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Adenylate Cyclase 1 dependent refinement of retinotopic maps in the mouse

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Abstract

Development of the retino-collicular pathway has served as an important model system for examining the cellular mechanisms responsible for the establishment of neuronal maps of the sensory periphery. A consensus has emerged that molecular or chemical cues are responsible for the initial establishment of gross topography in this map, and that activity dependent factors sharpen this initial rough topography into precision. However, there is little evidence available concerning the biochemical signaling mechanisms that are responsible for topographic map refinement in the retino-collicular system. Using a combination of anatomical and biochemical techniques in normal and mutant mice, we provide evidence that Ca^{2+}/Calmodulin regulated Adenylate Cyclase 1 (AC1), which is strongly expressed in the superficial layers of the colliculus, is an important downstream signaling agent for activity dependent map refinement in the superior colliculus.

Keywords: Development; Superior colliculus; Adenylate Cyclase

1. Introduction

The retino-collicular map has served as an important model system for understanding how neuronal maps of the sensory periphery develop. Over half a century of research, dating back at least to Roger Sperry's 'Chemoaffinity' hypothesis, support a model in which retinal ganglion cell axon guidance and the initial establishment of a course retinotopic map are guided by molecular cues. Strong evidence from non-mammalian vertebrates and recent studies in the mouse suggest that the coarse retinotopic map that is initially established via molecular cues is refined to precision by activity dependent factors (Debski & Cline, 2002; Goodhill & Richards, 1999; McLaughlin & Hindges, 2003; Peters, 2002).

It was originally suggested that the final precision of the retino-collicular map was achieved through neuronal activity driven by visual experience. This suggestion was challenged by the observation that even in animals born with their eyes closed the retino-collicular map is essentially complete by the time the eyes open. For example, in mice the map is considered to be mature by P8 (postnatal day 8), whereas the eyelids do not open until P13. It was then discovered that the neonatal retina generates an intrinsic activity, termed retinal waves, which results in patterns of electrical signals being sent to the colliculus via the optic tract even in the absence of visual experience (Meister & Wong, 1991). This pattern of spontaneous retinal activity provides an appropriate

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signal for driving retinal ganglion cell refinement even in the absence of visual experience because neighboring ganglion cells are likely to be spontaneously coactive, but distant retinal ganglion cells are not. Neighborhood relationships in the retina are therefore encoded by the pattern of spontaneous activity in the retinal ganglion cell afferents. The obvious proposition was that this spontaneous retinal activity was responsible for the final sharpening of the retino-collicular map (Meister et al., 1991).

Experimental evidence for the importance of retinal waves in visual system development has relied both on genetic manipulations (Muir-Robinson & Hwang, 2002) of the mechanisms that produce the waves and on direct pharmacological manipulations (Stellwagen & Shatz, 1999) of the waves. Genetic control of retinal waves takes advantage of the fact that the β2 subunit of the acetylcholine receptor is required for their generation in the retina. Retinal waves are mediated by nicotinic acetylcholine receptors (nAChRs) containing β2 and α3 subunits in synapses between amacrine cells (Bansal & Singer, 2000; Feller & Wellis, 1996; Penn & Riquelme, 1998; Rossi & Pizzorusso, 2001). It was recently shown that β2 mutant mice lack retinal waves until around P8 (Muir-Robinson et al., 2002), when glutamatergic activity becomes sufficient for their generation. Furthermore, retinotopic maps in the β2 mutant mice lack retinotopic map refinement (McLaughlin & Torborg, 2003; Plas, Gonzalez, She, & Crair, submitted for publication), which can be specifically attributed pharmacologically to the absence of β2 receptor-mediated activity in the eye (Plas et al., submitted for publication). This result is significant because even though the retina of β2 mutant mice lack spontaneous correlated activity, individual retinal ganglion cells still fire action potentials at the same average rate. Thus, the spontaneous waves of retinal activity are instructive, not just permissive in the process of retinotopic map refinement.

