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Neuronal Expression of Soluble Adenylyl Cyclase in the Mammalian Brain

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Abstract

Cyclic 3', 5'-adenosine monophosphate (cAMP) is a critical and ubiquitous second messenger involved in a multitude of signaling pathways. Soluble adenylyl cyclase (sAC) is a novel source of cAMP subject to unique localization and regulation. It was originally discovered in mammalian testis and found to be activated by bicarbonate and calcium. sAC has been implicated in diverse processes, including astrocyte-neuron metabolic coupling and axonal outgrowth of neurons. However, despite these functional studies, demonstration of sAC protein expression outside of testis has been controversial. Recently, we showed sAC immunoreactivity in astrocytes, but the question of neuronal expression of sAC remained. We now describe the generation of a second sAC knockout mouse model (C2KO) designed to more definitively address questions of sAC expression, and we demonstrate conclusively using immune-electron microscopy that sAC is expressed in neuronal profiles in the central nervous system.

Keywords

Cyclic AMP (cAMP); cerebellum; hippocampus; visual cortex; adenylyl cyclase

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1. Introduction

In mammals, signaling by the archetypal second messenger cAMP is determined by the balance between its synthesis by adenylyl cyclases and its degradation by phosphodiesterases (PDEs). The most widely studied source of cAMP in mammals is a family of G protein regulated, transmembrane adenylyl cyclases (tmACs). These enzymes contain two transmembrane domains and are localized at the plasma membrane. In 1999, a second source of cAMP was discovered that lacks transmembrane domains called “soluble adenylyl cyclase” (sAC) (Buck et al., 1999). Alternative splicing generates at least 2 sAC isoforms with distinct forms of regulation (Buck et al., 1999; Jaiswal and Conti, 2001). Plus, an alternative start site has been proposed to generate even greater isoform diversity (Fig. 1A) (Farrell et al., 2008). sAC is activated by HCO_3^- (Chen et al., 2000) and Ca^{2+} (Litvin et al., 2003), and it can be localized anywhere within the cell, even within organelles (Zippin et al., 2003; Zippin et al., 2004; Acin-Perez et al., 2009). sAC was found to be involved in the regulation of sperm capacitation and hyperactivated motility, bicarbonate-sensing in the eye, CO_2-sensing in lung cilia and mitochondria, and pH sensing in epididymis and kidney (reviewed in (Tresguerres et al., 2011)).

Roles for sAC have also been identified in the developing brain (Wu et al., 2006), in axonal outgrowth (Stessin et al., 2006; Corredor et al., 2012) and in astrocyte-neuron metabolic coupling (Choi et al., 2012). While these and other reports demonstrated sAC mRNA expression in the brain (Sinclair et al., 2000; Geng et al., 2005; Farrell et al., 2008; Moore et al., 2008), confirming sAC protein expression has been controversial. We reported sAC protein expression in rat dorsal root ganglia and spinal cord neurons by immunofluorescence and in total rat brain by immunoprecipitation (Wu et al., 2006). However, when antibodies were used to examine sAC expression in mice, supposed sAC signals did not disappear in homozygous $Sacy^{tm1Lex}$ knockout (C1KO) mice (Farrell et al., 2008; Corredor et al., 2012). We also showed that sAC was downstream from the neuronal guidance cue netrin-1 (Wu et al., 2006), but a different study contradicted this conclusion (Moore et al., 2008). An independent study showed sAC to be necessary for retinal ganglion cell survival and axon growth (Corredor et al., 2012); however, they were unable to show definitive sAC expression. They suggested this could be due to the proposal by Farrell *et al.* that neurons express C2-only isoforms derived from an alternate start site (Fig. 1A) (Farrell et al., 2008). Because of these contradicting reports, sAC expression in brain, and specifically neurons, has been questioned.

In this report, we describe generation of a sAC-C2 knockout mouse strain (C2KO), which we use to definitively demonstrate sAC expression in neurons of the cerebellum, hippocampus, and visual cortex, regions of the brain with proposed functions for sAC or cAMP biology. In these regions, we demonstrate sAC-immunoreactivity in wild type mice that is absent in equivalent regions of C1KO and C2KO mice.

2. Results

Despite reports showing sAC’s roles in multiple systems, definitive detection of sAC in mouse somatic tissues has proven difficult. The previous gold standard for sAC detection utilized the C1KO mouse, which interrupted a sequence in the C1 catalytic domain (Fig. 1A) (Esposito et al., 2004). Using this standard, sAC activity (Esposito et al., 2004; Hess et al., 2005; Xie et al., 2006) and expression of sAC_t and sAC_{fl} isoforms (Hess et al., 2005; Farrell et al., 2008) had been definitively demonstrated in mouse testis cytosol by the absence of a signal in parallel samples from C1KO animals.

