Published in final edited form as:

Neuroscience. 2015 April 02; 290: 518–529. doi:10.1016/j.neuroscience.2015.01.043.

THALAMIC ADENYLYL CYCLASE 1 IS REQUIRED FOR BARREL FORMATION IN THE SOMATOSENSORY CORTEX

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Abstract

Cyclic AMP signaling is critical for activity-dependent refinement of neuronal circuits. Global disruption of adenylyl cyclase 1 (AC1), the major calcium/calmodulin-stimulated adenylyl cyclase in the brain, impairs formation of whisker-related discrete neural modules (the barrels) in cortical layer 4 in mice. Since AC1 is expressed both in the thalamus and the neocortex, the question of whether pre- or postsynaptic (or both) AC1 plays a role in barrel formation has emerged.

Previously, we generated cortex-specific AC1 knockout (Cx-AC1KO) mice and found that these animals develop histologically normal barrels, suggesting a potentially more prominent role for thalamic AC1 in barrel formation. To determine this, we generated three new lines of mice: one in which AC1 is disrupted in nearly half of the thalamic ventrobasal nucleus cells in addition to the cortical excitatory neurons (Cx/pTh-AC1KO mouse), and another in which AC1 is disrupted in the thalamus but not in the cortex or brainstem nuclei of the somatosensory system (Th-AC1KO mouse). Cx/pTh-AC1KO mice show severe deficits in barrel formation. Th-AC1KO mice show even more severe disruption in barrel patterning. In these two lines, single thalamocortical (TC) axon labeling revealed a larger lateral extent of TC axons in layer 4 compared to controls. In the third line, all calcium-stimulated adenylyl cyclases (both AC1 and AC8) are deleted in cortical excitatory neurons. These mice have normal barrels. Taken together, these results indicate that thalamic AC1 plays a major role in patterning and refinement of the mouse TC circuitry.

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Keywords

mouse; conditional knockout; Cre/loxP system; neuronal circuit; refinement; somatosensory system

INTRODUCTION

Neuronal activity plays a critical role in the development of highly organized circuits of the sensory systems (Goodman and Shatz, 1993; Katz and Shatz, 1996; Yamamoto and Lopez-Bendito, 2012). Layer 4 of the primary somatosensory cortex (barrel cortex) of rodents has a patterned array of modules, called “barrels”, corresponding to the arrangement of whiskers on the snout (Woolsey and Van der Loos, 1970; Fox, 2008). In each barrel of the mouse neocortex, presynaptic thalamocortical (TC) afferents cluster in the center, and postsynaptic cortical neurons aggregate around these clusters, forming a cylindrical structure (barrel wall). Barrels form within a week after birth in a manner dependent on the inputs from the whiskers via the brainstem and thalamus (Wu et al., 2011; Erzurumlu and Gaspar, 2012; Narboux-Neme et al., 2012; Li et al., 2013).

Mouse genetics have contributed significantly to the understanding of the molecular bases of activity-dependent patterning in the neocortex (Erzurumlu and Kind, 2001; Erzurumlu and Iwasato, 2006; Li and Crair, 2011; Wu et al., 2011). Molecules that are involved in glutamate release and serotonergic regulation on the presynaptic side of TC connectivity, such as monoamine oxidase A (MAOA), serotonin (5-HT) transporter (5-HTT), and 5-HT receptor 1B (5-HT1B), Rim1 and Rim2, and VgluT1 and VgluT2, have been suggested to play important roles in barrel formation (Cases et al., 1996; Wu et al., 2011; Narboux-Neme et al., 2012; Li et al., 2013). On the other hand, glutamate receptors including *N*-methyl-D-aspartate receptor (NMDAR) and mGluR5 and their downstream signaling molecules on the postsynaptic side are also involved in barrel formation (Iwasato et al., 1997, 2000; Hannan et al., 2001).

Among many molecules, adenylyl cyclase 1 (AC1) has received much attention. AC1 is the neurospecific type of calcium-stimulated adenylyl cyclases, which produce cyclic AMP (cAMP) upon stimulation of calcium and G protein (Hanoune and Defer, 2001; Wang and Storm, 2003). The AC1 gene is highly expressed both in cortical layer 4 cells and their presynaptic partners, thalamic ventrobasal nucleus (VB) cells, during early postnatal stages (Nicol et al., 2005). In addition, the AC1 protein is located both at the postsynaptic density and presynaptic axons (Conti et al., 2007). Gene disruption of AC1 by spontaneous or targeted mutation causes complete loss of barrels (Abdel-Majid et al., 1998; Iwasato et al., 2008), indicating a critical role of AC1 in barrel formation. It is well established that NMDAR and cAMP-dependent protein kinase (PKA) in postsynaptic cortical neurons play important roles in barrel formation (Iwasato et al., 2000; Datwani et al., 2002; Lee et al., 2005; Inan et al., 2006; Watson et al., 2006), and therefore, postsynaptic AC1 has been a candidate molecule which could link NMDAR-mediated calcium influx and PKA signaling. On the other hand, AC1 could also function on the presynaptic side by activating PKA and

subsequently play a role in presynaptic glutamate release (Erzurumlu and Kind, 2001; Lu et al., 2006).

