-/-}) have disordered visual and somatotopic maps. However, the broad expression of AC1 in the brain and the promiscuous nature of cAMP signaling have frustrated attempts to determine the underlying mechanism of AC1-dependent map development. In the mammalian visual system, the initial coarse targeting of retinal ganglion cell (RGC) projections to the superior colliculus (SC) and lateral geniculate nucleus (LGN) is guided by molecular cues, and the subsequent refinement of these crude projections occurs via an activity-dependent process that depends on spontaneous retinal waves. Here, we show that AC1^{-/-} mice have normal retinal waves but disrupted map refinement. We demonstrate that AC1 is required for the emergence of dense and focused termination zones and elimination of inaccurately targeted collaterals at the level of individual retinofugal arbors. Conditional deletion of AC1 in the retina recapitulates map defects, indicating that the locus of map disruptions in the SC and dorsal LGN of AC1^{-/-} mice is presynaptic. Finally, map defects in mice without AC1 and disrupted retinal waves (AC1^{-/-};β2^{-/-} double KO mice) are no worse than those in mice lacking only β2^{-/-}, but loss of AC1 occludes map recovery in β2^{-/-} mice during the second postnatal week. These results suggest that AC1 in RGC axons mediates the development of retinotopy and eye-specific segregation in the SC and dorsal LGN.

INDEXING TERMS

retinal waves; superior colliculus; AC1; visual development; retinotopy; eye segregation; lateral geniculate nucleus

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*CORRESPONDENCE TO: Michael C. Crair, 333 Cedar St., SHM B301; Yale University School of Medicine; New Haven, CT 06510. michael.crair@yale.edu.

O.S. Dhande's current address is Neurosciences Department, School of Medicine, University of California, San Diego, La Jolla, CA 92093.

T. Iwasato's current address is National Institute of Genetics, Shizuoka 411-8540, Japan.

E.C. Swindell's current address is Department of Pediatrics, University of Texas-Houston Medical School, Houston, TX 77030.

Retinal ganglion cell (RGC) projections to the superior colliculus (SC) and dorsal lateral geniculate nucleus (dLGN) are segregated with respect to eye of origin and form a precise point-to-point (retinotopic) map of visual space. The development of these maps is guided by molecular cues (McLaughlin and O'Leary, 2005) and an activity-dependent competitive process that is sensitive to the disruption of spontaneous retinal waves (Chandrasekaran et al., 2005; Crair, 1999; Grubb et al., 2003; Huberman et al., 2008; McLaughlin et al., 2003; McLaughlin and O'Leary, 2005; Torborg and Feller, 2005). Mice lacking Ca^{2+} /calmodulin-dependent AC1 ($\text{AC1}^{-/-}$) have impaired retinotopic map formation and eye-specific segregation in both the SC and dLGN (Nicol et al., 2006a; Plas et al., 2004; Ravary et al., 2003).

The specific role of AC1 in visual map development is unclear, because AC1 is widely expressed in the retina and brain (Nicol et al., 2005; Plas et al., 2004). Broadly, there are three possibilities. First, because retinal waves are strongly modulated by cAMP/PKA signaling (Stellwagen et al., 1999) and disruptions in spontaneous retinal waves are associated with visual map defects (Chandrasekaran et al., 2005; McLaughlin et al., 2003; Mrsic-Flogel et al., 2005; Rossi et al., 2001; Muir-Robinson et al., 2002; Cang et al., 2005), deficits observed in $\text{AC1}^{-/-}$ mice may be due to disturbed retinal waves. Second, AC1-mediated cAMP activation in RGC axons may be required for normal structural changes in response to axon guidance factors (Ming et al., 1997; Nicol et al., 2006b, 2007). Third, the loss of AC1 signaling at retinocollicular synapses may interfere with synapse function or development during sensory map refinement (Constantine-Paton and Cline, 1998; Lu et al., 2003; Zhang and Poo, 2001).

Here, we clarify the role of AC1 in visual map development and establish its role in mediating retinofugal arbor and gross map refinement via presynaptic mechanisms independent of retinal waves. First, we assay the spatio-temporal properties of retinal waves in $\text{AC1}^{-/-}$ mice with multielectrode array recordings. Second, we use anatomical techniques to examine when the retinotopic map phenotype emerges and whether the map becomes more refined with age. Third, we employ an in vivo single RGC labeling technique to examine retinocollicular and retino-geniculate arbor maturation. Finally, we generate tissue-specific AC1 conditional knockout mice to determine directly the pre- vs. postsynaptic role of AC1 in visual map development. Our results suggest that presynaptic AC1 signaling drives visual map development in part by regulating the refinement and topographically appropriate elaboration of individual RGC arbors.

MATERIALS AND METHODS

Animals and genotyping

All experimental procedures were in accordance with NIH guidelines and approved by Institutional Animal Care and Use Committees. Animals were treated in compliance with the U.S. Department of Health and Human Services, Yale University School of Medicine, Baylor College of Medicine, and University of California guidelines. $\text{AC1}^{-/-}$ mice (Abdel-Majid et al., 1998), except for those used to generate tissue specific knockouts (see below), came from the eighth back-cross generation of the incipient C57BL/6J-Adcy1 (*barrelless*, *brl*) congenic inbred strain by breeding $\text{AC1}^{+/-}$ mice crossed to $\text{AC1}^{+/-}$ or $\text{AC1}^{-/-}$ mice. $\text{AC1}^{-/-}$ (*brl*) mice were discovered as a spontaneous mutation, resulting in a loss-of-function caused by an early retrotransposon inserted into the genetic locus in a line from ICR stock at Université de Lausanne (Abdel-Majid et al., 1998; Van der Loos et al., 1986). Littermate AC1 wild-type (WT) and heterozygous ($\text{AC1}^{+/-}$) mice were used as controls (except for quantitative comparison of single RGC projections, for which only nonlittermate C57BL/6 WT mice were used as controls). Genotypes were determined by genomic PCR using primer

sequences described previously (Lu et al., 2003). AC1; β 2 double knockout (DKO) mice were generated by crossing the above-mentioned AC1^{-/-} mice to β 2^{-/-} mice (Xu et al., 1999). Littermate AC1^{+/+}; β 2^{-/-} and AC1^{+/-}; β 2^{-/-} mice were used for comparison with DKO mice. β 2^{-/-} Mice were generated by deleting exons 1–5 (8-kb region) by homologous recombination with a neomycin resistance cassette (Neo) containing the targeting vector and maintained on a C57BL/6J background and genotyped as previously described (Xu et al., 1999).

To generate mice lacking retinal AC1 (RxAC1KO), Rx-Cre transgenic mice (Swindell et al., 2006) were crossed to AC1^{-/-} mice to generate Rx-Cre;AC1^{-/-} and Rx-Cre;AC1^{+/-} mice. These mice were then crossed to AC1^{flx/flx} (AC1^{f/f}, Iwasato et al., 2008) to obtain RxAC1KOs and littermate controls (AC1^{f/+}, AC1^{f/-}, and Rx-Cre;AC1^{f/+}). Mice lacking AC1 in the SC (EnAC1KOs) were generated using Engrailed1-Cre (En1-Cre) mice (Jackson Laboratory; stock No. 007916; Kimmel et al., 2000), with the same breeding scheme as described above for RxAC1KO mice. Rx-Cre mice were generated by random insertion of a transgenic construct containing Cre recombinase under the control of a 4-kb promoter region of the medaka fish Rx3 gene followed by an SV40 poly-A tail (Swindell et al., 2006). En1-Cre mice were generated by replacing part of the En1 coding sequence with Cre, resulting in endogenous En1 loss of function (Kimmel et al., 2000). Heterozygous En1-Cre mice are viable and fertile and were used in this study. The AC1^{flx} mice were generated by flanking AC1 exon 1, which contains the transcriptional and translational initiation sites, by a pair of loxPs (Iwasato et al., 2008). The AC1^{-/-} mice used to generate the conditional KOs were obtained by crossing AC1^{flx/flx} mice to a line that expresses Cre ubiquitously (Iwasato et al., 2008). The floxed, null, and WT allele of AC1 were detected using PCR as previously described (Iwasato et al., 2008). Primers for genotyping the Rx-Cre allele were 1) 5'-gttgggagaatgtccgtaa-3' and 2) 5'-gtatcccacaattccttgcg-3'. PCR with primers 1 and 2 detected the 362bp Rx-Cre allele. En1-Cre mice were genotyped using primers suggested by Jackson Laboratory.