More is known about the role of activity in the segregation of eye-specific lamina in both the lateral geniculate nucleus (Cook & Prusky, 1999; Shatz & Stryker, 1988) and superior colliculus (Rossi et al., 2001) than in retinotopic map development. For instance, spontaneous waves of activity in the neonatal retina are known to be essential for the segregation of RGC afferents into eye-specific lamina in the LGN of ferrets and mice (Feller, 2002; Huberman & Stellwagen, 2002; Muir-Robinson et al., 2002), where it is thought that Hebbian competition between retinal afferents is guided by independent waves of spontaneous activity in the two eyes (Penn et al., 1998; Stellwagen & Shatz, 2002). However, it has also recently been argued (Huberman & Wang, 2003) that spontaneous decorrelated retinal ganglion cell activity is sufficient to drive eye-specific segregation, but the results with the β2 mutant mice argue against this. The segregation of ON and OFF retinal afferents into sub-lamina in the ferret LGN is similarly thought to be mediated by spontaneous retinal waves that allows differences in firing patterns between ON and OFF retinal ganglion cells to drive segregation using Hebbian mechanisms (Lee & Eglen, 2002).

In fish and frogs, the role of activity in retinotopic map refinement has been explored more thoroughly than in mammals (Debski & Cline, 2002). In goldfish, the blockade of all retinal activity prevents the refinement of retino-collicular arbors (Schmidt, 1985). In zebrafish, a recent screen for genetic mutations affecting retinotopy in the optic tectum revealed that retinotopy is abnormal in the absence of spontaneous waves (Gnuegge & Schmid, 2001). In hamsters, it has been reported that NMDA receptor blockade causes increased receptive field size in a remapped colliculus (Razak & Huang, 2003), and in the rat, NMDA receptor blockade in the colliculus interferes with retino-collicular refinement (Simon & Prusky, 1992), suggesting that activity dependent factors may play an important role in shaping receptive field structure in mammals too. Some have argued that activity dependent retinotopic map refinement occurs through a process of selective programmed cell death of inappropriately targeted RGCs (Cellerino & Bahr, 2000; O'Leary & Fawcett, 1986). However, if spontaneous retinal waves are necessary for retinotopic map refinement in the colliculus, Hebbian competition by RGC afferents for collicular target space might be a better model. Consistent with this, blocking nAChR mediated retinal waves in the mouse causes a failure of eye-specific segregation of retinal afferents into sublamina in the superior colliculus (Rossi et al., 2001) in a process much like the formation of eye-specific lamina in the lateral geniculate nucleus (Feller, 2002) and ocular dominance column plasticity in visual cortex (Crair, 1999).

Very little is known about the mechanisms downstream from or in addition to activity that drive the development of the retioncollicular map. The apparent Hebbian nature of the process, and its reliance on NMDA receptors, suggests that there may be important parallels to synaptic mechanisms of learning and memory. There are several biochemical signaling pathways that are thought to play a role in cellular mechanisms responsible for learning and memory. One of the pathways involves PKA signaling through cAMP and Adenylate Cyclase (Storm & Hansel, 1998; Villacres & Wong, 1998). In particular, phosphorylation of glutamate receptors via cAMP and PKA is thought to be an essential step in LTP and LTD, putative synaptic mechanisms for learning and memory (Lu & She, 2003).

It is now generally accepted that molecular mechanisms play an important role in the initial establishment of retinotopic maps. It has also long been argued that activity dependent factors act in concert with molecular mechanisms to aid in the refinement of precise neighbor-

hood relationships in the map, and NMDA receptors are thought to be involved in the process of map refinement and plasticity. However, there is very little known about the cellular mechanisms responsible for this refinement, and almost nothing is known about the downstream biochemical pathways mediating retinotopic map refinement. With the use of a genetically manipulated mouse model, we report here that retinotopic map refinement is dramatically degraded in the absence Ca^{2+} stimulated signaling via Adenylate Cyclase.