To dissociate the pre- and postsynaptic roles of AC1 in barrel formation, we previously generated mice in which AC1 is disrupted specifically in the cortical excitatory neurons [cortex-specific AC1 knockout (Cx-AC1KO) mice] (Iwasato et al., 2008). Cx-AC1KO mice showed a mild impairment in the asymmetry of barrel cell dendrites, which usually have a strong orientation bias toward the barrel center, and abnormal postsynaptic physiological properties, demonstrating important roles of postsynaptic AC1 in TC circuit development. On the other hand, TC axonal clustering and barrel wall formation, which are completely disrupted in global AC1 null mutant mice, were apparently normal in Cx-AC1KO mice (Iwasato et al., 2008). These phenotypic discrepancies between global AC1 KO mice and Cx-AC1KO mice suggested that the presynaptic AC1 might play an important role in barrel formation. Here, by generating new lines of genetically altered mice, including thalamus-specific AC1 KO (Th-AC1KO) mice, we reveal thalamic roles of AC1 in cortical barrel formation.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed according to the guidelines for animal experimentation of the National Institute of Genetics (NIG) and RIKEN Brain Science Institute (BSI). AC1 flox, AC1 KO and Cx-AC1KO mice (Iwasato et al., 2008), Emx1-Cre KWN and Emx1-Cre KDN mice (Iwasato et al., 2000, 2004, 2008), 5-HTT-Cre mice (Tg208 and Tg810) (Arakawa et al., 2014) and TCA-GFP mice (Mizuno et al., 2014) were generated in RIKEN BSI and reported. AC8 KO mice (Schaefer et al., 2000), CAG-CAT-Z reporter mice (Sakai and Miyazaki, 1997), and RNZ reporter mice (Kobayashi et al., 2013) were reported previously. C57Bl/6J (B6) mice were purchased from CLEA Japan Inc. (Tokyo, Japan) for breeding. PCR primer sets used for genotyping are as follows: KS64(5'-GTG ACT TTG TGG TGT GGC TG-3')/KS66(5'-TGG CCA CCA AGG ACT CTA TG-3') for Emx1-Cre KWN and Emx1-Cre KDN mice (346 bp); AS76(5'-AGA GCT CTC AGT CTT GTC TC-3')/AS77(5'-TCA TCA CTC GTT GCA TCG AC-3') for 5-HTT-Cre mice (242 bp); CWC2(5'-ACC TGA TGG ACA TGT TCA GGG ATC G-3')/CWC3(5'-TCC GGT TAT TCA ACT TGC ACC ATG C-3') for Cre mice (108 bp); KS68(5'-TTCTCGTTGGGGTCTTTGCT-3')/KS69(5'-ACT TCTTCAAGTCCGCCATG-3') for GFP mice (397 bp); 133(5'-CAT GCC CTC TTG GGT ACT GTC TGT C-3')/135(5'-CTC CCT TCA GAC CCT GTC ACC TCT G-3')/139 (5'-AGG GAC CAA GAT CTG GCC TCT CAT C-3') for AC1^{flox} (273 bp), AC1^{wild-type} (101 bp) and AC1⁻ (165 bp) alleles; AC8WT5' (5'-GAC TCC AAA GAG CGA AGT GA-3')/AC8WT3' (5'-ACG TAT AGG CGG CTG CAG AA-3')/AC8KO3' (5'-GTT CAG ACA TCT GTG TCC AC-3')/pgkNeo (5'-GGT GGA TGT GGA ATG TGT GC-3') for AC8^{wild-type} (430 bp) and AC8⁻ (280 bp) alleles; R1295(5'-GCGAAGAGTTTGTCTCAACC-3')/R523(5'-GGAGCGGGAGAAATGGATATG-3')/R26(5'-AA AGTCGCTCTGAGTTGTTAT-3') for RNZ (330 bp) and WT (603 bp) alleles; 5'-TCGGCGGTGAAATTATCGATG AGC-3'/5'-CCACAGCGGATGGTTCGGATAATGC-3' for CAG-CAT-Z reporter mice.

Quantification of recombination efficiencies

The VB thalamus was excised from coronal slices (60- μ m-thick) of three each of Emx1-Cre KWN;AC1^{flox/+}, Emx1-Cre KDN;AC1^{flox/+}, AC1^{flox/+}, and AC1^{-/+} mice at P7. Then, 24 VBs from three mice with the same genotypes were pooled and genomic DNA samples were prepared from these pools using DNeasy Tissue Kit (Qiagen). Then genomic DNA samples of AC1^{flox/+} and AC1^{-/+} mice were mixed in 999:1, 99:1, 9:1, 8:2 or 5:5 ratios. PCR reaction using the 137(5'-CTT GGG TAC TGT CTG TCT AGC CAT C-3')/139(5'-AGG GAC CAA GAT CTG GCC TCT CAT C-3') primer set was performed to VB genomic DNA samples of Emx1-Cre KWN;AC1^{flox/+}, Emx1-Cre KDN;AC1^{flox/+}, AC1^{flox/+}, AC1^{-/+} and mixtures of AC1^{flox/+} and AC1^{-/+} to estimate the ratio of AC1⁻ allele in total AC1 alleles (AC1⁻ and AC1^{flox}). PCR conditions were 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s, and 72 °C for 7 min. To enhance the signals, Southern blot was also performed using a probe for the AC1⁻ allele. Experiments were repeated with a new set of Emx1-Cre KWN;AC1^{flox/+}, Emx1-Cre KDN;AC1^{flox/+}, AC1^{flox/+}, and AC1^{-/+} mice (19 VBs from three mice per genotype). For Southern blot analyses, an AC1⁻ allele fragment, which was amplified from AC1^{-/+} mouse genomic DNA by PCR using the primer set 137/139, was cloned into pGEM-TEasy vector (Promega, Madison, WI, USA) and used as the probe, and Gene Images Random Prime Labeling Module (Amersham RPN3540) and CDP-Star Detection Module (Amersham RPN3510) were used.