All experiments involving RxAC1KO and EnAC1KO mice used littermates as controls. Target zone size and percentage overlap of contralateral and ipsilateral projections were not significantly different between control animals generated from the RxAC1KO and EnAC1KO breeding schemes (data not shown) and are combined in Figure 10. Experimentation and data analysis were performed blind to the genotype. Data values are presented in the text as mean $\pm$ SEM, except for the multielectrode experiments, for which they are presented as mean $\pm$ SD. Differences in means were tested for significance using a Student's *t*-test, unless otherwise indicated.

Analysis of Cre expression pattern

Tissue-specific expression patterns of Rx-Cre and En1-Cre mouse lines were confirmed by crossing Cre-lines to a Rosa26 reporter (R26R; Soriano, 1999) and CAG-Cat-GFP (Kawamoto et al., 2000) reporter mouse lines (see Fig. 9). Reporter mice were genotyped using previously described primers and protocols (R26R: Soriano, 1999; CAG-Cat-GFP: Kawamoto et al., 2000). At appropriate ages brain and retina tissue were collected and stained for reporter expression (β -galactosidase [β -gal] and GFP respectively). β -Gal staining was performed using protocols modified from Hogan et al. (1994). Briefly, tissue was washed in 1 $\times$ PBS and subsequently incubated in a fresh staining solution containing 200 mM ferricyanide, 200 mM ferrocyanide, 4% X-gal in N-N-dimethylformamide (DMF), 5% NP40, 1 M MgCl₂ in 1 $\times$ PBS at 37°C, followed by rinses in 1 $\times$ PBS. Tissue was mounted in *Fluoromount G* (17984-25 Electron Microscopy Sciences, Hatfield, PA).

For analysis of GFP expression patterns, mice were transcardially perfused and tissues were fixed in 4% paraformaldehyde (PFA) overnight. Eyes were subsequently cryoprotected

overnight in 30% sucrose in 1× PBS. Eyes were then embedded in O.C.T. (Tissue-Tek, Sakura, Torrance, CA) and sectioned at 10 μm thick with a cryostat. Brains were vibratome sectioned at 150 μm. The tissue was permeabilized with 1% Triton X-100 in PBS for 20 min, incubated with 0.1 M glycine for 1 hour, and then blocked with 10% normal goat serum and 5% BSA in PBS containing 0.05% Triton X-100 overnight at 4°C. Tissue was then incubated in anti-GFP antibody in blocking solution at 4°C for ~48 hours. After being washed four times with 1× PBS, tissue was mounted with Fluoromount G. Rabbit polyclonal anti-GFP conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA; A21311) is a purified IgG fraction from rabbit serum raised against GFP. GFP was isolated directly from *Aequorea victoria*, and the IgG fraction was purified by ion-exchange chromatography (manufacturer's specifications). No immunofluorescent signal was detected from WT tissue with this antibody.

Focal retinal injections and quantification of projection size to SC

Injections of DiI and the quantification of retinal projections to the SC were performed as previously described (Chandrasekaran et al., 2005; Plas et al., 2004). Briefly, mice aged P1 and P3 were anesthetized on ice, and mice aged P6–7, P12–13, and P29 were anesthetized (0.7 ml/kg) with a mixture containing ketamine (4.28 mg/ml), xylazine (0.82 mg/ml), and acepromazine (0.07 mg/ml). A small (2.3 nl for P1 and P3 mice, 4.6 nl for P6–7 and P12–13 mice, and 9.2 nl for P29 mice) injection of dye (10% DiI; Molecular Probes, Eugene, OR; in 100% DMF) was then made beneath the scleral margin at the desired retinal location. Twenty-four to forty-eight hours after the injection, digital images of the colliculus were acquired under epifluorescent illumination using a CCD camera and associated software (Epix, Inc., Houston, TX; and Carl Zeiss Micro Image, Thornwood, NY). Temporal retinal injections were used to analyze the early retinal projection pattern. Dorsonasal retinal injections were used for quantification of projection size. The target zone size was quantified as the area above one-half of the maximal fluorescent intensity after background subtraction. Results are reported as the ratio of target area to total SC area, in percentage. Nasal and temporal injections were also made separately in P7 animals to ascertain the presence or absence of “ectopic” spots similar to those found in ephrin-A knockout mice (data not shown; Feldheim et al., 2000; Frisen et al., 1998).

To control for variations in target zone size resulting from systematic differences in retinal injections between genotypes, we quantified the area of DiI injections relative to the total retinal area (in percentage) as described above for target zone size. Retinal injection size was not significantly different between control mice (0.54 ± 0.06 , $n = 15$) and $AC1^{-/-}$ mice (0.56 ± 0.07 , $n = 12$, $P = 0.75$). Also, retinal injection size did not differ significantly between $AC1;\beta2$ DKO mice (0.63 ± 0.06 , $n = 6$) and their littermate controls (0.8 ± 0.16 , $n = 10$, $P = 0.45$). Finally, retinal injection size was not significantly different between control (0.89 ± 0.07 , $n = 24$) and $RxAC1KO$ (0.85 ± 0.04 , $n = 9$, $P = 0.75$) or $EnAC1KO$ (0.80 ± 0.09 , $n = 9$, $P = 0.5$). These data are consistent with previously published reports (McLaughlin et al., 2003; Xu et al., 2011) that, for small injections (such as those used here), the size of the labeled spot in the retina is not correlated with the size of the target zone in the SC and that differences in target zone size observed between genotypes are not the result of systematic differences in the size of retinal injections.

Bulk anterograde labeling and quantification of eye-specific segregation

RGC axons from the right eye were bulk labeled with Alexa-488 conjugated to cholera toxin subunit B (CTB; C-34775; Invitrogen) and from the left eye were bulk labeled with Alexa-555 conjugated to CTB (C-34776; Invitrogen). Mice were anesthetized and injected with 1–2 μl tracer per eye in a manner similar to the DiI injections described above. The tracer was diluted to 1 mg/ml in 0.9% saline. Injections were made at P6–7 or P12–13, and

48 hours later mice were transcardially perfused. Brains were fixed overnight in 4% PFA and sagittally sectioned at 150 μm thickness with a vibratome (Leica VT100S). Sections were mounted in Fluoromount G and imaged with a CCD camera as described above for DiI injections.

The fraction of the contralateral SGS layer (green) with RGC axon projections from the ipsilateral (magenta) eye was quantified based on previously described methods (Torborg and Feller, 2004). Briefly, images were background subtracted using a 200 radius roller ball filter (ImageJ; NIH, Bethesda, MD). The outline of the contralateral (SGS) layer was manually drawn based on the fluorescent signal, and the fraction of ipsilateral signal within the outlined area was computed at the various thresholds. The overlap (percentage) of the ipsilateral RGC axon projections with the contralateral RGC axon projections in the SGS layer was similarly quantified.

Analysis of segregation of contralateral and ipsilateral projections in dLGN was performed as previously described (Torborg and Feller, 2004). Briefly, images were background subtracted as described above, and the three sections with the largest ipsilateral (Alexa 555 labeled) area were used for analysis. The logarithm of the intensity ratio, $R = \log_{10}(\text{ipsilateral channel fluorescence intensity} / \text{contralateral channel fluorescence intensity})$, was determined for each pixel, and a segregation index for each animal was computed as the mean of the variance of the distribution of R values. A larger segregation index (higher variance) is indicative of better segregation.

