

# The Type 3 Adenylyl Cyclase Is Required for Novel Object Learning and Extinction of Contextual Memory: Role of cAMP Signaling in Primary Cilia

Zhenshan Wang,<sup>1</sup> Trongha Phan,<sup>2</sup> and Daniel R. Storm<sup>2</sup>

<sup>1</sup>College of Life Science, Hebei University, Baoding, People's Republic of China 071002, and <sup>2</sup>Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195

Although primary cilia are found on neurons throughout the brain, their physiological function remains elusive. Human ciliopathies are associated with cognition defects, and transgenic mice lacking proteins expressed in primary cilia exhibit defects in learning and memory. Recently, it was reported that mice lacking the G-protein-coupling receptor somatostatin receptor-3 (SSTR3), a protein expressed predominately in the primary cilia of neurons, have defective memory for novel object recognition and lower cAMP levels in the brain. Since SSTR3 is coupled to regulation of adenylyl cyclase, this suggests that adenylyl cyclase activity in primary cilia of CNS neurons may be critical for some forms of learning and memory. Because the type 3 adenylyl cyclase (AC3) is expressed in primary cilia of hippocampal neurons, we examined AC3<sup>-/-</sup> mice for several forms of learning and memory. Here, we report that AC3<sup>-/-</sup> mice show no short-term memory for novel objects and fail to exhibit extinction of contextual fear conditioning. They also show impaired learning and memory for temporally dissociative passive avoidance. Since AC3 is exclusively expressed in primary cilia, we conclude that cAMP signals generated within primary cilia contribute to some forms of learning and memory, including extinction of contextual fear conditioning.

## Introduction

Primary cilia are antenna-like organelles found on many animal cells that project from the apical surface of the cell into the extracellular space (for review, see Singla and Reiter, 2006). In mammals, almost every cell possesses a single primary cilium (Snell et al., 2004; Praetorius and Spring, 2005). A variety of receptors, ion channels, and transporter proteins, as well as their downstream effectors, are found in cilia or their basal bodies, suggesting that primary cilia are specialized structures for signal transduction (Satir et al., 2010). For example, components of the cAMP signal transduction pathway, including G-protein-coupled receptors, adenylyl cyclases (AC3, AC4, AC6, and AC8), and protein kinase A (RI- $\beta$  and RII- $\alpha$ ), have been found in primary cilia of bile cholangiocytes (Masyuk et al., 2008), bone cells (Kwon et al., 2010), and renal epithelial cells (Raychowdhury et al., 2009), implying that primary cilia may serve as a subcellular compartment for cAMP signaling (Johnson and Leroux, 2010).

Cognitive impairment is associated with several ciliopathy-related syndromes (Lee and Gleeson, 2010), suggesting that primary cilia in CNS neurons are important for cognition. The majority of neurons in the hippocampus, which plays an important role in several forms of memory, have primary cilia (Berbari

et al., 2007). Moreover, several G-protein-coupled receptors that couple to adenylyl cyclase, including SSTR3 (Händel et al., 1999; Schulz et al., 2000) and melanin-concentrating hormone receptor 1 (MCHR1) (Berbari et al., 2008), are located in primary cilia of hippocampal neurons. Recently, it was discovered that SSTR3<sup>-/-</sup> mice are severely impaired in memory for novel objects (Einstein et al., 2010). Furthermore, mice without a functional MCHR1 gene also exhibit defective learning and memory (Adamantidis et al., 2005). This supports the hypothesis that primary cilia in neurons play an important role in learning and memory and suggests that regulation of adenylyl cyclase activity in primary cilia of neurons may be critical for some forms of learning and memory.