Histology

Mice intraperitoneally injected with a lethal dose of tribromoethanol were perfused transcardially with saline and subsequently with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were removed, post-fixed in 4% PFA in 0.1 M PB and in some cases cryoprotected in 30% sucrose in 0.1 M PB until brains were completely sunk. Coronal and tangential slices were cut using freezing microtome (ROM-380; Yamato, Asaka, Japan) or microslicer (Dosaka, Kyoto, Japan). Cytochrome oxidase (CO) and Nissl staining and 5-HTT (DIASORIN 24330: 1:10,000) and VgluT2 (Synaptic Systems 135403: 1:1500) immunohistochemistry were described previously (Iwasato et al., 1997, 2000, 2008). For CO staining (100- μ m-thick tangential sections), 20 Cx-AC1KO (Emx1-Cre KDN;AC1^{flox/-}), 34 Cx/pTh-AC1KO (Emx1-Cre KWN;AC1^{flox/-}), 14 Th-AC1KO(Tg810) (5-HTT-Cre (Tg810);AC1^{flox/-}), 20 Th-AC1KO(Tg208) (5-HTT-Cre (Tg208);AC1^{flox/-}), 4 Cx-AC1;AC8 double KO (Emx1-Cre KDN;AC1^{flox/-};AC8^{-/-}) and 68 control (AC1^{flox/-}, AC1^{flox/-};AC8^{-/-} and other littermate) mice were used at various ages (P6-adult). For Nissl staining (50–100- μ m-thick tangential sections), 7 Cx-AC1KO, 21 Cx/pTh-AC1KO, 15 Th-AC1KO(Tg810), 9 Th-AC1KO(Tg208), 16 Cx-AC1;AC8 double KO, and 54 control (AC1^{flox/-}, AC1^{flox/-};AC8^{-/-} and other littermate) mice were used at various ages (P6-adult). For 5-HTT immunohistochemistry (100- μ m-thick tangential sections), two Cx-AC1KO, nine Cx/pTh-AC1KO, nine Th-AC1KO(Tg810), four Cx-AC1;AC8 double KO, and 13 control (AC1^{flox/-} and other littermate) mice were used at P5–P11. For CO staining with 60- μ m-thick coronal sections, 11 Cx/pTh-AC1KO, six Th-AC1KO(Tg810), and 18 control (AC1^{flox/-}) mice were used at P6–P9. For VgluT2 immunohistochemistry (50- μ m-thick coronal sections), two Th-AC1KO(Tg208), and two control (AC1^{flox/-}) mice were used at P7. For GFP imaging with 30–60- μ m-thick tangential sections, 13 TCA-GFP;Th-

AC1KO(Tg208), and 13 control (TCA-GFP;AC1^{flx/-}) mice were used at P7. For GFP imaging (50- μ m-thick coronal sections), four TCA-GFP;Th-AC1KO(Tg208), and four control (TCA-GFP; AC1^{flx/-}) mice were used at P7. LacZ staining was described (Arakawa et al., 2014). In brief, mice were perfused with saline followed by 10% formalin. The brains were removed and cut using Microslicer or BrainMatrix (RBM-2000C; ASI Instruments, Warren, MI, USA). Slices were stained in LacZ solution [5 mM K₃FeCN₆, 5mM K₄FeCN₆, 2 mM MgCl₂, 1 mg/ml X-gal, 0.05 M PR (7.4)] at 37 °C overnight.

Quantitative analysis of TC axonal segregation

P7 mice were anesthetized with a lethal dose of tribromoethanol (Avertin, Wako) and perfused with 0.9% NaCl solution. Brains were removed and postfixed overnight in 4% PFA with 0.1 M PB. Flattened cortex was cryoprotected for 3 days in 30% sucrose in 0.1 M PB and sectioned in the tangential plane on a freezing microtome (ROM-380, Yamato) at 30- μ m thickness. Slices were mounted on glass slides with VECTASHIELD (Vector Laboratories). TCA-GFP fluorescence images were collected with a Leica TCS SP5 confocal scanning microscope (Leica, Nussloch, Germany) using a 10 $\times$ objective with channels for Alexa 488. Images that covered whole posterior medial barrel subfield (PMBSF) area of the barrel cortex were used for TCA-GFP quantification. PMBSF images were edited by using Photoshop CS4. GFP intensities were measured in 150- μ m $\times$ 300- μ m rectangles within a row (C1 and C2) and arc (B2 and C2) using ImageJ software. Statistical analysis was conducted using unpaired t-test in Excel and SPSS.

Single TC axonal tracing

Brains were removed from 4% PFA-perfused P7 mice and kept in the same fixative. The forebrains were split in half along the sagittal plane, embedded in 2% agar, and cut in an angle optimized for intact TC pathway (Lee et al., 2005) by using a vibratome (VT1000S, Leica, Nussloch, Germany) at a thickness of 300 μ m. The origin (VB) and the target (barrel cortex) of the TC axons were preserved in these sections. A tiny (20–30 μ m in diameter) crystal of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Eugene, OR, USA) was inserted into the large whisker barreloid area of the VB. The sections were kept in 4% PFA (in 0.1 M PB) at 37 °C in the dark for 1–2 weeks for dye diffusion. DiI-labeled sections were then examined under a Nikon epifluorescence microscope. Images at different focal planes were acquired using a CoolSnap digital camera (US Photometrics, Tucson, AZ, USA) or a digital CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) with the MetaVue image software (Universal imaging Corporation, Downingtown, PA, USA). Multiple images of axonal processes from serial images were superimposed by using Adobe Photoshop 7.0 software (San Jose, CA, USA) and image J (<http://imagej.nih.gov/ij/>) on a PC. Single TC axons were then reconstructed and traced. All measures were performed blinded to genotypes. For statistical analyses of characteristics of 11, 11, and 17 single TC axons from 6 Cx-AC1KO, 5 Cx/pTh-AC1KO, and 10 control (AC1^{flx/-}) mice, respectively, SPSS was used for one-way ANOVA followed by Dunnett's test. For statistical analyses of 18 and 14 single axons from seven Th-AC1KO and five control mice, respectively, Excel was used for two-tailed unpaired *t*-test. Values were given as mean $\pm$ standard errors.