2. Methods

2.1. Animals

Mutant *Adcy1*^{brl} mice were discovered as a spontaneous mutation in a line from ICR stock at Université de Lausanne (Van der Loos & Welker, 1986). *Adcy1*^{brl} nulls (homozygous mutants) survive to adulthood and are good breeders despite the fact that they are incapable of making functional Adenylate Cyclase 1 (AC1) protein, and have significantly reduced Adenylate Cyclase activity (Villacres et al., 1998; Villacres & Wu, 1995). We used mice from the eighth backcross generation of the incipient C57BL/6J-*Adcy1*^{brl} congenic inbred strain. Wild type (WT) and heterozygous (Het) littermates were used as controls. Experimentation and data analysis were performed blind to the genotype. Genotypes were determined by genomic polymerase chain reaction (PCR), using a mixture of primers 1, 3, and 4, and a mix of primers 1, 2, and 5. The PCR products of the first primer mix are 372 bp for the wt *Adcy1* allele and 299 bp for the *Adcy1*^{brl} mutant allele. Primer mix II yields 380 and 692 bp PCR products for the wt and mutant alleles, respectively. The primer sequences are: Primer 1: 5′-TCC CAA CCC AAG TTG CCC AGA-3′; Primer 2: 5′-TAC AGT GGA CGG ACA GTC GA-3′; Primer 3: 5′-AGA CCA CGA TCG ATG CTA CC-3′; Primer 4: 5′-CAG AGA TTA TAC GGC GGG AA-3′; Primer 5: 5′-CGA AGA TCC TTT CCT GTG GA-3′. Animals were treated in compliance with the US Department of Health and Human Services and Baylor College of Medicine guidelines.

2.2. Retinal injections of lipophilic dye

Neonatal (P6/7) mice are anesthetized using a combination of ketamine, xylazine, and acepromazine supplemented with hypothermia. After opening the eyelid, the eye is protruded and a small (9.2 nL) injection of dye ('DiI'; Molecular Probes, 10% in dimethylformamide) is made beneath the sclera at the desired location in the eye. The injection is through a glass pipette attached to a microinjector (Nanoject II, Drummond Scientific). Animals are sacrificed 24–48 h later. Injection sizes are held constant by using the same volume and concentration of dye, and checking each eye after each experiment to insure the dye injection is confined to the retina and does not invade the vitreous or lens. Samples from DiI injections that are not confined to the retina are discarded. We also measure the physical size of the injection in the retina under bright field illumination and verify that injection sizes do not vary systematically between mutant and Het or WT controls.

2.3. Image acquisition and quantification of projection size to superior colliculus

Digital Images are acquired under fluorescence microscopy using a CCD camera and associated software (Epix, Inc., Houston, TX). Image analysis is performed blind to genotype. The superior colliculus is outlined manually, and a point within the superior colliculus (SC), but far from the labeled target in the SC, is chosen as background level. The background is subtracted from the entire image and a threshold is set at one-half the maximum fluorescent signal. The area above threshold is taken as the target area labeled by the injection. Results are reported as the ratio of target area to total SC area, in percent. Errors are standard error of the mean.

2.4. In situ hybridization

Expression patterns are determined by means of non-radioactive in situ hybridization (ISH) on frozen sagittal sections of P7 mouse brains using published methods (Visel & Thaller, 2004). Briefly, specimens are serially sectioned and arranged in sets on standard microscope slides. Each set is hybridized in a flow-through chamber using digoxygenin-labeled antisense and sense riboprobes (non-radioactive) generated by in vitro-transcription from DNA templates using T3-, T7- or Sp6-bacteriophage RNA polymerases. The detection of hybridized riboprobes includes a tyramide-biotin based amplification step without which digoxygenin-based ISH would not be as sensitive as radioactive ISH. Specifically, digoxygenin-tagged RNA hybridized to cellular mRNA is first detected with a peroxidase-coupled anti-digoxygenin antibody. The peroxidase moiety of the anti-digoxygenin antibody activates a biotin-tyramide conjugate resulting in covalent attachment of biotin to proteins in the vicinity of the peroxidase enzyme. Biotin is subsequently detected by an alkaline phosphatase-based BCIP/NPT color reaction. Gene expression patterns are digitally photographed at a resolution of 3.2 μm/pixel using a customized compound microscope equipped with a motorized scanning stage and a digital camera.

3. Results

3.1. Retino-collicular refinement is impaired in *Adcy1*^{brl} mice while topography is normal

A focal injection of DiI into the retina causes the uptake of dye by a small group of RGCs. This results, within 24–48 h, in a spot of label in the superior colliculus that reflects the final pattern of labeled RGC axon arbors (Fig. 1). The spot of label in the SC is initially large early in postnatal development, and becomes progressively smaller as the animals age. This mirrors the degree of RGC axon arbor refinement, which is essentially complete by PND 8 (Simon & O'Leary, 1992).