In vivo electroporation and quantitative analysis of single RGC axonal arbors

Single $\text{AC1}^{-/-}$ RGC axons were labeled, imaged, and quantitatively compared with previously described WT (C57BL/6J) arbors (Dhande et al., 2011). Briefly, ~2.3–4.6 nl of plasmid solution containing a combination of pCAG-Cre (Cre recombinase driven by a CAG [chicken β -actin promoter with a CMV enhancer] promoter; ~0.15–1 ng/ μl) and pCAG-LNL-gapEGFP (membrane-targeted enhanced green fluorescent protein [EGFP] preceded by a floxed STOP sequence; ~2–3 $\mu\text{g}/\mu\text{l}$) plasmids was injected into the temporal retina, and RGCs were subsequently transfected using in vivo electroporation. Mice were anesthetized and electroporated at P2 and P4–5 and killed at P4, P8–9, and P14–15 respectively.

Mice were transcardially perfused, and brains were post-fixed in 4% PFA. Fixed brains were sectioned at 250 μm with a vibratome (VT1000S; Leica). EGFP signal was enhanced by immunohistochemistry with anti-GFP conjugated to Alexa488 (described above). RGC axon arbors were imaged with an Ultima IV two-photon microscope (Prairie Technologies) at 920 nm with a $\times 20$ water immersion objective (Carl Zeiss; NA = 1.0). Three-dimensional (3-D) reconstructions of RGC arbors were generated in Neurolucida (MicroBrightField, Colchester, VT).

Total length and number of branch points were computed in NeuroExplorer (MicroBrightField). Average branch length was computed as the ratio of total length to number of branch points + 1. Quantification of total length, number of branch points, and average branch length for retinogeniculate arbors was performed without the main trunk and from the first node in the SC for retinocollicular arbors. For calculating area and density of retinogeniculate arbors, coronal (x-y axis) and sagittal (y-z axis) projection images without the main trunk were blurred using a circular filter of 10 μm . Arbor area is defined as sum of area of total nonzero pixels. Arbor density is defined as the ratio of the sum of all nonzero pixel values to the total number of nonzero pixels. Retinocollicular arbor area and density was computed in a similar manner using surface view projections of axonal projections from entry into the SC. The mediolateral (ML) and rostrocaudal (RC) extents of retinocollicular arbors were measured from the point of entry into the SC.

Multielectrode array recording

Eyes were obtained from terminally anesthetized neonatal control (heterozygous, AC^{+/-}) and AC1^{-/-} mice according to institutional guidelines for the care and use of animals. The comparisons are made with littermate controls to account for strain differences. After enucleation, the eyes were transferred to oxygenated Ames' medium and hemisected posterior to the ora serrata. The cornea, lens, and vitreous were removed from the eyecup, and the retinal pigment epithelium was detached from the retina. The isolated retinas were placed ganglion cell side downward onto a flat, hexagonal array of 61 extracellular electrodes spaced 60 μm from each other, with a total diameter of 480 μm (Litke et al., 2003). A dialysis membrane was used to hold the retina in place on the array (Spectra/Por 6 RC dialysis tubing; MWCO 25,000). The array was superfused with Ames' solution (~220 ml/hour, pH 7.4, gassed with 95% O₂ and 5% CO₂) and maintained at 34–35°C.

Voltage traces recorded simultaneously by the individual electrodes were bandpass filtered between 80 and 2,000 Hz, sampled with a temporal resolution of 0.05 msec, and stored for offline analysis. Spikes were detected in individual voltage traces by thresholding at 3.5 standard deviations of the mean. Each electrode typically picked up the activity of more than one cell. The spikes detected on a given electrode were sorted into single-cell clusters by a semiautomated procedure based on principal components analysis of spike waveforms (modified for 61 electrodes from Litke et al. [2003]). Only sufficiently large clusters, with the number of spikes corresponding to an average firing rate of more than 0.5 Hz, were included into the analysis. To verify that each cluster contained spikes from a single cell, the rate of refractory period violations was estimated using the number of interspike intervals between 0.5 and 1.2 msec. Only the clusters for which refractory period violations did not exceed 10% were used in further analysis. In some cases, several neighboring electrodes picked up the activity of the same cell. Such duplicate recordings were identified using a temporal cross-correlation and removed. Fourteen to forty-two unique cells were identified for each retina.

We computed several measures of spiking properties of each identified single cell. The average firing rate was calculated by summing the total number of spikes during a 20–60-min recording and dividing by the length of the recording. The firing rate as a function of time was computed by counting the spikes in successive 1-sec bins and dividing these counts by the bin width. Bursts were defined as increases in the binned firing rate that exceeded 3.5 standard deviations from the mean for a particular cell. The percentage of time bursting was computed as the number of bins with over-the-threshold firing relative to the total number of bins, multiplied by 100%. Interburst interval was measured as the time from the beginning of one burst to the beginning of a subsequent burst. The interburst interval and the burst duration were computed for each burst in the cell's spike train and then averaged over the length of the recording. The medians and quartiles of all of these measures were computed by grouping the cells according to age and genotype. Differences between medians of different genotypes at a given age were evaluated for statistical significance using a two-sided Wilcoxon rank sum test, which rejected or confirmed the null hypothesis of equal medians at the 1% significance level.

The correlation index was calculated for all possible pairs of cells in each retina using a method described previously (Torborg et al., 2005; Wong et al., 1993). Briefly, the correlation index measures the factor by which the firing rate of cell B increases over its mean value within ± 100 msec of a spike from a reference cell A:

$$\text{Correlation index} = \frac{N_{AB(-0.1s, +0.1s)} \cdot T}{N_{A(0,T)} \cdot N_{B(0,T)} \cdot (0.2s)}$$

where $N_{AB(-0.1 \text{ second}, +0.1 \text{ second})}$ is the number of spike pairs from cells A and B that are separated by no more than 100 msec, T is the total recording time, $N_{A(0,T)}$ is the total number of spikes in cell A, and $N_{B(0,T)}$ is the total number of spikes in cell B. Each cell was considered to be at the position of the electrode on which its activity had been recorded, and the cell pairs were grouped according to the distance between cells. The discrete set of possible distances was determined by the geometry of the multielectrode array. The correlation index was then averaged over all cell pairs in a given distance group.

Quantitative RT-PCR

Quantitative RT-PCR was performed to confirm tissue-specific loss of AC1 in RxAC1KO and En1AC1KO mice. Retinal and collicular tissue was microdissected from P5 animals. Tissue was homogenized in Trizol reagent (15596-018; Invitrogen), and total RNA was isolated by using chloroform phase separation followed by precipitation with 75% alcohol and resuspended in water. cDNA was synthesized with a QuantiTect Reverse Transcription Kit (205311; Qiagen, Valencia, CA). Quantitative PCR was performed with an iQ SYBR Green Supermix Kit (170-8880; Bio-Rad, Hercules, CA) for AC1 and β -tubulin, used for normalization, with an iCycler system (170-8740; Bio-Rad). Quantification was performed as previously described by Schmittgen and Livak (2008). Photomicrograph figures were adjusted only for brightness and contrast in Adobe Photoshop CS2 and prepared in Adobe Illustrator CS2 (Adobe Systems, San Jose, CA).

RESULTS

Retinal waves in AC1^{-/-} mice are preserved

The distinct spatial and temporal properties of retinal waves are thought to be critical in their ability to mediate normal visual map development (Crair, 1999; Huberman et al., 2008; Xu et al., 2011). Retinal wave activity patterns are strongly modulated by cAMP/PKA signaling (Dunn et al., 2006; Stellwagen et al., 1999; Stellwagen and Shatz, 2002), so it is possible that the visual map phenotypes observed in AC1^{-/-} mice are due to a disruption in spontaneous retinal waves (Plas et al., 2004; Nicol et al., 2006a; Ravary et al., 2003). To determine whether AC1^{-/-} mice have altered spontaneous firing patterns, we used a multielectrode array to record extracellularly from several RGCs simultaneously (Meister et al., 1991; Wong et al., 1993). In WT mice, retinal neurons spontaneously fire bursts of action potentials that are correlated with neighboring cells and are separated by long periods of silence (Demas et al., 2003; McLaughlin et al., 2003; Torborg et al., 2005). The multielectrode array recordings showed that the retinas of AC1^{-/-} mice have spontaneous firing patterns similar to those of littermate controls (Fig. 1A–B”).