In contrast, in mouse brain, immunoprecipitation followed by Western blotting and cyclase assays yielded protein bands and activity profiles that did not disappear in parallel samples from C1KO mice (Farrell et al., 2008). Identification of mRNA transcripts from mouse brain that appeared to use a novel transcription initiation site in the intronic region between exons 7 and 8 (called exons 4 and 5 in their paper) suggested the existence of C2-only sAC isoforms that would be unaffected by the design of the C1KO. Therefore, we developed a new transgenic knockout mouse targeting the C2 domain of sAC (genetic deletion of exons 11 to 13) named “sAC-C2 KO or C2KO” (Fig. 1B, C). This design would definitively test for sAC expression by affecting all known and proposed isoforms.

C2KO mice demonstrate male specific sterility (data not shown), the original phenotype seen in the C1KO. It remains to be seen if other phenotypes reported for the C1KO (Lee et al., 2011; Choi et al., 2012) are also seen in the C2KO, and whether there are any different phenotypes between the two.

Molecular verification of the knockout was performed in testis, where sAC is expressed at its highest levels. Adenylyl cyclase activity assays of testis cytosol (Fig. 2A) and immunoprecipitation followed by Western blotting (Fig. 2B) using non-overlapping anti-sAC monoclonal antibodies confirmed absence of sAC activity and protein (specifically, the two isoforms sAC_t, 48kD, and sAC_{fl}, 187 kD), respectively. Interestingly, slightly different results were seen with the two different monoclonal antibodies used for immunoprecipitation, R5 and R37. While both antibodies immunoprecipitated a ≈187kDa band (corresponding to sAC_{fl}) from wild type (WT) testis that disappeared in both C1KO and C2KO, only R5 visibly immunoprecipitated a ≈48kDa band (corresponding to sAC_t) that disappeared in the knockouts. In the R37 immunoprecipitate, a non-specific doublet appears between 45 and 50 kD in WT and both C1KO and C2KO. These intense non-specific bands mask the sAC_t band, which is still being bound to R37 based on the positive adenylyl cyclase activity in WT R37 immunoprecipitate compared to negative activity in C1KO and C2KO immunoprecipitates (Fig. 2C). R5 and R37 are our two most effective antibodies at immunoprecipitation. While R5 immunoprecipitates from testis show sAC_{fl} and sAC_t at the correct sizes and appropriately disappearing in both sAC-KOs, it has not been able to immunoprecipitate specific protein bands or cyclase activity in somatic tissues (data not shown). In somatic tissues, sAC is not found in the cytosol but is in particulate fractions that require solubilization methods detrimental to R5’s ability to immunoprecipitate. In addition, R5 has been shown to be an inhibitory antibody (Kamenetsky, 2006), and thus cyclase activity cannot be detected following R5 immunoprecipitation. Finally, in our hands, R5 has not proven suitable for immunostaining. Thus, we had relied upon R37 immunoprecipitations for exploring sAC expression in somatic tissues (Wu et al., 2006; Farrell et al., 2008; Lee et al., 2011). However, due to the contaminating bands obscuring sAC_t in R37 immunoprecipitates (Fig. 2B), we turned to immunohistochemistry to investigate whether sAC is expressed, and where it is localized in neurons and/or glia, in select regions of the mouse brain. sAC immunoreactivity was analyzed by light and electron microscopy in the cerebellum, hippocampus, and visual cortex of male WT, C1KO, and C2KO mice.

First, we used the sAC antibody R52 to identify topographic distribution of sAC in the cerebellum by light microcopy. Cell processes containing sAC immunoperoxidase labeling were detected in the molecular layer of the cerebellum of WT mice (Fig. 3A), but absent in C1KO or C2KO mice (Fig. 3B, C, respectively).

Second, to determine the cellular as well as subcellular localization of sAC, we analyzed R21 sAC immunolabeling in the molecular layer of the cerebellum. Consistent with morphological criteria described by Peters *et al.* (Peters et al., 1991), sAC-immunoreactivity

was seen in astrocytic profiles in WT mice (Fig. 3D) but not in the C1KO or C2KO mice (Fig. 3G,H). Although to a lesser extent possibly reflecting the relative abundance of the two cell types in brain, sAC-immunogold silver labeling was also found in neurons in axon terminals, dendrites and dendritic spines (Fig. 4C). Moreover, quantitative examination revealed that the number of immunogold silver particles in both glia and neurons is significantly greater in WT mice versus the C1KO and C2KO mice (Fig. 3E,F). Interestingly, in both neuronal and glial profiles, the majority of immunogold silver particles were in the cytoplasm (76% and 87%, respectively), with the remainder localizing to the plasma membrane and endomembranes. These data suggest that in neurons and glia, sAC is distributed to distinct subcellular locations, similar to its previously reported distribution in cell lines (Zippin et al., 2003).