We developed a quantitative procedure to examine the degree of refinement in retino-collicular projections (Fig. 1). First, digital images are acquired under fluorescence microscopy at 2.5X (Zeiss Axioscope) using standardized gain and offset settings with a CCD camera (1300×1030 pixels) and associated software (Focus Instruments, Inc., Houston, TX). The superior colliculus is then outlined manually (Fig. 1B, white outline), converted to gray scale and a point far from the labeled target in the SC is chosen to derive the background fluorescence level (small white circle in Fig. 1C). The background fluorescence level is subtracted from the image of the SC (Fig. 1D), and the image is then gently smoothed (Fig. 1E, 5×5 gaussian filter). A threshold is then set at one-half the maximum fluorescence signal of the target zone (Fig. 1F), and the area of fluorescence above this threshold (the 'target area') is measured, and results are reported as the ratio of target area to total SC area in percent.

We examined the retino-collicular projection in *Adcy1*^{brl} homozygous mutants and their Het and WT littermates using focal retinal injections of DiI on P6/7. The gross topography of the retino-collicular projection was preserved in the *Adcy1*^{brl}–/– mice, with injections into ventral/nasal retina resulting in labeled spots in the caudal/lateral colliculus (Figs. 1B and 2). Similarly, injections into dorsal/temporal retina result in labeled spots in the rostral/medial colliculus (data not shown), reflecting the preservation of gross topography. Typical focal targeted retinal injections in WT mice resulted in label in a few percent of the colliculus (Figs. 1 and 2D). However, the fraction of colliculus labeled by similar injections in *Adcy1*^{brl}–/– was qualitatively larger (Fig. 2A and B). Injections in heterozygote mice resulted in target areas that were similar to WT mice, but more variable in size (Fig. 2C). Consistent with this phenotype, focal retinal injections in WT (five of seven injections) and sometimes Het mice (two of five injections) resulted in horseshoe shaped spots in the colliculus (Fig. 2C for example). This likely reflects the small retinal scotoma produced by the injection. Horseshoe shaped spots in the colliculus of *Adcy1*^{brl}–/– were never seen (zero of six injections), presumably because in the absence of map refinement, structures on this spatial scale in the colliculus could not be revealed. A quantitative analysis of the projection sizes in the colliculus of WT, Het and *Adcy1*^{brl}–/– mutant mice were consistent