To analyze the spatiotemporal structure of the spontaneous firing, we focused on three different age ranges: P3/P4, P6/P7, and P10/P11. Spikes produced by individual RGCs were separated as described in Materials and Methods (P3/P4: control, n = 34 cells from two retinas; AC1^{-/-}, n = 29 cells from two retinas; P6/P7: control, 73 cells from three retinas, AC1^{-/-}, 156 cells from four retinas; P10/P11: control, 104 cells from three retinas, AC1^{-/-}, 95 cells from five retinas). The overall level of activity (the average number of spikes per second) was similar between the RGCs of control and AC1^{-/-} mice. At P6/P7 and P10/P11 (but not P3/4), AC1^{-/-} mice had a small but significant increase in firing rate (Fig. 1G, Table 1), although this small difference in firing rate is unlikely to alter activity-dependent

refinement as we have previously demonstrated that elevated firing rates are correlated with structural refinement of retinogeniculate afferents (Torborg et al., 2005).

To characterize the spatial structure of spontaneous activity, we computed the correlation index as a function of pairwise distance between RGCs (Meister et al., 1991; Torborg et al., 2005; Wong et al., 1993) and found no significant differences in control and AC1^{-/-} mice. In all retinas, the correlation index was highest for neighboring neurons and fell off as a function of distance between cell pairs (Fig. 1C–F; P3/P4: control, 288 pairs from two retinas, AC1^{-/-}, 196 pairs from two retinas; P6/P7: control, 852 pairs from four retinas, AC1^{-/-}, 3,015 pairs from four retinas; P10/P11: control, 1,866 pairs from three retinas, AC1^{-/-}, 931 pairs from five retinas).

We next analyzed the temporal structure of the firing patterns and found that the temporal structure of RGC bursting during retinal waves was also similar between the two genotypes. The interburst interval (defined as the interval between bursts of activity in individual cells), burst duration, and percentage of time bursting were all similar in AC1^{-/-} mice relative to littermate controls in all three age ranges (Fig. 1H–J, Table 1). The increase in variance in interburst interval observed at P10 is due to the irregular initiation of glutamatergic waves (Blankenship et al., 2009). Combined, these recordings reveal that the spontaneous firing patterns (and, hence, the instructive signal for retinotopic map refinement) were not disrupted in AC1^{-/-} mice, except for a 10% increase in mean firing rate during the second postnatal week. This suggests that the observed map phenotype may be due to an early, activity-independent process involved in RGC axon guidance and/or a later failure to read out this instructive signal at retinocollicular synapses.

Anatomical phenotype emerges between P2 and P4 in AC1^{-/-} mice

To clarify the potential role of AC1 in an early, activity-independent phase of map formation, we examined how early in development the map phenotype emerged. To study the RGC projection patterns in the SC early in development, we made focal DiI injections into the temporal retina at P1 and P2 in AC1^{-/-} and control mice and analyzed the projection patterns at P2 and P4, respectively (see Materials and Methods). Our *in vivo* analysis revealed no difference in the projections of temporal RGC axons in AC1^{-/-} mice relative to controls at P2, when temporal axons overshoot their eventual target zone and extend through most of the rostrocaudal length of the colliculus (Fig. 2A,B; AC1^{-/-}, *n* = 6; control, *n* = 3). The rostrocaudal extents of DiI-labeled projections at P2 were not significantly different between control and AC1^{-/-} mice (Fig. 2C; AC1^{-/-}, 0.84 ± 0.02 ; control, 0.85 ± 0.04 ; *P* = 0.89), suggesting that AC1^{-/-} mice do not have initial gross topographical targeting errors. A difference in the projection pattern, however, did become apparent in AC1^{-/-} mice by P4, when a clear target zone in anterior colliculus is evident (Fig. 2D–F; AC1^{-/-}, $5.6\% \pm 0.8\%$, *n* = 5; control, $2.9\% \pm 0.3\%$, *n* = 5; *P* < 0.01), suggesting that map refinement, but not initial RGC axon targeting, is impaired in AC1^{-/-} mice. The absence of an early, gross anatomical phenotype in AC1^{-/-} mice at P2, with defects emerging at P4 when retinal waves are indistinguishable from those of control mice, suggests a role for AC1 signaling in driving RGC axon arbor refinement that is independent of retinal waves.

Map refinement failure persists after the first postnatal week in AC1^{-/-} mice

We have previously shown that, in mice that lack the $\beta 2$ subunit of the nicotinic acetylcholine receptor ($\beta 2^{-/-}$ mice), correlated retinal waves are disrupted during the first postnatal week, and retinocollicular projections fail to refine by P8 (Chandrasekaran et al., 2005; McLaughlin et al., 2003). After the onset of ionotropic glutamate receptor-mediated waves, significant refinement occurs between P8 and P14 in $\beta 2^{-/-}$ mice, suggesting that the delayed onset of an instructive signal drives “corrective” refinement during the second

postnatal week (Chandrasekaran et al., 2005). To investigate whether similar “corrective” map refinement occurs in $AC1^{-/-}$ mice, we made focal DiI injections in dorsal-nasal retina at P6–7, P12–13, and P29 and analyzed the size of their projections in the SC 48 hours later (see Materials and Methods). In $AC1^{-/-}$ mice, retinocollicular target zones at P8 were enlarged (Plas et al., 2004; Fig. 3A,D,G), but, unlike in $\beta2^{-/-}$ mice, no corrective refinement occurred during the second postnatal week or after eye opening (Fig. 3B,C,E–G; $AC1^{-/-}$ P8–9, $2.7\% \pm 0.2\%$, $n = 5$; $AC1^{-/-}$ P14–15, $2.7\% \pm 0.2\%$, $n = 9$; $AC1^{-/-}$ P31, $2.9\% \pm 0.4\%$, $n = 9$).

Because retinocollicular projections fail to refine after P8 in $AC1^{-/-}$ mice, we next used DKO mice to examine whether the partial refinement observed in $\beta2^{-/-}$ during the second postnatal week requires AC1 signaling. To examine map refinement in AC1 and $\beta2$ DKO ($AC1^{-/-}; \beta2^{-/-}$) relative to littermate mice ($AC1^{+/-}; \beta2^{-/-}$ and $AC1^{+/-}; \beta2^{+/-}$), we made focal DiI injections in the dorsal-nasal retina at P6–7 and P12–13 and analyzed the size of their projections 48 hours later. Unlike their littermates, $AC1^{-/-}; \beta2^{-/-}$ DKO mice failed to undergo further refinement during the second postnatal week (Fig. 4B,D,E; $AC1^{-/-}; \beta2^{-/-}$: P8–9: $6.6\% \pm 0.5\%$, $n = 9$; P14–15: $6.2\% \pm 0.9\%$, $n = 7$, $P = 0.7$). Furthermore, the loss of AC1 on a $\beta2^{-/-}$ background did not have an additive effect on the disruption in map refinement in $\beta2^{-/-}$ mice at P8–9 (Fig. 4A,B,E; combined $AC1^{+/-}; \beta2^{-/-}$ and $AC1^{+/-}; \beta2^{-/-}$: P8–9, $6.7\% \pm 0.5\%$, $n = 20$, $P = 0.9$), demonstrating a genetic interaction between AC1 and $\beta2$. Together these results suggest that AC1 is involved in an ongoing activity-dependent refinement process and is not limited to a role during early map formation.

Maturation of single RGC arbors in the SC and dLGN of $AC1^{-/-}$ mice

In the mouse visual system, loss of AC1-mediated signaling results in defects in both retinotopic map refinement (Plas et al., 2004; Ravary et al., 2003) and eye-specific segregation (Nicol et al., 2006a; Ravary et al., 2003). We fluorescently labeled RGCs in vivo and reconstructed individual RGC arbors in the SC and dLGN to examine the structural basis of map defects in $AC1^{-/-}$ mice.