RESULTS

Partial deletion of thalamic AC1 causes deficits in barrel formation

We previously reported that cortex-specific AC1 KO (Cx-AC1KO) mice have histologically normal appearing barrels (Iwasato et al., 2008). The Cx-AC1KO mice are generated by crossing the AC1^{flox} mice with a line of Emx1-Cre knock-in (KI) mice [Emx1-Cre KI Neo (Emx1-Cre KDN) mice], in which Cre recombinase gene is expressed under the control of the dorsal telencephalon-specific *Emx1* promoter and the pgk-neo cassette used for positive selection in embryonic stem (ES) cell clones is removed from the KI allele (Iwasato et al., 2004, 2008). However, when we generated and analyzed conditional AC1 KO mice (here we call this line Cx/pTh-AC1KO mice for the reason elaborated below) using another line of Emx1-Cre KI mice [Emx1-Cre KI w/Neo (Emx1-Cre KWN) mice] that retains the pgk-neo cassette in the KI allele (Iwasato et al., 2000, 2004), unexpectedly, we observed defects in barrel formation (Fig. 1). CO staining revealed cortical barrels (Wong-Riley and Welt, 1980), which is a most widely used method to visualize the patterned distribution of barrels. Unlike the barrel cortex phenotype in Cx-AC1KO mice, the barrel patterns were partially impaired in Cx/pTh-AC1KO mice. CO-stained patches in the PMBSF, which corresponds to the whiskers, were present but slightly obscured. CO-patches corresponding to the sensory hairs in the anterior snout were mostly diminished (Fig. 1A, B). TC axons were revealed by 5-HTT immunohistochemistry and we found that these phenotypes were similar to those revealed by CO staining: the TC clusters corresponding to the sensory hairs in the anterior snout were mostly diminished; while those corresponding to the whiskers were mostly present albeit obscured (Fig. 1C, D). The ring-like patterns (barrel walls) composed of layer 4 neurons were obscured even in the PMBSF (Fig. 1E, F). On the other hand, no abnormality was observed in the barreloids, which are whisker-related modules in the VB (Fig. 1G, H).

It is likely that the differences of barrel patterning between Cx-AC1KO and Cx/pTh-AC1KO mice were derived from differences of specificities of Cre-mediated recombination between Emx1-Cre KDN and Emx1-Cre KWN mice. We tested this possibility (Fig. 2). When we examined adult Emx1-Cre KDN and Emx1-Cre KWN mice crossed with CAG-loxP-CAT-loxP-LacZ (CCZ) reporter mice (Sakai and Miyazaki, 1997), Emx1-Cre KDN mice showed Cre-mediated recombination only in the dorsal telencephalon including the cortex, amygdala and hippocampus (Fig. 2A); whereas, Emx1-Cre KWN mice showed ectopic LacZ signals in a few non-dorsal telencephalon areas such as dorsal thalamus, including the VB (Fig. 2B), and cerebellar molecular layers (data not shown) in addition to the dense LacZ signals in the dorsal telencephalon. When we used CCZ reporter mice, the ectopic Cre-mediated recombination was detected in the late postnatal period such as 3 weeks (data not shown) and adulthood (Fig. 2B) but not in neonatal stages (data not shown). However, it should be noted that the efficiencies of Cre-mediated recombination is often different among the target floxed loci. For example, a line of CaM-KII-Cre Tg mice, which gives hippocampal CA1-specific Cre-mediated recombination when tested with a LacZ reporter line, shows forebrain-wide deletion with a CNB1^{flox} allele (Zeng et al., 2001). Therefore, it is possible that Emx1-Cre KWN mice may delete the floxed AC1 gene efficiently in the neonatal period, which is important for barrel formation. To test this possibility, we evaluated

efficiencies of Cre-mediated recombination in the floxed AC1 gene in the VB thalamus in neonates. By semi-quantitative analyses, we found that Emx1-Cre KWN mice induced ectopic Cre-mediated recombination in approximately 20%–50% of VB cells at P7 (Fig. 2C, D); on the other hand, Emx1-Cre KDN mice induces no (less than 0.1%) recombination in the VB (Fig. 2C', D'). These results clearly showed that conditional AC1 KO mice generated by crossing AC1^{flox} mice with Emx1-Cre KWN mice had partial AC1 disruption in the VB thalamus in addition to the cortex: thus we call Emx1-Cre KWN;AC1^{flox/-} mice as cortex- and partial thalamus-specific AC1 KO (Cx/pTh-AC1KO) mice.

Partial deletion of AC1 in the thalamus causes defects in TC axonal refinement

Global AC1 mutant mice have larger lateral extent of TC axons in layer 4 of the barrel cortex (Welker et al., 1996; Gheorghita et al., 2006). To test whether a similar morphological abnormality can be observed in TC axons of Cx-AC1KO and/or Cx/pTh-AC1KO mice, we examined morphological features of single TC axon arbors in the barrel cortex by applying DiI into the VB nucleus in TC slices (Fig. 3). We found that DiI-labeled TC axons were predominantly distributed in layer 4 in all three groups (Fig. 3A–C), and their lateral extent was significantly larger in Cx/pTh-AC1KO mice, but not in Cx-AC1KO mice, than in control mice (Fig. 3F). Total axonal arbor length was also larger in Cx/pTh-AC1KO mice, but not in Cx-AC1KO mice, than in control mice (Fig. 3E), and the total branch points were comparable among control, Cx-AC1KO and Cx/pTh-AC1KO mice (Fig. 3D). These results suggest the importance of thalamic AC1 for TC axonal refinement, which is an important component of barrel formation.

Th-AC1KO mice show severe deficits in barrel formation

To directly investigate the role of thalamic AC1 for barrel formation, we generated Th-AC1KO mice (Fig. 4). For this, we used bacterial artificial chromosome (BAC) transgenic (Tg) mice expressing the Cre recombinase gene under the control of 5-HTT promoter (Arakawa et al., 2014; Mizuno et al., 2014). The endogenous 5-HTT gene expression is specific to the sensory thalamus and raphe during early postnatal development (Allen-Developing-Mouse-Brain-Atlas; Lebrand et al., 1998; Narboux-Neme et al., 2008). In this work, we used Tg810 and Tg208 lines of 5-HTT-Cre mice, which had similarly high levels of Cre-mediated recombination in the VB and recombination was negligible in the somatosensory cortex or PrV brainstem nucleus (Arakawa et al., 2014) (Fig. 4A–D). In our recent paper (Arakawa et al., 2014), we showed that virtually all of neurons (97.45% $\pm$ 0.63%) in the VB of 5-HTT-Cre (Tg810) mice exhibit Cre-mediated recombination at P5.