In the hippocampus and cortex of brain, AC3 is expressed exclusively in the primary cilia of neurons; its concentration in primary cilia is a striking example of subcellular compartmentalization of a signaling protein (Bishop et al., 2007). Given that SSTR3<sup>-/-</sup> mice have impaired memory for novel object recognition (Einstein et al., 2010) and SSTR3 couples to regulation of adenylyl cyclase, we wondered whether AC3 is required for specific types of learning and memory, including novel object recognition. Here we report that AC3<sup>-/-</sup> mice exhibit deficits in memory for temporally dissociative passive avoidance (TDPA), novel object recognition, and contextual fear extinction. We conclude that AC3 generates a cAMP signal in primary cilia that is required for some forms of learning and memory.

## Materials and Methods

*Mice.* AC3<sup>+/+</sup> and littermate AC3<sup>-/-</sup> mice were bred from heterozygotes and genotyped as previously reported (Wang et al., 2009b). Basal

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Correspondence should be addressed to Dr. Daniel R. Storm, Department of Pharmacology, University of Washington, Seattle, WA 98195. E-mail: dstorm@u.washington.edu.

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adenylyl cyclase activity in the hippocampus of AC3<sup>+/+</sup> and AC3<sup>-/-</sup> mice is  $175 \pm 5$  and  $155 \pm 8$  pmol of cAMP  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, respectively. The age of animals used in this study was 3–8 months old. All of the mice used in this study were males. Mice were maintained on a 12 h light/dark cycle at 22°C, and had access to food and water *ad libitum*. Mice were housed individually and handled daily for at least 1 week before behavioral experiments to minimize stress. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington and performed in accordance with their guidelines.

**Immunohistochemistry.** The immunohistochemistry of hippocampal slices was performed as described previously (Wang et al., 2009b).

**TDPA paradigm.** This procedure was performed as described previously (Wang et al., 2004a, 2009a; Zhang et al., 2008). On the training day, mice were placed into the light side of a shuttle box consisting of two compartments connected by a door; one side was well lit, and the other side was dark (Coulbourn Instruments). The mice were allowed to explore the environment for 2 min before the door between the two compartments was opened. The time that each mouse crossed over into the dark compartment was recorded. After the mouse crossed over into the dark compartment (all four paws in the dark compartment), the door was closed, and 10 min later, the mouse received a mild footshock (0.7 mA, 2 s). Twenty-four hours after the first training session, the trained mice were placed into the light compartment again. Their crossover latencies into the dark side were monitored and they received a footshock 10 min after they entered into the dark side. This procedure was repeated several days until their crossover latencies reached a cutoff time of 300 s. The mice were removed if they failed to enter the dark side after 300 s, and a crossover latency of 300 s was recorded. For analysis of short-term memory, the animals were trained only once, and their crossover latencies were recorded 10 min later after training.

**Contextual fear conditioning.** Contextual fear conditioning was performed as we described previously (Wang et al., 2004b). Contextual memory extinction was monitored by returning the mice to the original training box on successive days without shock and monitoring the percentage of time spent freezing during a 2 min period.

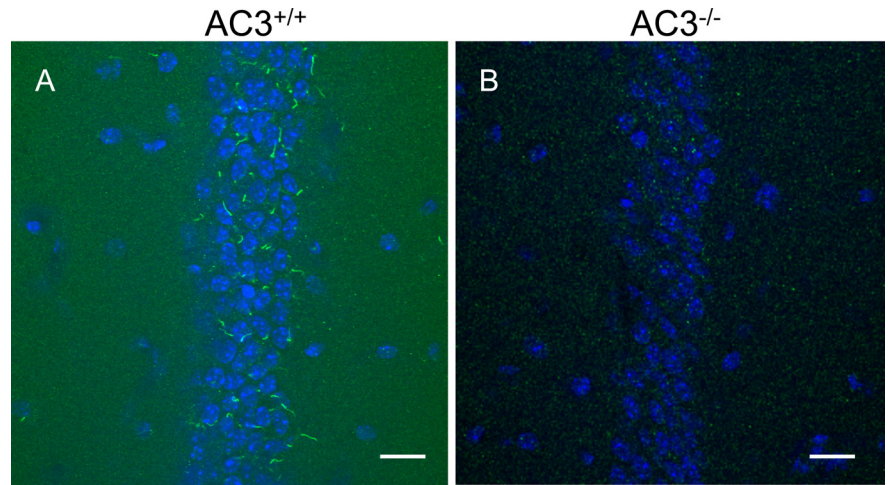
**Novel object recognition test.** The novel object recognition test was performed as described previously (Wang et al., 2004b; Shimizu et al., 2007).