We analyzed the development of single temporal contralateral RGC arbors in the SC of $AC1^{-/-}$ mice at P4, P8–9, and P14–15 and compared their maturation with that of age-matched WT arbors. Qualitatively, single arbors were clearly perturbed at all ages analyzed in comparison with WT arbors (Fig. 5). $AC1^{-/-}$ arbors appeared to elaborate inappropriately as early as P4 (Fig. 5B–F), consistent with the disrupted retinotopic maps observed with bulk DiI injections (Fig. 2). WT contralateral RGC axons form a dense restricted termination zone in the anterior aspect of the SC at the end of the first postnatal week and continue to ramify locally during the second postnatal week (Fig. 5G,Q; Dhande et al., 2011). Qualitatively, single retinocollicular arbors in $AC1^{-/-}$ are enlarged and less dense at both P8–9 (Fig. 5H–P) and P14–15 (Fig. 5R–Y) in comparison with WT arbors and appear to maintain and elaborate inappropriately located branches. Quantitative analysis supports these qualitative assessments as evidenced by a significantly larger arbor area, lower arbor density, and larger RC and ML extent in $AC1^{-/-}$ mice (Fig. 6D–F, Table 2). Although not statistically significant, total length tended to be larger in comparison with WT arbors at P14–15 (Fig. 6A, Table 2). These results suggest that AC1 signaling is required for elimination of inappropriately located branches and ramification within the correct termination zones consistent with previous in vitro data (Nicol et al., 2006b).

We next analyzed the development of single contralateral RGC arbors in the dLGN of $AC1^{-/-}$ mice during the first 2 postnatal weeks in a manner similar to retinocollicular arbors. WT temporal retinogeniculate arbors undergo significant reorganization and focused elaboration during the first 2 postnatal weeks, leading to dense and restricted termination zones in the dorsal half of the dLGN (Fig. 7A,H,P; Dhande et al., 2011). Individual RGC

arbors in the dLGN in $AC1^{-/-}$ are disrupted in comparison with WT retinogeniculate axons, particularly at P4 and P8–9 (Fig. 7). Total length and total number of branch points are significantly larger at P4 and P8–9 but not at P14–15 (Fig. 8A,B, Table 3). The retinogeniculate arbor area is also significantly larger at P4 and tends to be larger at P8–9 and P14–15 (Fig. 8D,F, Table 3), but $AC1^{-/-}$ arbors are significantly less dense at the end of the second postnatal week in comparison with WT mice (Fig. 8G, Table 3).

In summary, these results suggest that defects in map refinement and eye-specific segregation in $AC1^{-/-}$ mice are not simply a consequence of mistargeting of RGCs and reduction in overlap among neighboring RGC arbors but rather a consequence of enlarged arbors resulting from the elaboration of inappropriately located axon collaterals. Also, individual retinofugal arbors in $AC1^{-/-}$ mice are disrupted as early as P4, with abnormally large arbor area and density in comparison with WT mice, suggesting that AC1 might be required for the suppression of inappropriate and premature branching in the dLGN and SC during development.

Retinotopic map refinement and eye-specific segregation require pre- but not postsynaptic AC1

AC1 is normally expressed in both the retina and the brain during the period when visual maps undergo dramatic reorganization. To understand better the pre- vs. postsynaptic role of AC1 during the development of a normal retinocollicular map, we generated conditional mutant mice with AC1 deleted in the retina or SC (Fig. 9; RxAC1KO and EnAC1KO, respectively; for details see Materials and Methods). We confirmed retina- and SC-specific loss of AC1 in the two lines, respectively, by using quantitative RT-PCR (for details see Materials and Methods). AC1 transcript levels were reduced 12.5-fold in the retina relative to control retinas in RxAC1KO ($n = 5$; $P \ll 0.01$) and 260-fold in the SC in EnAC1KO ($n = 3$; $P \ll 0.01$). AC1 transcripts levels were not significantly different in the SC in RxAC1KO mice and in the retina in EnAC1KO mice in comparison with controls (data not shown).

We first examined whether the retinotopic map undergoes normal refinement in both RxAC1KO and EnAC1KO mice. Focal injections of DiI were made in the dorsolateral retina at P6–7, and after 48 hours the area occupied by DiI-labeled projections in the SC was quantified (see Materials and Methods). At P8–9, RGC projections in EnAC1KO mice were indistinguishable from controls (Fig. 10A,B,D; control: P8–9, $1.3\% \pm 0.04\%$, $n = 57$; EnAC1KO: P8–9, $1.4\% \pm 0.1\%$, $n = 15$). However, target zones in RxAC1KO mice occupied a significantly larger area in comparison with controls (Fig. 10C,D; RxAC1KO: P8–9, $2.1\% \pm 0.08\%$, $n = 12$; $P \ll 0.01$), although the target zone in littermate $AC1^{-/-}$ mice was more severely disrupted than in RxAC1KO mice ($AC1^{-/-}$: P8–9, $4.0\% \pm 0.14\%$, $n = 4$; $P \ll 0.01$). As in $AC1^{-/-}$ mice, the anatomical phenotype in RxAC1KO mice emerged at P4 (data not shown).

We next examined whether pre- or postsynaptic AC1 is required for normal eye-specific segregation by bulk labeling RGCs with fluorescent dyes and analyzing the projection pattern of contralateral (CTB-Alexa488; green) and ipsilateral (CTB-Alexa555; magenta) axons in the SC (see Materials and Methods). At P8–9, RxAC1KO mice had a greater degree of binocular overlap within the SGS layer relative to control mice (Fig. 10E,G,H; $P < 0.01$ at multiple imaging thresholds; data not shown), but EnAC1KO mice were not different from controls (Fig. 10F,H). The disruption in eye-specific segregation in RxAC1KO mice was clear, but it was less severe than in $AC1^{-/-}$ mice (overlap at threshold 10: $24.14\% \pm 0.02\%$, $n = 6$; $P \ll 0.01$). The partial phenotype in RxAC1KO mice likely is due to the incomplete excision of AC1 resulting from the mosaic expression of Rx-Cre in the retina (Cai et al., 2010). EnAC1KO mice show no abnormality in map refinement even though the

deletion of AC1 transcript is much higher than in RxAC1KO mice (260- fold vs. 12.5-fold; see above).

Finally, we used the RxAC1KO mice to examine whether presynaptic AC1 plays a role in eye-specific segregation in the dLGN. Indeed, similarly to our findings in the SC, loss of presynaptic AC1 disrupted the emergence of segregated eye-specific domains in the dLGN (Fig. 10I–O). Quantitatively, the segregation index (see Materials and Methods and Torborg and Feller, 2004) is significantly lower in RxAC1KO (0.45 ± 0.02 , $n = 8$; $P \ll 0.01$) mice relative to littermate controls (0.59 ± 0.01 , $n = 8$), suggesting that projections from the two eye are less segregated in RxAC1KO mice relative to controls. Together, these results show in vivo that presynaptic AC1 is preferentially involved in retinocollicular map refinement and eye-specific segregation in both the SC and dLGN.

DISCUSSION

Summary

AC1 is known to play a role in the emergence of eye-specific segregation and the refinement of retinotopy in both the LGN and the SC (Nicol et al., 2006a; Plas et al., 2004; Ravary et al., 2003). Here, we examined the structural basis of map defects at the level of individual RGCs and the potential mechanisms by which AC1 influences visual map development. First, we showed that spontaneous retinal waves are preserved in AC1^{-/-} mice, providing a clear demonstration of impaired map development in animals with mostly normal retinal waves. Second, we demonstrated that, in contrast to that in $\beta 2^{-/-}$ mice, the visual map phenotype in AC1^{-/-} mice does not improve after the first postnatal week and persists in adults (P31). Furthermore, the partial refinement observed in $\beta 2^{-/-}$ mice is inhibited in AC1^{-/-}; $\beta 2^{-/-}$ DKO mice, suggesting that AC1 plays a role in both the initial emergence and the later refinement of visual maps. Third, we showed that individual RGC arbors in AC1^{-/-} mice do not form a dense termination zone in their topographically appropriate location and fail to eliminate mistargeted axon collaterals. Moreover, loss of AC1 signaling leads to the formation of premature and aberrant RGC arbors. Finally, by using a Cre-lox strategy to knock out AC1 selectively in the retina or SC, we demonstrated that deletion of retinal (presynaptic) AC1, but not collicular (postsynaptic) AC1, recapitulates much of the whole-animal AC1 knockout phenotype. Below we discuss the implications of these findings in the context of the primary mechanisms that have been proposed to guide visual map development.