We obtained Th-AC1KO mouse lines by crossing 5-HTT-Cre (Tg810 or Tg208) mice and AC1^{flox} mice. Both types of Th-AC1KO mice were healthy and viable as global AC1 KO mice. The thickness of the barrel cortex was similar between genotypes [911.5 μ m $\pm$ 31.5 μ m for control (n = 7) and 931.3 μ m $\pm$ 24.2 μ m for Th-AC1KO (Tg208, n = 8) at P7] (Fig. 4K, L). The layer thickness ratios were also similar between genotypes [layer 1: 4.8% $\pm$ 0.3% and 4.9% $\pm$ 0.3%; layer 2/3: 19.7% $\pm$ 0.9% and 21.9% $\pm$ 1.1%; layer 4: 14.3% $\pm$ 0.8% and 14.5% $\pm$ 0.9%; layer 5: 30.6% $\pm$ 1.2% and 28.8% $\pm$ 0.6%; layer 6: 30.6% $\pm$ 1.1% and 29.9% $\pm$ 0.6%, for control and Th-AC1KO mice, respectively].

Firstly, we analyzed barrel patterning in the Th-AC1KO mice (both Tg810 and Tg208-derived lines) with CO histochemistry. CO-patches corresponding to the anterior snout were completely absent (Fig. 4E, F). In the PMBSF area, most patterns were diminished and only a few patches corresponding to relatively large whiskers were visible, albeit obscured. It looked that larger barrels were harder to be impaired than smaller barrels, as many lines of previous evidence suggested (e.g. Iwasato et al., 2000; Watson et al., 2006; Ballester-Rosado et al., 2010). These phenotypes of Th-AC1KO mice were severer than those of Cx/pTh-AC1KO mice. To analyze TC axonal clustering directly, we used immunohistochemistry for 5-HTT (Fig. 4G, H). We also used TCA-GFP Tg mice, in which enhanced GFP is expressed in TC axons (Mizuno et al., 2014), to observe TC axonal patterns. We generated Th-AC1KO (Tg208);TCA-GFP mice and analyzed GFP signals in these mice. The boundary of TC axonal patterns was largely diminished in the area corresponding to the large whiskers and almost completely diminished in the area corresponding to the small sensory hairs in the anterior snout in Th-AC1KO;TCA-GFP mice (Fig. 4M, N). We quantified TC axonal segregation in areas corresponding to the large whiskers (Fig. 5). We defined the segregation index as the difference between GFP intensities in barrels and septa, and compared it between Th-AC1KO and control mice. We found that both the segregation index between C1 and C2 barrels and that between B2 and C2 barrels were significantly smaller in Th-AC1KO mice than in control mice (Fig. 5F, L). These results indicated that even in the large whisker-corresponding areas in the PMBSF, TC axonal segregation was severely impaired both within a row (C1 and C2) and also between rows (B2 and C2). On the other hand, in these mice layer 4 and layer 6 targeting of TC axons looked normal, although again there was no whisker-specific segregation in layer 4 (Fig. 4O, P). We next analyzed ring-like structures corresponding to the barrel wall using Nissl staining and found that only rudimentary patterns were visible only for the area corresponding to the large whiskers in the Th-AC1KO cortex (Fig. 4I, J). In the coronal plane, barrel walls were detected in layer 4 of control mice but not in that of Th-AC1KO mice (Fig. 4K, L). On the other hand, thalamic barreloids appeared normal in Th-AC1KO mice (Fig. 4Q–T). Thus, barrel morphologies were notably impaired in Th-AC1KO mice. We found no differences in these phenotypes between Th-AC1KO mice generated using 5-HTT-Cre Tg810 line and those generated using 5-HTT-Cre Tg208 line. These results clearly demonstrate that thalamic or presynaptic AC1 signaling is critical for barrel formation.

Th-AC1KO mice show defects in TC axonal refinement

To directly investigate the role of thalamic AC1 for the TC axonal refinement, we characterized morphologies of single TC axons of Th-AC1KO mice and their controls. We applied DiI into the thalamic VB nucleus in TC slices and examined morphological characteristics of single TC axonal arbors in the barrel cortex (Fig. 6). The lateral extent of TC axons in layer 4 was significantly larger in Th-AC1KO mice than in control mice (Fig. 6E), suggesting that TC axonal refinement was impaired in Th-AC1KO mice. These results were consistent with those from Cx/pTh-AC1KO mice (Fig. 3F). On the other hand, the total length of TC axons was not different between Th-AC1KO (Tg208) mice and control mice (Fig. 6D), and the number of branch points was smaller in Th-AC1KO mice than in control mice (Fig. 6C). These results were different from those of Cx/pTh-AC1KO mice (Fig. 3D, E). The discrepancies may suggest important roles of combination of cortical and thalamic

AC1 for aspects of TC axon development. In summary, our results indicate that thalamic AC1 is important for TC axonal refinement in cortical layer 4.

Ca²⁺-stimulated adenylyl cyclases in the cortex do not have a major role in barrel formation

Lastly, we examined the possibility that lack of AC1 activity in the cortex could be compensated by the activity of AC8, the other Ca²⁺/calmodulin-stimulated adenylyl cyclase (Hanoune and Defer, 2001; Wang and Storm, 2003), which is also richly expressed in the developing cortex (Nicol et al., 2005). Double KO mice of AC1 and AC8 sometimes show more severe phenotypes than AC1 or AC8 single KO mice. For example, long-term memory and late-phase long-term potentiation (LTP) in the hippocampus are normal in either of AC1 and AC8 single KO mice but they are severely impaired in AC1;AC8 double KO mice (Wong et al., 1999); thus AC8 can compensate functional deficits caused by AC1 deletion at least partially. This consideration is important; since KO mice of PKA regulatory subunit II β and cortex-specific KO of NR1, the essential subunit of NMDAR, show similar barrel defects in the cortex (Iwasato et al., 2000; Datwani et al., 2002; Inan et al., 2006; Watson et al., 2006). Therefore, calcium-stimulated adenylyl cyclases (AC1 and AC8), which produce cAMP upon stimulation of calcium, could be mediators that link NMDAR-mediated calcium influx and PKA activation in the cortex (Inan et al., 2006; Watson et al., 2006; Iwasato et al., 2008).

To test the possibility that AC8 function may compensate AC1 deficiency in barrel formation, we generated Cx-AC1KO mice in the background of AC8 global KO mice [Cx-AC1;AC8 double KO (Emx1-Cre KDN;AC1^{flox/-};AC8^{-/-} mice)] and analyzed them. We found that both TC axonal clustering and barrel wall formation appeared normal in the Cx-AC1;AC8 double KO mice (Fig. 7). These results show that calcium-stimulated adenylyl cyclases (AC1 and AC8) in the cortex do not have a major role in barrel formation.