Next, to determine if sAC labeling in the brain extended beyond the cerebellum, we used the R21 sAC antibody in stratum radiatum of the CA1 region of the hippocampus and visual cortex. Consistent with our findings in the cerebellum, sAC-immunoreactivity was present in glial profiles (Fig. 4A) exclusively in WT mice. In the hippocampus, sAC immunoreactivity was observed in virtually all glial profiles in WT mice, but was absent in C1KO (Fig. 4B and (Choi et al., 2012)) and C2KO (Fig. 4C) brains. sAC immunoreactivity in WT hippocampus was predominantly found in glial processes (96% of the greater than 600 immune-reactive profiles examined); however, sAC-immunoreactivity was also seen in axon terminals (4% of immunoreactive profiles), as identified by the presence of small synaptic vesicles, in WT mice (Fig. 4A), but not in C1KO or C2KO mice (Fig. 4B,C, respectively). Additionally, sAC immunogold-silver particles were found in axon terminals within the visual cortex of WT mice (Fig. 4D), but not in C1KO or C2KO mice (Fig. 4E,F). Collectively, these results definitively demonstrate that sAC is expressed in neurons and glia within the cerebellum, hippocampus, and visual cortex of adult mice.

3. Discussion

The C2KO mouse strain is a new tool that allows for definitive investigation of sAC expression, especially with the possibility of C2-only isoforms (Farrell et al., 2008). Studies with this new strain showed that non-specific bands might interfere with attempts to detect sAC expression via immunoprecipitation. However, utilizing both C1KO and C2KO mice, we confirm expression of sAC in brain, specifically in astrocytes, and we definitively demonstrate for the first time its expression in neurons, specifically in axon terminals, dendrites and dendritic spines.

Studying cAMP signaling has long been dominated by investigations into transmembrane adenylyl cyclases. Research on this family of obligate membrane-bound cyclases has yielded a staggering wealth of insight into cellular signaling, and tmACs are now known to play critical roles in a number of diverse and widespread biological processes. However, there remain many questions posed by the inherent limitations of tmACs. One issue is that there are signaling pathways that include a rise in cAMP levels without evidence for involvement of extracellular ligands, G-protein coupled receptors, or tmACs (Sayner and Stevens, 2006). A second issue is their restricted localization to the plasma membrane. It has been proposed that once cAMP is generated, it can only diffuse a short distance as PDEs are thought to form barriers or “firewalls” throughout a cell that limit diffusion of the signal (Zaccolo, 2011). This is hypothesized to be a mechanism for achieving cAMP signaling specificity. Therefore, it may be essential to produce cAMP locally near downstream effectors, especially for signaling pathways in membrane delimited compartments such as mitochondria and the nucleus (Zippin et al., 2004; Acin-Perez et al., 2009; Sample et al., 2012).

Soluble adenylyl cyclase represents a fundamentally different way to think about cAMP production, as it is uniquely regulated by bicarbonate and calcium and can be localized anywhere in a cell. Although only recently discovered (Buck et al., 1999), a rapidly growing field of research has uncovered many roles for this new family of cyclases (reviewed in (Tresguerres et al., 2011)). However, while the molecular identification of sAC in sperm and testis has been established for many years (Hess et al., 2005), sAC in somatic tissues had heretofore been refractory to definitive molecular detection.

RT-PCR data have showed sAC mRNA being transcribed in many somatic tissues, including brain, but only after 40 cycles (Farrell et al., 2008; Moore et al., 2008). Interestingly, detection of sAC protein isoforms in testis cytosol by Western blotting required prior enrichment by immunoprecipitation. Thus, even in testis cytosol, where sAC activity was originally detected (Braun and Dods, 1975), ultimately purified (Buck et al., 1999), and is believed to be at the highest levels of any tissue, the individual isoforms are rare. This observation explains the requirement to start with cytosols from 950 rat testes to purify < 5 μg of sAC_t protein during the original purification (Buck et al., 1999), and may suggest one reason why detection in somatic tissues has been so difficult. For signaling enzymes functioning within microdomains, such as sAC in the cAMP signaling cascade, low levels of expression are functionally relevant. When the signaling machinery is compartmentalized into microdomains, physiological effects require only small changes in local cAMP concentrations.

Another hypothesis suggested by Farrell *et al.* was that an alternative transcription initiation site led to C2-only isoforms that defined the molecular identity of sAC in somatic tissues (Farrell et al., 2008). This proposal led us to develop the C2KO mouse strain. The C2KO design targeted the second catalytic domain (Fig. 1), ensuring that all known and proposed isoforms would be disrupted, especially in combination with the C1KO. This useful tool allows for more unequivocal investigation of sAC expression. Additionally, this new KO will allow for further investigation into the hypothesis that C2-only isoforms of sAC are expressed in the brain. Immunostaining did not reveal any significant evidence of staining that was in the WT and C1KO but absent from the C2KO. However, absence of data does not refute the existence of C2-only isoforms, and it remains possible that they are expressed in other, as yet unexplored, regions of the brain. It is also possible that the alternative initiation site leads to mRNA species that play a direct function without translation into polypeptides.