Spontaneous firing patterns in the retina are preserved in AC1^{-/-} mice

Retinal wave propagation depends on cAMP signaling in RGCs (Stellwagen et al., 1999), and, because AC1 catalyzes the production of cAMP, we hypothesized that the loss of AC1 may disrupt the spatiotemporal properties of retinal waves, thereby interfering with topographic mapping. However, we found that retinal waves in AC1^{-/-} mice were not disrupted. During the first postnatal week, retinal wave propagation is mediated by nicotinic acetylcholine receptors, and RGCs receive compound postsynaptic currents through cholinergic receptors from starburst amacrine cells (Feller et al., 1996; Zhou, 1998, 2001). Current clamp recordings from starburst amacrine cells show that they undergo spontaneous depolarizations mediated by calcium action potentials that are followed by long afterhyperpolarizations (AHPs; Zheng et al., 2006). The AHP in starburst amacrine cells is mediated by a calcium-dependent potassium channel that is strongly gated by cAMP and increasing cAMP levels may increase retinal wave frequency by shortening the duration of the AHP. Our data presented here indicate that the interplay between calcium and cAMP levels in starburst cells does not depend on AC1. We recently demonstrated that calcium-dependent, depolarization-induced activation of cAMP/PKA signaling is intact in RGC

bodies of $AC1^{-/-}$ and $AC1^{-/-};AC8^{-/-}$ DKO mice (Dunn et al., 2009), which suggests that a soluble adenylate cyclase rather than AC1 or AC8 plays a role in the depolarization induced activation of the cAMP pathway in RGCs.

Role of AC1 signaling in retinofugal arbor development

Recently, we demonstrated that individual retinofugal arbors undergo dramatic structural reorganization during the first 2 postnatal weeks when both retinotopic maps and eye-specific domains emerge in the mouse dLGN and SC (Dhande et al., 2011). We also showed that disruptions in eye-specific segregation and map refinement in $\beta2^{-/-}$ mice are in part a result of enlarged individual RGC arbors that fail to ramify within the correct termination zone and do not eliminate topographically incorrect collaterals (Dhande et al., 2011). Similarly to the case in $\beta2^{-/-}$ mice, $AC1^{-/-}$ retinofugal arbors did not have a defect in their ability to grow and elaborate, but individual retinofugal arbors were enlarged and sparser in $AC1^{-/-}$ mice relative to controls at the end of the second postnatal week. These results are consistent with our hypothesis that AC1 signaling is involved, in part, downstream of cholinergic retinal waves during maturation of visual maps. Interestingly, the complexity of $AC1^{-/-}$ arbors is comparable to that of WT arbors at P8–9, unlike RGC arbors in $\beta2^{-/-}$ mice, which are much sparser in comparison with controls at the same time point (Dhande et al., 2011). This suggests that the “delay” in maturation of $\beta2^{-/-}$ RGCs at the end of the first postnatal week is independent of AC1 signaling. We also observed that the structure of individual retinofugal arbors was disrupted as early as P4 in $AC1^{-/-}$ mice, suggesting that AC1 is part of the biochemical machinery involved in providing a “stop” signal for growth of topographically inaccurate collaterals during both an early, activity-independent and a later retinal wave-dependent phase of development. Retinogeniculate arbor structure at P14–15 in $AC1^{-/-}$ mice was considerably more variable than in WT or $\beta2^{-/-}$ mice. A few arbors (4/10) in $AC1^{-/-}$ mice were extremely simple, with very little branching, which we never observed in WT or $\beta2^{-/-}$ mice. Our in vivo electroporation protocol results in random labeling of RGC subtypes, and the large variation between arbor structures in $AC1^{-/-}$ could reflect a subtype-specific role of AC1. Moreover, several RGC subtypes were recently shown to terminate in specific lamina within the mouse dLGN (for review see Hong and Chen, 2011). It is tempting to speculate that AC1 in concert with other genetic determinants might play a role in lamination of RGC subtypes in the LGN by suppressing their branching in inappropriate lamina.

Presynaptic AC1 is sufficient for map refinement and eye-specific segregation

Presynaptic AC1 signaling is implicated in the ephrinA5- dependent retraction of RGC growth cones, which may account for the mapping phenotype observed in $AC1^{-/-}$ mice (Nicol et al., 2006b, 2007). EphrinA5 mutant mice have coarse topographic targeting errors consisting of ectopic spots along the rostral-caudal axis of the SC (Feldheim et al., 2000; Frisen et al., 1998). EphrinAs and other axon guidance factors act cumulatively with $\beta2$ nicotinic acetylcholine receptors to regulate retinotopic map development (Chandrasekaran et al., 2005; Pfeifferberger et al., 2006). For $AC1^{-/-}$ mice, we observed disruptions in the fine-scale refinement of the retinotopic map without any coarse topographic mapping defects, and the loss of AC1 did not have an additive effect on the $\beta2^{-/-}$ phenotype. Moreover, disruption of AC1 in the retina, but not in the SC, impaired visual map development. These results show that presynaptic but not postsynaptic AC1 is necessary for retinofugal map refinement. However, the dissimilar phenotypes in AC1 and ephrinA mutants and the disparate consequences of disrupting spontaneous retinal activity in these two mutant strains suggest a model in which AC1 signaling downstream of retinal waves and partially independent of ephrin-mediated repulsion of RGC axons influences visual map development.

In the somatosensory cortex of rodents, whisker-derived “barrel” maps are completely absent in AC1^{-/-} mice (Abdel-Majid et al., 1998; Lu et al., 2003). Deleting AC1 only from neurons postsynaptic to thalamocortical axons, which form the barrels in cortex, impairs thalamocortical LTP and synaptic maturation but has only modest effects on barrel formation (Iwasato et al., 2008), suggesting that presynaptic AC1 is the locus for barrel map deficits in AC1^{-/-} mice. AC1 at the thalamocortical synapse mediates the phosphorylation of presynaptic active zone molecules that control neurotransmitter release, and deleting AC1 from the synaptic terminal results in an abnormally low probability of neurotransmitter release (Lu et al., 2003, 2006), similar to what we have observed at the retinocollicular synapse (Shah and Crair, unpublished data).

For visual map development, we envision two possible presynaptic roles for AC1 in sensory map development. First, presynaptic AC1 could modulate neurotransmitter release, affecting activity-dependent processes involved in retinocollicular synapse and map development. Second, presynaptic AC1 may regulate molecular mechanisms involved in axon guidance or branching processes responsible for map refinement. Both theoretical modeling and experimental data suggest that inadequacies in neurotransmitter release can disrupt the topographic representation of mouse facial whiskers in the somatosensory cortex (Lu et al., 2006). Recent in vivo imaging in zebrafish and tadpole tectum also show that synapse formation and maturation are tightly correlated with RGC axon branch dynamics and may play an instructive role in arbor remodeling during map formation through activity-dependent competitive mechanisms (Ben Fredj et al., 2010; Hua et al., 2005; Meyer and Smith, 2006; Ruthazer et al., 2003, 2006). Growing numbers of studies have demonstrated that signaling via axon guidance cues is influenced by activity and neurotransmission during circuit formation (Argaw et al., 2011; Carrillo et al., 2010; Nicol et al., 2007). For example, the specific pattern of activity at the *Drosophila* neuromuscular junction during development influences the chemorepellant semaphorin–plexin interaction (Carrillo et al., 2010). We propose a model in which AC1 plays a multifaceted role in retinofugal development by sculpting visual map refinement, presynaptically, through the regulation of synaptic function such as neurotransmitter release in concert with the modulation of axon guidance and branching pathways.

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Figure 1.