DISCUSSION

Thalamic AC1 is important for barrel formation

Since it was found that disruption of the gene encoding AC1 is causative of spontaneous barrelless mutation (Welker et al., 1996; Abdel-Majid et al., 1998), AC1 and cAMP-signaling have been central in studies of molecular mechanisms of neuronal circuit refinement in early postnatal development. While it was clear that AC1 is important for barrel formation, there has been a debate whether AC1 functions in postsynaptic cortical neurons or in presynaptic TC axons in cortical patterning (Erzurumlu and Kind, 2001; Wu et al., 2011; Nicol and Gaspar, 2014). We previously generated Cx-AC1KO mice, in which AC1 is disrupted in all cortical excitatory neurons, and found that these mice have apparently normal barrel patterns (Iwasato et al., 2008). Here we generated Cx/pTh-AC1KO and Th-AC1KO mice, in which the AC1 gene is disrupted in the thalamic VB, and found that in these mice, barrel formation was impaired. Thus, thalamic AC1 is indispensable for normal barrel formation in the somatosensory cortex. 5-HTT Cre mice (both Tg208 and Tg810) that were used for generation of Th-AC1KO mice show Cre-mediated recombination in the raphe in addition to the VB (Arakawa et al., 2014). However, it is unlikely that AC1

deletion in the raphe results in barrel impairment in Th-AC1KO mice, because AC1 expression is undetectable in the raphe during postnatal development (Allen-Developing-Mouse-Brain-Atlas; Nicol et al., 2005). In the visual system of mammals, recent evidence by *in vitro* (Ravary et al., 2003) and *in vivo* (Dhande et al., 2012) experiments suggest that presynaptic retinal AC1 is important for the postnatal refinement of retinotectal and retinogeniculate projections. Our results in the somatosensory TC circuit are consistent with their results in the visual system, and further support presynaptic roles of AC1 for sensory circuit refinement.

Discrepancies between barrel phenotypes of Th-AC1KO mice and those of barrelless and global AC1 KO mice

In barrelless and global AC1 KO mice, barrel patterns are completely absent (Welker et al., 1996; Iwasato et al., 2008); while in Th-AC1KO mice, there were still patterns, albeit faint (Fig. 4E–J, M, N). A possible explanation for this discrepancy is that AC1 expression in non-VB neurons may contribute to formation of the rudimentary barrel patterns seen in Th-AC1KO mice. Brainstem AC1 is a strong candidate for the role. In wild-type brains during neonatal stages, the AC1 gene is highly expressed not only in the cortex and thalamus but also in the brainstem trigeminal nuclei (Nicol et al., 2005). Barreloids are partially impaired in global AC1 KO mice (Welker et al., 1996; Iwasato et al., 2008) but they were normal in Th-AC1KO mice (Fig. 4O–R). Therefore, it is likely that, in Th-AC1KO mice, normal barreloids are formed at least partly by function of presynaptic brainstem AC1; and in turn normal barreloids contribute to the formation of cortical barrels, albeit immature, even in the absence of thalamic AC1. To test these possibilities, generation and analyses of brainstem-specific AC1 KO mice would be helpful. Another possible explanation is that, in Th-AC1KO mice, the timing of AC1 deletion was not early enough to completely diminish AC1 protein in the neonatal stages, which are important periods for barrel formation. We first detected 5-HTT-Cre-mediated gene deletion in the VB thalamus as early as at embryonic day 15 (E15), but at this age, recombination was not as efficient as later embryonic stages such as at E17 (Arakawa et al., 2014). On the other hand, expression of the AC1 gene in VB is already high at E15 (Nicol et al., 2005). Therefore, we cannot exclude the possibility that the AC1 protein is remained in the VB of Th-AC1KO neonates, and this residual AC1 may contribute to the formation of rudimentary patterns in the cortex. To test this possibility, a Cre mouse line in which floxed alleles can be deleted completely in the VB at E15 or earlier stages is required.

How does presynaptic AC1 play a role in cortical barrel formation?

It is an important question of how functions of presynaptic thalamic AC1 form barrels in the cortex. A possibility is that thalamic AC1 contributes to barrel formation by regulating neurotransmitter release from the TC axon terminal. Recently, lines of mice in which TC neurotransmission is blocked by thalamus-specific KO of Rim1 and Rim2 (Narboux-Neme et al., 2012), VgluT1 and VgluT2 (Li et al., 2013), or Munc18 (Li et al., 2013) were reported. These mice show barrel defects similar to Th-AC1KO mice, suggesting an important role for TC neurotransmission in barrel formation. In barrelless mice, release probability of TC synapses is substantially decreased (Lu et al., 2006); while in Cx-AC1KO mice, it is intact (Iwasato et al., 2008). Therefore, it is likely that TC neurotransmission is decreased in Th-AC1KO mice, which could result in impaired barrel formation. Indeed,

some molecules involved in transmitter release are targets of PKA (Evans and Morgan, 2003; Lu et al., 2006), which could be activated by cAMP produced by presynaptic AC1. In addition, cAMP-GEFII also functions as a direct target of cAMP to induce exocytosis (Kawasaki et al., 1998; Ozaki et al., 2000). Another possibility is that thalamic AC1 contributes to cortical barrel formation through a mechanism that involves spontaneous activity and axon guidance molecules, but not synaptic transmission, in TC axons. This scenario is inspired by studies of retinotectal projection, which is also refined during early postnatal period in an activity-dependent manner. In organotypic retinotectal coculture experiments, AC1 deletion in retina impairs retinal axon refinement in the tectum, which also depends on ephrin-A5, a guidance molecule (Nicol et al., 2006). The ephrin-A5-dependent refinement of retinotectal projection requires cAMP oscillations and spontaneous retinal activity in the retina but does not require retinotectal synaptic transmission (Nicol et al., 2007).