Our data support the idea that failure to detect sAC in somatic tissues was due to technical limitations. We have shown here that immunohistochemistry and in particular immuno- EM comparing WT with C1KO and C2KO mice may be the optimal methods for definitively detecting sAC expression in somatic tissues.

Choi *et al.* identified a role for sAC in brain by demonstrating sAC expression in astrocytes and elucidating its role in mediating metabolic coupling between glia and neurons (Choi et al., 2012). We now show that sAC protein is also present in neurons of the brain, at least in cerebellum, hippocampus, and visual cortex. Multiple cAMP signaling roles have been reported in neurons in these three regions: synaptic plasticity, cytoskeletal reorganization and neuronal differentiation and maturation in cerebellum (Evans, 2007); neuronal differentiation and maturation, neuroprotection, and learning and memory (Frey et al., 1993; Wong et al., 1999) in hippocampus; and synaptic plasticity (Berardi et al., 2003; Cancedda et al., 2003) and ocular dominance plasticity (Rao et al., 2004) in the visual cortex. Our data demonstrating sAC expression in these regions raises the possibility that it contributes to the cAMP controlling these processes, and it suggests each of these processes should be evaluated in C1KO and C2KO mice.

As an ever-increasing number of roles are discovered for sAC and its still unknown number of isoforms, it will be imperative to definitively establish whether sAC is expressed in the tissues of interest. The immunostaining techniques developed here represent a new gold standard for demonstrating sAC expression.

4. Experimental Procedures

All animal work was performed with approval from the Institutional Animal Care and Use Committee of Weill Cornell Medical College (IACUC) and conforms to NIH guidelines for the Care and Use of Laboratory Animals.

Development of the sAC-C2 KO mice

Targeting vector construction and knock-out strategy has been designed and performed by GenOway (Lyon, France) (Fig. 1B). A genomic 2.2-kb region comprising exons 11 to 13 (encoding much of the C2 catalytic domain) of mouse Adcy10 was flanked by a Neo cassette (*FRT* site-PGK promoter-Neo cDNA-FRT site-*Lox*P site) and a *lox*P site. FRT recombination sites allowed removal of the selection cassette while loxP sites enabled generation of both a constitutive and a conditional knockout line. Linearized targeting vector was electroporated into 129Sv ES cells, and clones were selected with G418. Accurately targeted clones were microinjected into C57BL/6 blastocysts, and gave rise to male chimeras. Mice were first bred to C57BL/6 mice expressing Flp recombinase to remove the Neo cassette ($Adcy10^{lox}$ mice) and then to C57BL/6 mice expressing Cre recombinase to generate a germline deletion of *Adcy10* ($Adcy10^{del}$ or C2KO mice). Genotypes of the C2KO mice were determined by PCR using the following primers: Forward: 5'-GGACAGAAAGTAGAATGACTATCCCCCATTG, Reverse: 5'-CCGCTCACCTCTTTTCGGATTACATC (Fig. 1C).

Monoclonal antibodies—The anti-sAC monoclonal antibodies were developed in our laboratory (Zippin et al., 2003). R21 is directed against amino acids 203–216 of human sAC; R5, R37 and R52 are targeted against amino acids 436–449, 436–466 and 450–466, respectively, of human sAC (Fig. 1A). The R21 epitope is within the first catalytic domain but is not affected in the C1KO, and the R5, R37, and R52 epitopes are within the second catalytic domain but not affected in the C2KO.

Immunoprecipitation followed by Western blots were performed as previously described (Hess et al., 2005).

In vitro adenylyl cyclase assays were performed in 100 mM Tris pH 7.5, 20 mM creatine phosphate, 100 U/ml creatine phosphokinase, 1 mM DTT, 2.5 mM ATP, 10 mM $MnCl_2$, and 1 mM IBMX at 37°C for 20 minutes. cAMP was determined using the Correlate-EIA Direct Assay (Assay Designs, Inc.).

Immunocytochemistry—Details of the procedure have been previously described (Milner et al., 2011). Briefly, adult male mice were anesthetized with sodium pentobarbital, perfused with heparin/saline followed by 3.75% acrolein and 2% paraformaldehyde in 0.1 M phosphate buffer (PB). Coronal sections (40μm) were cut and stored in 30% ethylene glycol and 30% sucrose in PB at 20°C. Free-floating sections were washed in PB followed by 1% sodium borohydride in PB (30 min.) and blocked in 0.5% BSA in 0.1M Tris–saline, pH 7.6 (TS) (30 min.). Sections were incubated with monoclonal anti-sAC antibodies, R21 (0.6–3.0 μg/ml) or R52 (0.6 mg/ml in 0.1% bovine serum albumin in TS for 1 day at room temperature (RT) followed by 3 days at 4°C. Sections were incubated in biotinylated goat anti-mouse IgG in TS (1:400; Vector Laboratories) for 30 minutes and labeled with ABC using the Vectastain Elite kit (Vector Laboratories) for 30 minutes. Labeling was visualized