$AC1^{-/-}$ mice have intact spontaneous retinal activity. **A–B''**: Single unit spike trains recorded from retinas isolated from a P4 control (A–A'') and a P4 $AC1^{-/-}$ (B–B'') littermate. Hexagons above the spike train show the position (solid circle) of the electrode on the multielectrode array that recorded spikes. Hexagons shown in A – A'' apply for B – B'' as well. The maximum extent of the array is 480 μm . **C–F**: Summary of spatial correlations detected in the spontaneous firing patterns for retinas isolated from control (circles) and $AC1^{-/-}$ (triangles) littermates at three different ages: P3/P4 (C), P6/P7 (D), and P10/P11 (E). Correlation index was computed for pairs of spike trains (see Materials and Methods), averaged across mice of the same age and genotype, and plotted as a function of the distance between the cells that produced these spike trains. The size of the error bars for the P6/P7 $AC1^{-/-}$ group (F) was representative of all other populations. Error bars in all other plots were omitted for clarity. **G–J**: Summary of the temporal properties of spontaneous firing patterns for retinas isolated from control (gray) and $AC1^{-/-}$ (black) littermates at three different ages: P3/P4, P6/P7, and P10/P11. Data are averaged over all mice of a given age and genotype. Bursts in the firing of individual cells correspond to waves propagating across the retina. The boxes correspond to medians and quartiles, and the whiskers to 5% and 95%, with outliers denoted by crosses. Asterisks imply a significant

difference in medians at the 1% significance level as determined by a Wilcoxon rank sum test. *P* values for all measures are listed in Table 1.

Figure 2.

Map phenotype is not apparent until P4 in $AC1^{-/-}$ mice. **A,B:** Examples of DiI-labeled temporal retinal projections to the superior colliculus (outlined in white) in a P2 control (A) and P2 $AC1^{-/-}$ (B) mouse. No qualitative differences in the pattern of projections in the SC were apparent in $AC1^{-/-}$ mice relative to controls. **C:** DiI-labeled projections at P2 in control and $AC1^{-/-}$ mice have a similar rostrocaudal extent. **D,E:** Examples of retinal projections (target zone, outlined with dotted lines) from temporal retina in a P4 control (D) and P4 $AC1^{-/-}$ (E) mouse. The refinement phenotype is apparent at this age in $AC1^{-/-}$ mice; the size of their target zones remains larger than controls. **F:** Quantification of the target zone size at P4 shows that anatomical refinement of RGC axons in $AC1^{-/-}$ mice is reduced relative to control mice (* $P < 0.05$). L, lateral; C, caudal. Error bars are SEM. Scale bar = 100 μ m.

Figure 3.

No further refinement occurs after the first postnatal week in AC1^{-/-} mice. **A–F:** Example of retinal projections (target zones, outlined with dotted lines) to the superior colliculus (outlined in white) as the result of small focal injections of DiI into dorsonasal retina in control and AC1^{-/-} mice at P8 (A,D), P14 (B,E), and P31 (C,F), respectively. **G:** Quantification of percentage of SC occupied by labeled projections reveals significantly larger target zones in AC1^{-/-} mice relative to controls at P8–9, P14–15, and P31 (* $P < 0.01$). The target zone size did not change significantly in AC1^{-/-} mice from P8–9 to P14–15 to P31. L, lateral. C, caudal. Error bars are SEM. Scale bar = 100 μ m.

Figure 4.

Partial refinement of the retinocollicular map in $\beta2^{-/-}$ mice during the second postnatal week requires AC1 signaling. **A–D:** Example of retinal projections (target zones, outlined with dotted lines) to the superior colliculus (outlined in white) at P8 and P14 in $AC1^{+/-}; \beta2^{-/-}$ (A,C) and $AC1^{-/-}; \beta2^{-/-}$ (B,D) mice as the result of small focal injections of DiI into dorsonasal retina. **E:** Quantification of target zones reveals that the loss of AC1 does not have an additive effect on the $\beta2^{-/-}$ phenotype at P8–9, and it disrupts the partial refinement observed in $\beta2^{-/-}$ mice during the second postnatal week. C, caudal; L, lateral. Error bars are SEM. Scale bar = 500 μ m.

Figure 5.

Single retinocollicular arbors in $AC1^{-/-}$ mice are enlarged and sparser relative to WT mice during the period of map maturation. Reconstructions (surface view) of all $AC1^{-/-}$ and example WT (**A,G,Q**; Dhande et al. 2011) temporal RGC arbors in the SC at P4 (**B–F**), P8–9 (**H–P**), and P14–15 (**R–Y**). Individual $AC1^{-/-}$ RGC arbors fail to eliminate inappropriately located branches and form a dense accurate termination zone at all ages. Scale bars = 100 μ m.

Figure 6. Quantitative analysis of development of individual WT and AC1^{-/-} retinocollicular arbors during the first 2 postnatal weeks. Quantitative analysis of 3-D RGC arbor reconstructions in WT (white bars) and AC1^{-/-} (gray bars) mice. **A:** Total arbor length. **B:** Total number of branch points. **C:** Average branch length. **D:** Mean arbor area. **E:** Average arbor density. **F:** Quantification of rostrocaudal (RC) and mediolateral (ML) projection extent of individual RGC arbors in the SC of WT and AC1^{-/-} mice. Bars represent mean, and circles are individual data points. Error bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 7.

Loss of AC1 leads to enlarged and sparser single retinogeniculate arbors. Reconstructions of all AC1^{-/-} retinogeniculate arbors (coronal plane) at P4 (**B–G**), P8–9 (**I–O**), and P14–15 (**Q–Z**) shows that individual RGC arbors are disrupted as early as P4 in the dLGN (outline) in comparison with age-matched WT example arbors (**A, H, P**; Dhande et al. 2011). AC1^{-/-} arbors elaborate inappropriately located collaterals and fail to ramify within the correct target area as they continue to grow, with the net result of larger and sparser arbors at P14–15. Scale bars = 100 μ m.

Figure 8.

Quantitative and comparative analysis individual WT and AC1^{-/-} retinogeniculate arbor maturation. Comparative analysis of 3-D RGC arbor reconstructions in WT (white bars) and AC1^{-/-} (gray bars) mice. **A:** Total arbor length. **B:** Total number of branch points. **C:** Average branch length. Mean arbor area (**D**) and density (**E**) of coronal projections of 3-D arbor reconstructions. Mean arbor area (**F**) and density (**G**) of sagittal projections of retinogeniculate reconstructions. Bars represent mean, and black circles are individual data points. Error bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 9.

Examination of Cre expression pattern in Rx-Cre and En1-Cre mice. **A,B:** Retinal whole-mount β -gal staining shows LacZ expression throughout the retina in Rx-Cre mice (Rx-Cre⁺/LacZ⁺) but not in controls (Rx-Cre⁻/LacZ⁺; B) at P8. **C:** Rx-Cre expression was analyzed by visualizing GFP reporter expression pattern at P4 in retinal sections. GFP expression (green) was present in RGCs as well as other retinal cell types. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). **D,E:** At P8 no LacZ expression was detected in the SC (D) or dLGN (E) in Rx-Cre mice (Rx-Cre⁺/LacZ⁺). **F:** En1-Cre expression, detected by LacZ staining in whole-mount P2 brain, is restricted to the posterior region of the brain, including SC. **G,H:** At P2, in sagittal sections (G), GFP was clearly expressed by collicular neurons, and no expression was observed in the dLGN or cortex (Ctx). No Cre expression was observed in whole-mount retina (H) in En1-Cre⁺/GFP⁺ mice. Scale bars = 500 μ m.

Figure 10.