In the formation of patterned neuronal circuits in the vertebrate brain, numerous molecules and several types of neuronal activity work in concert on both presynaptic and postsynaptic sides (Mizuno et al., 2010; Wu et al., 2011; Erzurumlu and Gaspar, 2012; Yamamoto and Lopez-Bendito, 2012). Our current genetic study reveals an important role for presynaptic AC1 in TC circuit refinement in the mouse somatosensory cortex.

Acknowledgments

We thank R. Ando, Y.M. Saito, T. Sato, S. Kouyama, and M. Kanbayashi for their technical assistance. A.S. was supported by the JSPS. This work was supported by the Center for the Promotion of Integrated Sciences (CPIS) of Sokendai, KAKENHI 25-5985 (to A.S.); FIRST program and NIG Collaborative Research (to S.I.); NIH grants NS039050 and NS037070 (to R.S.E.); and KAKENHI 22115009, the Naito Foundation, the Mitsubishi Foundation, Yamada Science Foundation, and the Uehara Memorial Foundation (to T.I.).

Abbreviations

5-HTT	serotonin transporter
AC1	adenylyl cyclase 1
CO	cytochrome oxidase
Cx-AC1KO	cortex-specific AC1 knockout
NMDAR	<i>N</i> -methyl-D-aspartate receptor
PB	phosphate buffer
PFA	paraformaldehyde
PKA	protein kinase A
PMBSF	posterior medial barrel subfield
TC	thalamocortical
VB	ventrobasal nucleus

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Fig. 1.

Partial impairment of barrel formation in Cx/pTh-AC1 KO mice. (A–F) Tangential sections (100- μ m-thick) of layer 4 of the barrel cortices of control (A, C, E) and Cx/pTh-AC1KO [Emx1-Cre KI w/Neo (KWN);AC1^{flox/-}] (B, D, F) mice at P6 (C–F), P7 (A) and P9 (B) stained for CO (A, B), 5-HTT immunohistochemistry (C, D) and Nissl (E, F). C' and D' show adjacent sections of C and D, respectively. Results of CO staining (B) and 5-HTT immunohistochemistry (D, D') show that barrel patterns in posterior medial barrel subfield (PMBSF), which are corresponding to the large whiskers, are visible but they are less clear than those of control mice, and patterns corresponding to the anterior snout (AS) are completely diminished in Cx/pTh-AC1KO mice. Results of Nissl staining (F) show that barrel patterns are almost diminished even in the PMBF in Cx/pTh-AC1KO mice. These results are in contrast with normal patterns in Cx-AC1KO [Emx1-Cre KI Neo (KDN);AC1^{flox/-}] mice (Iwasato et al., 2008). (G, H) Coronal sections (60- μ m-thick) of ventrobasal (VB) thalamus of control (G) and Cx/pTh-AC1KO (H) mice at P7 stained with CO. There are no differences in barreloids between genotypes. Control mice shown in this figure are AC1^{flox/-} (A, G) and Emx1-Cre KI w/Neo (KWN);AC1^{flox/+} (C, E). Scale bar = 250 μ m.

Fig. 2.

Partially disrupted AC1 gene in the VB thalamus of Cx/pTh-AC1 KO mice. (A, B) LacZ-stained coronal sections (400- μ m-thick) of Emx1-Cre KDN (A) and Emx1-Cre KWN (B) mice crossed with CAG-CAT-Z reporter mice (Sakai and Miyazaki, 1997) in adulthood. Ectopic Cre-mediated recombination in the VB thalamus is detected in Emx1-Cre KWN mice but not in Emx1-Cre KDN mice. (C) Semi quantification of efficiencies of Emx1-Cre KDN-induced and Emx1-Cre KWN-induced AC1 gene deletion (i.e. conversion from the AC1^{flox} allele to AC1⁻ allele) in the VB thalamus. Genomic DNA is extracted from the VB thalamus collected from three each of Emx1-Cre KWN;AC1^{flox/+}, Emx1-Cre KDN;AC1^{flox/+}, AC1^{flox/+}, and AC1^{-/+} mice at P7. In genomic DNA samples of Emx1-Cre KWN;AC1^{flox/+} (lane 1), Emx1-Cre KDN;AC1^{flox/+} (lane 2), AC1^{flox/+} (0% deletion: lane 3), AC1^{-/+} (100% deletion: lane 10) and mixture of 0.1% AC1^{-/+} and 99.9% AC1^{flox/+} (0.1% deletion: lane 4), 1% AC1^{-/+} and 99% AC1^{flox/+} (1% deletion: lane 5), 10% AC1^{-/+} and 90% AC1^{flox/+} (10%: lane 6), 20% AC1^{-/+} and 80% AC1^{flox/+} (20% deletion: lane 7), 30% AC1^{-/+} and 70% AC1^{flox/+} (30% deletion: lane 8), and 50% AC1^{-/+} and 50% AC1^{flox/+} (50% deletion: lane 9), AC1⁻ allele was amplified by PCR. The PCR band density of lane 1 is between those of lanes 7 and 9, suggesting that Emx1-Cre KWN mice induce AC1 gene deletion in approximately 20–50% of VB cells. (C') To enhance the signals, Southern blot was performed using a probe for an AC1⁻ fragment. No band is detected in lane 2, even though a weak band is visible in lane 4 (0.1% deletion), suggesting that AC1 gene deletion in VB thalamus in Emx1-Cre KDN mice is negligible (less than 0.1%). (D, D') Experiments were repeated with a new set of Emx1-Cre KWN;AC1^{flox/+}, Emx1-Cre

KDN;AC1^{flox/+}, AC1^{flox/+}, and AC1^{-/+} mice (three mice per genotype) and similar results were obtained.

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Fig. 3.