with 3,3-diaminobenzidine and hydrogen peroxide in TS (4–8 min.). Labeled sections were either mounted on slides, dehydrated in an alcohol series, cover-slipped and visualized in light microscope, or processed for EM. For EM, sections were incubated in colloidal gold (1 nM) conjugated goat anti-mouse IgG (1:50; Electron Microscopy Sciences, EMS) in 0.08% BSA, 0.01% gelatin in PBS for 2 hours at RT, and post-fixed in 2% glutaraldehyde in PBS for 10 minutes followed by 0.2 M sodium citrate buffer, pH 7.4. Gold labeling was enhanced using a silver solution (IntenSE; GE Healthcare) for 4–10 minutes.

Statistical analysis was performed using one-way ANOVA with Bonferroni post-hoc tests (for EM) and Student's t-test (for cyclase assays) was performed for pairwise comparisons.

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Abbreviations

cAMP	Cyclic 3', 5'-adenosine monophosphate
PDE	phosphodiesterase
sAC	Soluble adenylyl cyclase (ADCY10, Sacy)
HCO_3^-	bicarbonate
WT	wild type
C1KO	$Sacy^{tm1Lex}/Sacy^{tm1Lex}$ or sAC-C1 domain knockout mice
C2KO	$ADCY10^{del}/ADCY10^{del}$ or sAC-C2 domain knockout mice

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Highlights

In this manuscript, we:

- describe a second sAC knockout model (sAC-C2 KO).
- confirm sAC expression in astrocytes.
- show sAC labeling in neurons of WT mice but not in C1KO or C2KO mice.
- show sAC is expressed in cerebellum, hippocampus, and visual cortex.
- show sAC is expressed in axon terminals and dendritic spines.