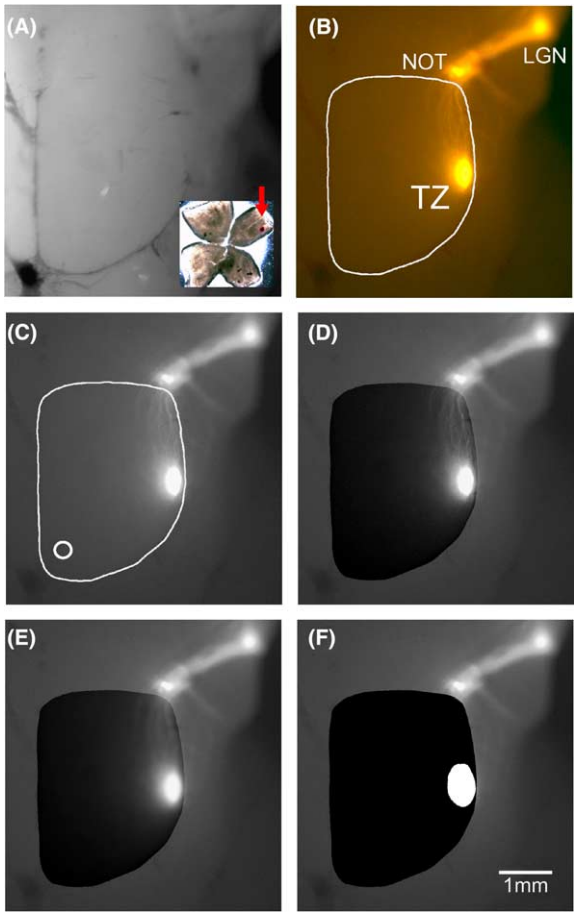

Fig. 1. Small retinal DiI injections lead to focal labeling in the superior colliculus. (A) Bright field image of the superior colliculus. Midline is on the left; caudal is down for all images in this figure. Inset is a bright field image of a retina injected with DiI; note the small spot of dye at the tip of the red arrow. (B) Flourescent image of the superior colliculus. The superior colliculus is outlined in white. The target zone (TZ) of the focal DiI injection is clearly visible on the lateral edge of the colliculus. The axons from the retina first pass through the lateral geniculate (LGN; somewhat out of focus bright spot at the top right of the image) and the nucleus of the optic tract (NOT; bright spot just rostral to the colliculus) before entering the colliculus. (C) Image converted to gray scale for quantification. Small circle at the bottom left indicates position where the background fluorescent level is measured. (D) Image resulting from subtraction of the background fluorescence. Subtraction is performed only in the superior colliculus, so the image is unchanged outside the boundaries of the superior colliculus, but darker within the boundaries. (E) Image is gently smoothed with a 5×5 gaussian filter. (F) The target zone area is determined by identifying the region of the superior colliculus with fluorescent level greater than one-half of the maximum fluorescence. This area is depicted as a bright white spot. Scale bar (1 mm) in (F) applies to all images in figure, except for the inset of (A), where the scale is actually 4 mm. Rostral is up and Medial is to the left for all images.

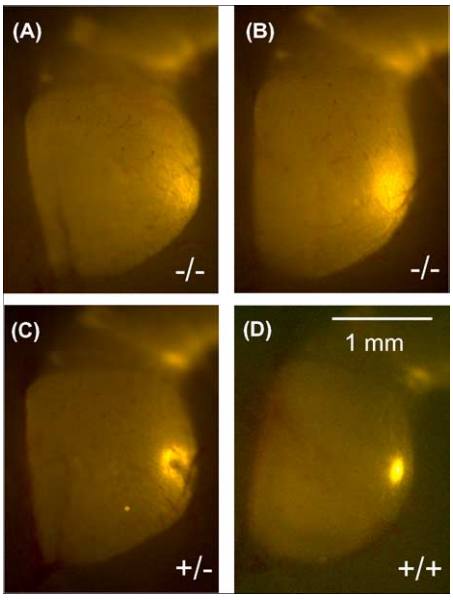

Fig. 2. Example *Adcy1*^{brl}–/– and WT target zones. (A,B) Examples of labeling in the superior colliculus as a result of focal injection of DiI into the retina of two homozygous mutant *Adcy1*^{brl}–/– mice. Target zones are relatively large and diffuse in comparison to wild type mice. (C) Target zones in *Adcy1*^{brl}+/– (heterozygous) mice are somewhat less affected than in *Adcy1*^{brl}–/– mice. Note the horseshoe shaped target zone in the superior colliculus. The horseshoe shape always has its open end facing out (to the right or lateral in this figure). We infer that this shape is the result of the small retinal scotoma caused by the injection, which may locally kill retinal ganglion cells and axons passing through the injection spot from more peripheral regions of the retina. This horseshoe shape was often found in the superior colliculus of wild type and *Adcy1*^{brl}+/– mice, but never in *Adcy1*^{brl}–/– mice. This is probably because the projections in the *Adcy1*^{brl}–/– were too diffuse to reveal structures at these small scales, which is consistent with the lack of refinement of retinal afferents in the *Adcy1*^{brl}–/– mice. (D) In wild type mice, focal injections result in small and compact target zones in the superior colliculus.

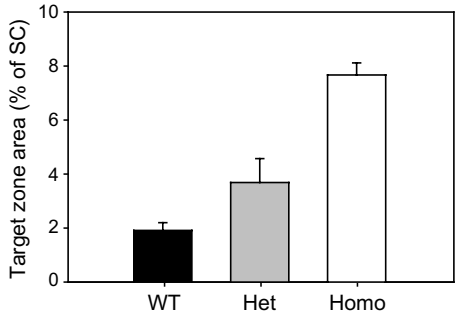

Fig. 3. Quantification of *Adcy1*^{brl}–/– phenotype. Quantitative analysis of the projection area in *Adcy1*^{brl}–/–, heterozygote and WT control mice reveals that the size of the projection or target zones in the superior colliculus of *Adcy1*^{brl}–/– mice (white bar; $n = 6$ Homo mice; $7.7 \pm 0.29\%$) is much larger than in control (black bar; $n = 7$ WT mice; $1.92 \pm 0.29\%$; $p \ll 0.01$) animals. The projection area in heterozygous mice (grey bar; $n = 5$ Het mice; $3.69 \pm 0.88\%$) is also smaller, but more variable, than in *Adcy1*^{brl}–/– mice ($p < 0.01$). Error bars are standard error of the mean.