**Statistical analysis.** All data were presented as means  $\pm$  SEM. We analyzed the data by Student's *t* test for two-sample comparison and one-way ANOVA for multiple-sample comparisons, with *p* < 0.05 considered as statistically significant.

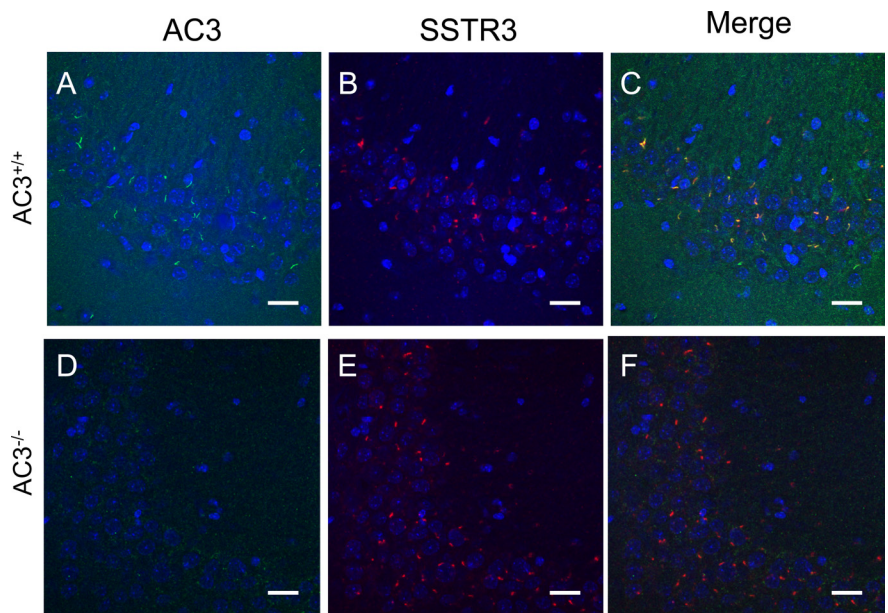
## Results

### AC3 is highly expressed in the primary cilia of the hippocampus

Although it was reported that AC3 is almost a universal marker for primary cilia throughout the mouse brain (Bishop et al., 2007), it was important to verify the specificity of the antibody