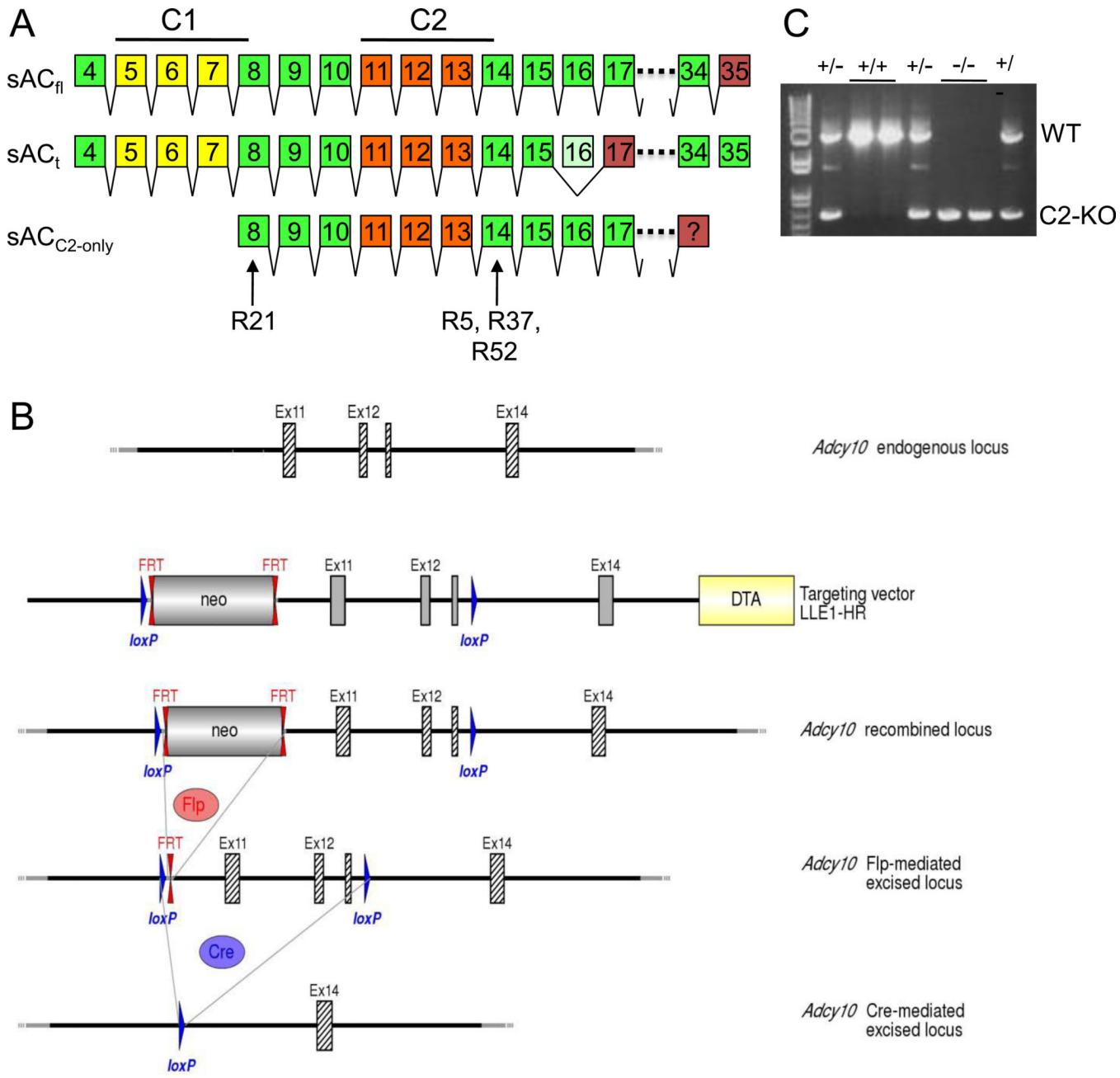

Figure 1.

sAC genomic organization and design of C2KO mouse. **A**. Schematic of the soluble adenylyl cyclase coding exons, numbered according to Genbank. The locations of epitopes for various monoclonal antibodies used in this study are shown, as well as the regions targeted by the two knockout mouse models (yellow = C1KO, orange = C2KO). **B**. Schematic of development of the C2KO mouse. Neo is neomycin resistance cassette; DTA is the Diptheria Toxin A negative selection marker; Frt is Flippase recognition target site; Flp is Flippase; and Cre is Cre recombinase. **C**. PCR identifying genotype of C2KO mice.

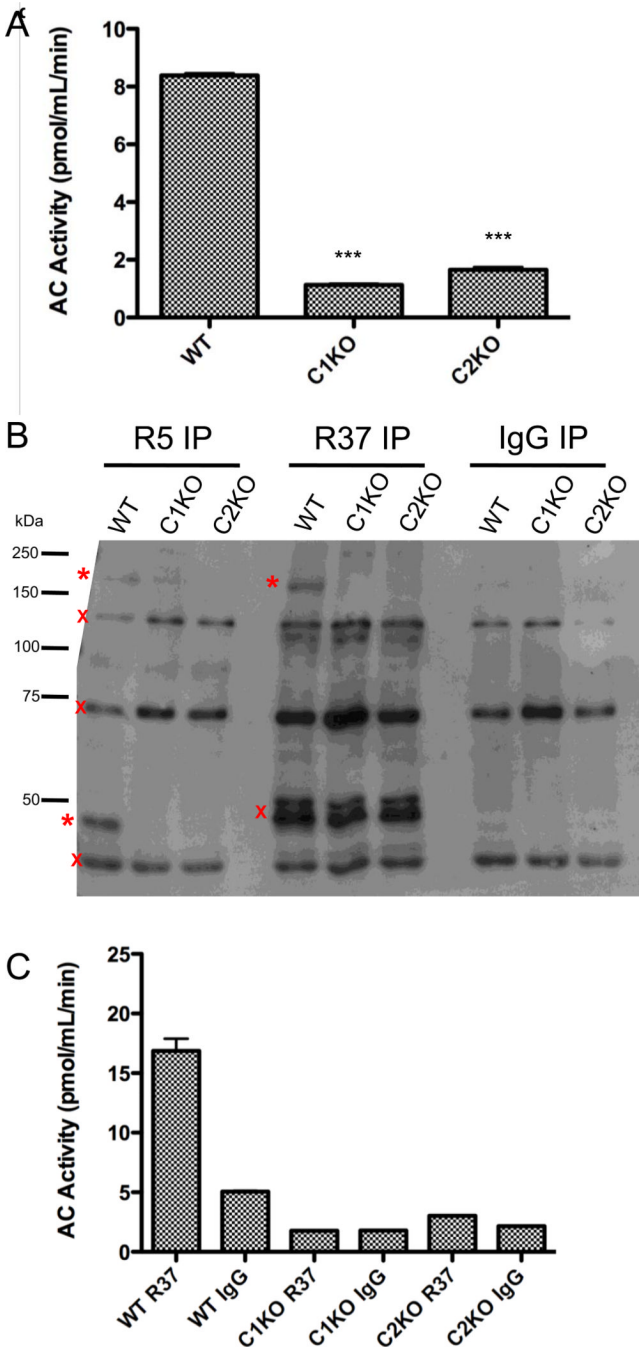

Figure 2.