Impaired TC axonal refinement in Cx/pTh-AC1 KO mice. (A–C) Examples of DiI-labeled single TC axon arbors of control (AC1^{flox/-}: A), Cx-AC1KO (B), and Cx/pTh-AC1KO (C) mouse barrel cortex at P7. Original images (left), converted black-and-white images (middle) and traces (right) are shown. (D–F) Numbers of branch points (D) and total length of axonal arbors (E) in cortical layers (layers 1–6) and lateral extent of axonal arbors in layer 4 (F) were measured in Cx-AC1KO ($n = 11$ axons), Cx/pTh-AC1KO ($n = 11$ axons) and control ($n = 17$ axons) mice. Numbers of branch points are not different among genotypes (one-way ANOVA). Total dendritic length and lateral extent of single TC axons of Cx/pTh-AC1KO mice are larger than those of control mice (one-way ANOVA, followed by Dunnett's test). ns: not significant ($p > 0.05$). Values are given as mean $\pm$ standard errors. Scale bar = 100 μm .

Fig. 4.

Severe impairment of barrel formation in Th-AC1KO mice. (A–D) Examples of LacZ-stained coronal sections (1-mm-thick) of 5-HTT-Cre (Tg810) mouse crossed with CAG-CAT-Z reporter mouse. Sensory thalamus such as VB and lateral geniculate nucleus (LGN) and raphe nucleus (raphe) show extensive Cre-mediated recombination but somatosensory cortex (S1) and principal nucleus of the somatosensory brainstem (PrV) did not show recombination. These images show the pattern in adulthood (4.5-month-old). Detailed analyses of spatial and temporal specificities and efficiencies of Cre-mediated recombination induced by 5-HTT-Cre (Tg810 and Tg208) mice are described (Arakawa et al., 2014). (E–J) Tangential sections of barrel cortices of control (AC1^{lox/-}) (E, G, I) and Th-AC1KO (F, H, J) mice stained for CO (E, F: 100- μ m-thick), 5-HTT immunohistochemistry (G, H: 100- μ m-thick) and Nissl (I, J: 50- μ m-thick). Whisker-related patterning is severely impaired in CO⁻ and 5-HTT-stained sections and almost completely disappeared in Nissl-stained sections. (K, L) Coronal sections of the barrel cortex of control (K) and Th-AC1KO (L) mice stained for Nissl (50- μ m-thick) in adulthood. Barrels (arrows) were clearly observed in layer 4 of control mice but not in Th-AC1KO mice. (M–P) Severely impaired whisker-related TC axonal patches of Th-AC1KO mice are further confirmed by analyzing tangential (M, N: 60- μ m-thick) and coronal (O, P: 50- μ m-thick) sections of control (AC1^{lox/-};TCA-GFP) (M, O) and Th-AC1KO;TCA-GFP (N, P) mice at P7. TCA-GFP mice (Mizuno et al., 2014) were used to label TC axons. Note that axonal targeting to layer 4 (L4) and layer 6 (L6) is not altered in Th-AC1KO;TCA-GFP mice (N). (Q–T)

Barreloids in VB thalamus were examined in coronal sections of control (AC1^{flax/-}; Q, S) and Th-AC1KO mice (R, T) with Vglut2 IHC (Q, R) and CO staining (S, T). No differences were detected between genotypes. Scale bar=200 μ m.

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Fig. 5.

Quantitative analyses of TC axonal segregation in the Th-AC1KO mouse cortex. (A–D) Examples of 30- μ m-thick tangential sections of the barrel cortex derived from control (AC1^{flox/-};TCA-GFP) (A) and Th-AC1KO;TCA-GFP (B) mice at P7, and fluorescence intensity in 150- μ m $\times$ 300- μ m rectangles containing two barrels (C1 and C2 barrels) within a row of control (C) and Th-AC1KO;TCA-GFP (D) mice are shown. S: septal region. Fluorescence intensity was normalized by the average intensity in the rectangle. (E) Fluorescence intensities in C2, S and C1 areas of 10 control mice and 6 Th-AC1KO;TCA-GFP mice at P7 are shown. (F) The TC axonal segregation index, which is defined as $[(I_{C1}+I_{C2})/2 - I_S]$, was significantly lower in Th-AC1KO mice than in control mice. I_{C1} : average intensity in C1 (C1-side 100 μ m); I_{C2} : average intensity of C2 (C2-side 100 μ m); I_S : average intensity of S (center 100 μ m). (G–J) The same images shown in A and B (G and H, respectively), and examples of fluorescence intensity in 150- μ m $\times$ 300- μ m rectangles containing two barrels (B2 and C2 barrels) within an arc of control (I) and Th-AC1KO;TCA-GFP (J) mice are shown. Fluorescence intensity was normalized by the average intensity in the rectangle. (K) Fluorescence intensities in B2, S and C2 of 10 control mice and those of six Th-AC1KO;TCA-GFP mice are shown. (L) The TC axonal segregation index, which is defined as $[(I_{B2}+I_{C2})/2 - I_S]$ was significantly lower in Th-AC1KO mice than in control mice. I_{B2} : average intensity in B2 (B2-side 100 μ m); I_{C2} : average intensity of C2 (C2-side 100 μ m); I_S : average intensity of S (center 100 μ m). Scale bar=150 μ m.

Fig. 6.

Impaired TC axonal refinement in Th-AC1KO mice. (A, B) Example traces of DiI-labeled single TC axon arbors of control (AC1^{flox/-}; A) and Th-AC1KO (B) mouse barrel cortex at P7. (C–E) Numbers of branch points (C) and total lengths of axonal arbors (D) in cortical layers (layers 1–6) and lateral extent of axonal arbors in layer 4 (E) were measured in control ($n=14$ axons from five mice) and Th-AC1KO ($n=18$ axons from seven mice) mice. Numbers of branch points were smaller in Th-AC1KO mice than in control mice. Total dendritic lengths were not different between genotypes. Lateral extent of axonal arbor was larger in Th-AC1KO mice than in control mice. Values are given as mean $\pm$ standard errors. * $p<0.05$, *** $p<0.001$; t -test.

Fig. 7.

Normal barrel formation in Cx-AC1:AC8 double KO mice. (A–C) Tangential sections (100- μ m-thick) of layer 4 of the barrel cortices of Cx-AC1:AC8 double KO mice at P7 (A, B) and in adulthood (C) stained for CO (A), 5-HTT immunohistochemistry (B) and Nissl (C). Whisker-related patterns are apparently normal in Cx-AC1:AC8 double KO mice. Scale bar=500 μ m.