with this qualitative picture (Fig. 3). The projection size is $1.92 \pm 0.29\%$ in WT mice ($n = 7$), and not significantly larger in *Adcy1*^{brl}+/– (Het) mice at $3.69 \pm 0.88\%$ ($n = 5$). However, the fraction of SC labeled in the *Adcy1*^{brl}–/– (Homo) mice, at $7.7 \pm 0.29\%$ ($n = 6$) was significantly larger than both WT ($p \ll 0.01$) and *Adcy1*^{brl}+/– (Het) littermates ($p < 0.01$). The difference in the relative area of the SC labeled in the different genotypes was neither due to a systematic difference in the size of the retinal injections nor a difference in the total area of the SC between genotypes (data not shown).

3.2. In situ hybridization of AC1

In order to examine the possible role of AC1 in retinotopic map refinement, in situ hybridization for AC1 mRNA was performed on P7 mice, when retinotopic map refinement is nearly complete. The in situ hybridization shows that AC1 is expressed in a wide variety of areas in the CNS during the first postnatal week. There is heavy expression in specific areas of the cortex, hippocampus (CA2 and dentate gyrus) and thalamus (Ventrobasal thalamus). In addition, there is heavy expression in the superficial (retino-recipient) layers of the SC and in the inferior colliculus at P7 (Fig. 4), as well as the retina (data not shown). The strong AC1 expression in both the superior colliculus and retina means that the deficit in topographic refinement we observe in the *Adcy1*^{brl}–/– mice could be due to either presynaptic affects of AC1 in the retinal afferents, or postsynaptic effects of the AC1 in the SC, or both.

4. Discussion

Adenylate Cyclase 1 (AC1) is one of a family of adenylate cyclases that upon activation catalyze the production of cAMP. AC1 and AC8, which are both expressed strongly in the brain, are unique among the family of adenylate cyclases because they are activated by Ca^{2+}/Calmodulin. The rest of the AC family is G-protein coupled. AC1 and AC8 are thus ideally positioned to directly link changes in neuronal activity, perhaps acting through NMDA receptors or voltage gated Ca^{2+} channels, to intracellular biochemical signaling pathways downstream of cAMP, such as PKA.

Previously, it was shown that mutations in AC1 (*Adcy1*^{brl}) have deficits in the development of topographic maps for other sensory modalities. In particular, *Adcy1*^{brl} mice have no barrels ('barrelless') in the primary somatosensory cortex, though rough topography is preserved in these mutants (Van der Loos et al., 1986; Welker & Armstrong-James, 1996). The barrel deficit is found in the pattern of presynaptic thalamic afferents, which do not cluster into barrels, and is also evident in the loss of cortical 'barrel' cytoarchitecture,