Absence of sAC activity and protein in C2KO testis. **A**. Adenylyl cyclase activity in testis lysates from WT, C1KO, and C2KO mice. Activities reflect averages of at least triplicate determinations with standard error of the mean. (*** $p<0.001$). **B**. Western blot using biotinylated R21 of immunoprecipitations with R5, R37, or IgG control from testis cytosol from WT, C1KO, and C2KO mice. Red asterisks indicate specific sAC bands; red X indicate nonspecific bands. **C**. Adenylyl cyclase activities in R37 immunoprecipitations.

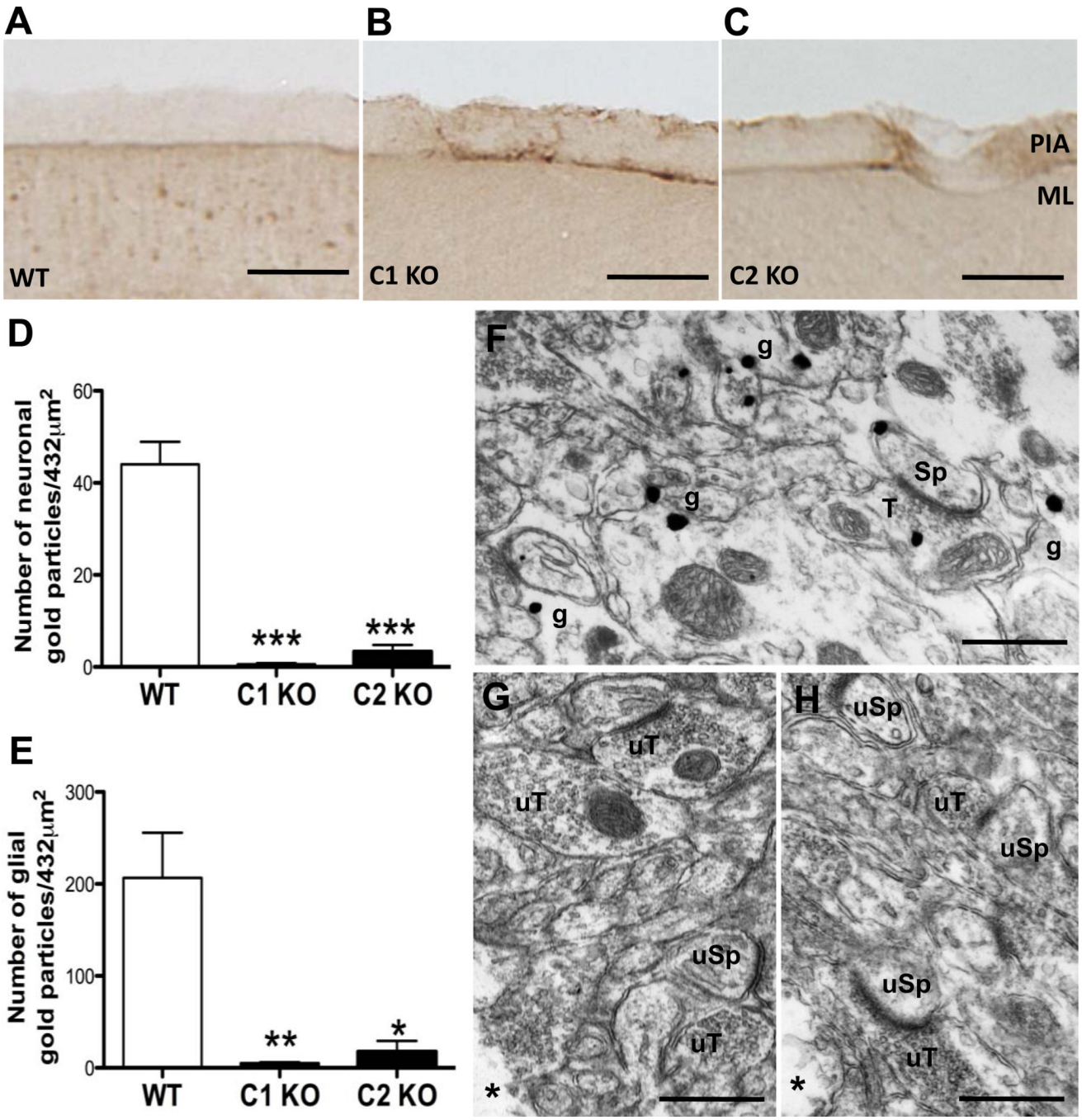

Figure 3.

sAC immunoreactivity is found in the cerebellum of the mouse brain. **A–C**. By light microscopy, sAC (R52) immunoperoxidase labeling is seen in the molecular layer (ML) of the cerebellum of WT mice (A), but absent from C1KO (B) or C2KO (C). PIA= pia mater. Scale bars= 50 μm. **D**, **E**. The number of sAC (R21) immunogold-silver particles in cerebellar neurons (D) and glia (E) is significantly higher in WT mice than in C1KO or C2KO mice. n=3 ***P<0.0001, **P<0.005, *P<0.05. **F–H**. Representative electron micrographs from the cerebellum of WT mouse (F) demonstrates sAC (R21) immunogold-silver particles in glia (g), axonal terminals (T) and dendritic spines (Sp), but not in C1KO

(G) or C2KO (H) mice. Asterisk indicates Epon/tissue interface. uT= unlabeled terminals, uSp= unlabeled spines. Scale bars=500 nm.