Retinotopic map refinement and eye-specific segregation depends on presynaptic AC1. **A–C:** Example of DiI labeled dorsolateral retinal projections (target zone, outlined in black) to the superior colliculus (outlined in white) at P8 in control (A), EnAC1KO (B) and RxAC1KO (C) mice. **D:** Quantification reveals that the target zones at the end of the first postnatal week in RxAC1KO but not EnAC1KO mice are significantly larger in comparison with control mice ($*P < 0.01$), showing that presynaptic AC1 is required for normal retinotopic map refinement. **E–G:** Example of sagittal SC sections showing contralateral (green) and ipsilateral (magenta) RGC axon projections at P8 in control (E), EnAC1KO (F), and RxAC1KO (G) mice. **H:** Overlap (%) between the ipsilateral (magenta) and contralateral (green) RGC axon projections in the SGS layer of the SC at threshold 10 at P8–9. This quantification shows that eye-specific segregation is disrupted only in RxAC1KO mice relative to controls ($*P < 0.01$) and not in EnAC1KO mice. **I–N:** Example of coronal dLGN sections showing contralateral (green) and ipsilateral (magenta) RGC projections at P15 in control (I,K,M) and RxAC1KO (J,L,N) mice. **O:** The segregation index (mean of variance of the distribution of R values) is significantly lower in RxAC1KO mice ($*P < 0.01$) in comparison with controls (dLGN is outlined with dashed white line), suggesting that retinal projections are less segregated in RxAC1KO mice. D, dorsal; R, rostral; L, lateral; C, caudal; SO, stratum opticum; SGS, stratum griseum superficiale. Error bars are SEM. Scale bars = 500 μ m in E (applies to E–G); 500 μ m in I (applies to I–N).

TABLE 1
Temporal Properties of the RGC Firing Patterns Are Similar Between $AC1^{-/-}$ and Control Mouse Retinas¹

	P3/P4		P6/P7		P10/P11		
	Control	AC1 ^{-/-}	Control	AC1 ^{-/-}	Control	AC1 ^{-/-}	
Mean firing rate, spikes/second	Lower quartile	0.20	0.19	0.41	0.63	0.81	1.32
	Median	0.26	0.27	0.59	0.78	1.33	2.11
	Upper quartile	0.52	0.42	1.03	1.08	3.31	2.99
<i>P</i>	0.80		<0.001*		<0.01*		
Interburst interval, seconds	Lower quartile	66.19	62.62	53.40	53.03	94.58	97.45
	Median	68.83	68.71	59.63	58.19	112.19	128.91
	Upper quartile	78.64	75.68	65.52	66.24	140.00	186.69
<i>P</i>	0.23		0.52		0.07		
Burst duration, seconds	Lower quartile	1.30	1.32	1.38	1.45	1.38	1.33
	Median	1.54	1.48	1.56	1.58	1.68	1.57
	Upper quartile	1.73	1.58	1.74	1.45	2.00	1.84
<i>P</i>	0.43		0.28		0.03		
Percentage of time bursting	Lower quartile	1.89	1.78	2.22	2.50	1.08	0.73
	Median	2.17	2.00	2.50	2.64	1.50	1.14
	Upper quartile	2.39	2.35	2.89	2.86	1.89	1.60
<i>P</i>	0.34		0.02		0.02		
No. of cells (No. of retinas)	34 (2)	29 (2)	73 (3)	156 (4)	104 (3)	95 (5)	

¹ *P*-values are computed based on a rank sum test; asterisks indicate the difference between $AC1^{-/-}$ and control at the 1% significance level.

TABLE 2

Quantification of Structural Parameters Based on 3-D Reconstructions of Single Retinocollicular Arbors in WT and AC1^{-/-} Mice at Multiple Developmental Stages¹

	P4	P8/9	P14/15
No. of reconstructed arbors			
WT	8	6	8
AC1 ^{-/-}	5	9	8
Total length (μm)	4,307.41 ± 585.95	*** 9,127.77 ± 707.42	# 12,512.79 ± 1,280.52
WT			
AC1 ^{-/-}	5,680.58 ± 1,018.00	* 10,844.56 ± 1,416.17	* 17,373.03 ± 2,071.40
No. of branch points	60.75 ± 8.03	*** 200.83 ± 15.10	** 499.50 ± 73.71
WT			
AC1 ^{-/-}	77.40 ± 21.41	## 213.00 ± 50.68	** 556.00 ± 87.88
Avg. branch length (μm)	73.14 ± 8.13	* 46.21 ± 4.58	** 28.18 ± 3.62
WT			
AC1 ^{-/-}	87.73 ± 14.79	67.62 ± 12.12	* 32.36 ± 1.23
Arbor density	0.19 ± 0.01	*** 0.31 ± 0.02	** 0.45 ± 0.03
WT	*		*
AC1 ^{-/-}	0.23 ± 0.01	0.27 ± 0.02	** 0.36 ± 0.03
Arbor area (μm ²)	26,060.29 ± 2,857.49	29,653.36 ± 2,293.9	24,011.97 ± 2,258.68
WT	#	**	***
AC1 ^{-/-}	37,555.69 ± 5,357.62	# 56,758.86 ± 6,218.26	57,835.89 ± 4,481.58
Rostrocaudal axis (μm)	732.42 ± 164.75	421.28 ± 132.18	400.60 ± 74.60
WT		##	*
AC1 ^{-/-}	832.57 ± 83.54	741.29 ± 103.54	646.00 ± 87.82
Mediolateral axis (μm)	276.28 ± 55.52	304.60 ± 20.73	* 229.46 ± 23.00
WT		*	*
AC1 ^{-/-}	320.96 ± 68.91	## 483.51 ± 50.12	366.44 ± 47.47

¹Mean ± SEM for all measurements for WT and AC1^{-/-} retinocollicular arbors. Asterisks identify statistically significant differences.

* $P < 0.05$,

** $P < 0.01$,
 *** $P < 0.001$.

For completion, all P values less than 0.1 are reported. The following symbols indicate P values for age and genotype groups indicated.

$P = 0.06$,
 ## $P = 0.08$.

Quantification of Structural Parameters Based on 3-D Reconstructions of Single WT and AC1^{2/2} RGC Arbors in the dLGN at Multiple Developmental Time Points¹

TABLE 3

	P4	P8/9	P14/15
No. of reconstructed arbors			
WT	10	7	9
AC1 ^{-/-}	6	7	10
Total length (μm)	498.70 ± 186.30	# 984.36 ± 154.85	* 3,627.01 ± 842.37
WT	***	*	
AC1 ^{-/-}	2,054.32 ± 356.58	2,355.13 ± 541.07	4,083.11 ± 1,032.93
No. of branch points	2.70 ± 1.50	*** 14.14 ± 2.24	* 60.89 ± 17.47
WT	**	*	
AC1 ^{-/-}	19.0 ± 6.00	30.71 ± 5.56	75.10 ± 25.69
Avg. branch length (μm)	184.91 ± 42.50	* 66.64 ± 6.25	65.47 ± 8.65
WT			
AC1 ^{-/-}	150.96 ± 46.45	78.11 ± 11.84	88.98 ± 16.55
Arbor area (μm ²) (coronal plane)	4,787.21 ± 1,542.14	7,507.60 ± 1,367.55	## 15,491.57 ± 3,263.96
WT	**		
AC1 ^{-/-}	13,265.64 ± 2,022.05	12,615.54 ± 3,617.65	18,318.43 ± 4,860.52
Arbor density (coronal plane)	0.15 ± 0.01	** 0.19 ± 0.01	** 0.27 ± 0.02
WT	***	**	
AC1 ^{-/-}	0.24 ± 0.01	0.25 ± 0.01	0.24 ± 0.01
Arbor area (μm ²) (sagittal plane)	3,551.08 ± 1,215.81	5,703.62 ± 1,331.42	* 12,673.87 ± 2,282.74
WT	***	##	
AC1 ^{-/-}	11,352.43 ± 1,394.18	13,764.03 ± 3,890.28	17,726.65 ± 4,228.60
Arbor density (sagittal plane)	0.18 ± 0.01	*** 0.25 ± 0.01	* 0.32 ± 0.02
WT	**		*
AC1 ^{-/-}	0.23 ± 0.02	0.24 ± 0.01	0.25 ± 0.01

¹ Mean ± SEM of various structural parameters for WT and AC1^{-/-} retinogeniculate arbors at different ages. Asterisks between columns and rows identify statistical difference between ages and genotypes.

* $P < 0.05$,

$P < 0.01$,
 *** $P < 0.001$.

For completion all P values less than 0.1 are reported. The following symbols indicate P values for indicated age and genotype groups.

$P = 0.07$,
 ## $P = 0.06$.