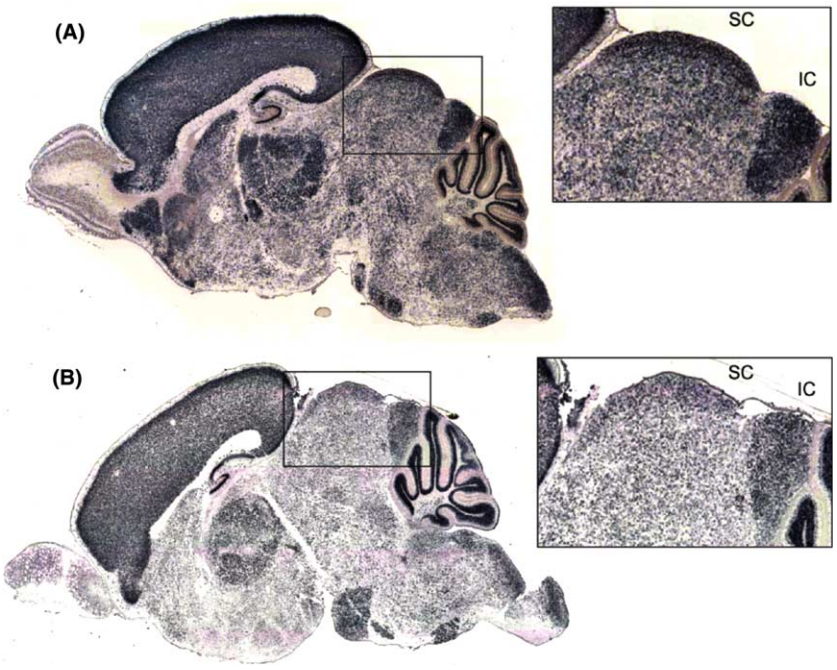

Fig. 4. *Adcy1*^{brl}−/− in situ shows strong expression in the superior colliculus at P7. (A,B) Example in situs using AC1 probes in P7 mice. These are sagittal sections about 1 mm from the midline (rostral is to the left). Insets show the boxed area at higher magnification. Superior colliculus (SC) and inferior colliculus (IC) are identified in the insets. Note the overwhelming similarity in the labeling pattern in these examples, taken from in situ runs made at different times in different mice. The pattern is highly specific. For example, there is strong label in the dentate gyrus (DG) and CA2 areas of the hippocampus, but low label in CA3 and CA1. Also note the strong label in the superficial (retino-recipient) layers of the superior colliculus and inferior colliculus.

which is due to the arrangement of cell bodies and dendrites of thalamo-recipient cells in layer 4 of the cortex. This barrelless phenotype is very reminiscent of the phenotype observed in the retino-collicular projections in the *Adcy1*^{brl} mice, which have intact rough topography but disturbed small-scale retinal ganglion cell afferent refinement. Using *Adcy1*^{brl} mice, it was previously shown that AC1 plays an important role in the development of fundamental aspects of postsynaptic development in the excitatory thalamocortical synapse, which makes up the somatosensory barrel (Lu et al., 2003). Those data suggest that AC1 may play an important role in mediating postsynaptic activity dependent signals that guide the development and refinement of thalamocortical afferents and their recipient dendrites.

Adcy1^{brl} mice, and their analogs, have behavioral deficits related to hippocampal learning as well as motor function mediated by the cerebellum (Storm et al., 1998; Villacres et al., 1998). In the hippocampus and cerebellum, AC1 has been specifically implicated in cellular pathways mediating LTP and LTD. In the cerebellum, for instance, LTP in the parallel fiber-Purkinje cell synapse, which is thought to be a presynaptic phenomenon, is completely absent in Adenylate Cyclase 1 mutants (Storm et al., 1998). Both LTP and LTD are also disturbed at the thalamocortical synapse in AC1 mutant barrel cortex, though at this synapse, it is thought that AC1 activity in the postsynaptic cell is responsible for the synaptic plasticity deficits (Lu et al., 2003). In addition to LTP and LTD deficits, thalamocortical synapses in *Adcy1*^{brl} mutant mice have significantly altered glutamate receptor expression and function (Lu et al., 2003).

Retinal ganglion cell axon refinement in the superior colliculus likely occurs through a Hebbian process that is triggered by Ca^{2+} influx through NMDA receptors or Voltage dependent Ca^{2+} channels. This Hebbian process requires patterned retinal waves to instruct the refinement of retinal ganglion cell arbors into a map that mirrors the neighborhood relationship of coactive retinal ganglion cells. Neighboring retinal ganglion cells that are coactive are likely to form synapses on the same or nearby neurons in the superior colliculus, thereby refining the course topography established by molecular or chemical cues during early development. Based upon the data presented here, we propose that an important signaling pathway downstream of Ca^{2+} influx in this activity dependent refinement process is likely to be cAMP mediated PKA activity in superior colliculus neurons. This PKA activity may be responsible for regulating the activity dependent development of retinocollicular synapses through its role in trafficking of glutamate receptors at the synapse.

A presynaptic role for AC1 in the barrel cortex and the superior colliculus cannot be ruled out. Recently, it was suggested that AC1 acts specifically in retinal

afferents to affect retinotopic map development (Ravary & Muzerelle, 2003). This argument is strengthened by the known role of cAMP in modulating retinal waves (Stellwagen et al., 1999). This suggests that AC1 mutant mice may have lower levels of cAMP in the retina, and therefore less frequent retinal waves than in control mice. A dramatic decrease in the frequency of retinal waves may be deleterious for the activity dependent refinement of retino-collicular synapses. Whatever the locus of the retinotopic map refinement deficit in the *Adcy1*^{brl} mice, be it pre- or postsynaptic, Adenylate Cyclase is ideally positioned downstream of neuronal activity to trigger signaling events that mediate retinotopic map refinement in the superior colliculus.

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