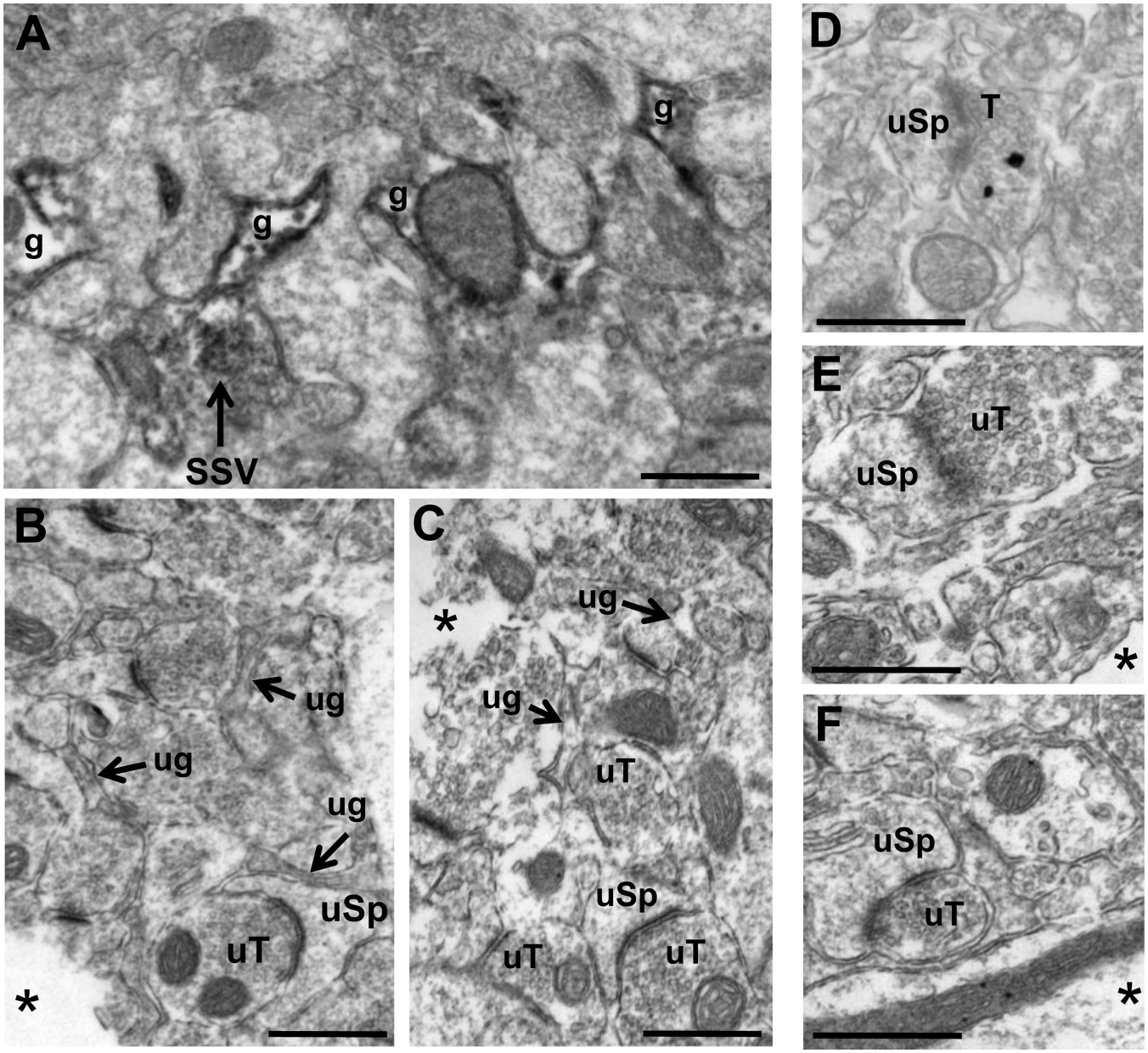

Figure 4.

sAC is found in glia and neurons in the hippocampus and visual cortex of the mouse brain. **A–F**. Representative electron micrographs from the CA1 region of the hippocampus (A–C; Scale bars=500 nm) demonstrates sAC (R21) immunoperoxidase staining (A) and in the visual cortex (D–F; Scale bars=250 nm) shows sAC (R21) immunogold-silver particles (D) in WT mice, but no staining in C1KO (B, E) or C2KO (C, F) mice. Regions are labeled as in Figure 3; in the hippocampus, ug = unlabeled glial processes.