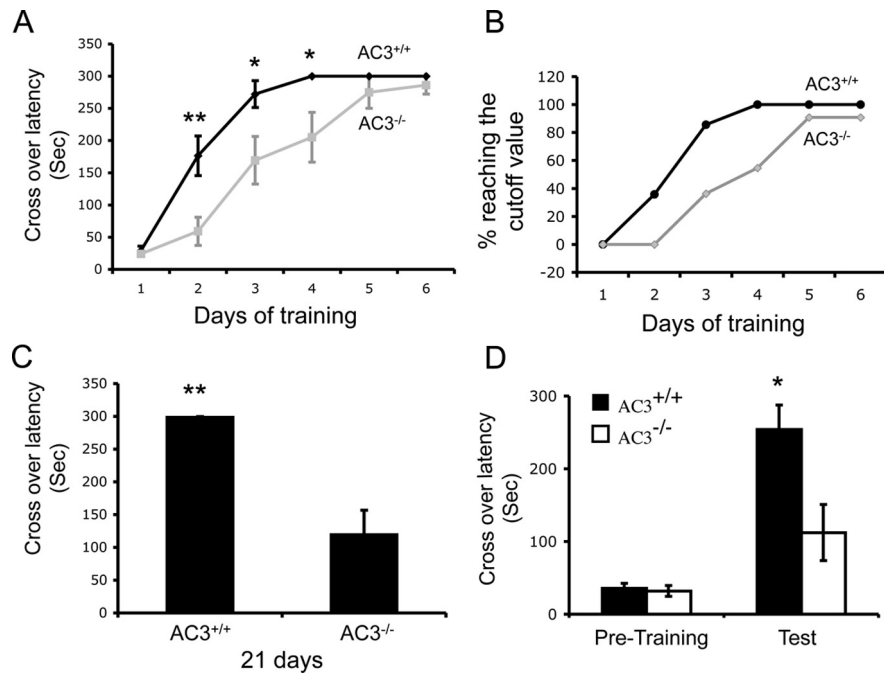


**Figure 1.** AC3 is highly expressed in the primary cilia of neurons in the CA1 region of the hippocampus. **A, B**, Representative images of staining for AC3 in primary cilia of neurons in the CA1 region of the hippocampus for AC3<sup>+/+</sup> mice (**A**) and AC3<sup>-/-</sup> mice (**B**). Scale bar, 20  $\mu$ m. *n* = 4 for each genotype. Green, AC3; blue, Hoechst nuclear staining.

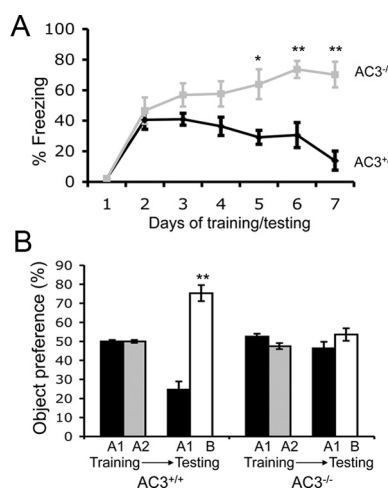


**Figure 2.** Hippocampal neurons in AC3<sup>-/-</sup> mice express primary cilia. **A–C**, Representative images of AC3-immunoreactive cilia in the CA3 region of the hippocampus in AC3<sup>+/+</sup> mice labeled with antibodies against AC3 (green) (**A**), SSTR3 (red) (**B**), and colocalization of AC3 and SSTR3 (merge) (**C**). Scale bar, 20  $\mu$ m. **D–F**, Representative images of immunoreactive cilia in the CA3 region of the hippocampus in AC3<sup>-/-</sup> mice labeled with antibodies against AC3 (green) (**D**), SSTR3 (red) (**E**), and colocalization of AC3 and SSTR3 (merge) (**F**). Scale bar, 20  $\mu$ m. *n* = 4 for each genotype. Blue, Hoechst nuclear staining.

used in those studies using AC3<sup>-/-</sup> mice as a null control. We visualized the localization of AC3 in the hippocampus by immunohistochemical staining with an antibody against AC3. Our data indicate that AC3 in wild-type mice, but not AC3<sup>-/-</sup> mice, is present exclusively in primary cilia and is especially abundant in the pyramidal cell layer of the CA1 region (Fig. 1) and CA3 region (Fig. 2), as well as in the granular cell layer of the dentate gyrus region of the hippocampus (data not shown). These data establish the specificity of the AC3 antibody for AC3 and the presence of AC3 in primary cilia of hippocampal neurons. To determine whether AC3 is required for the formation or maintenance of primary cilia, hippocampal sections from AC3<sup>+/+</sup> and AC3<sup>-/-</sup> mice were immunostained for another cilia marker, SSTR3. Immunostaining of hippocampal slices from AC3<sup>+/+</sup> mice with



**Figure 3.**  $AC3^{-/-}$  mice show impaired TDPA learning and memory. **A**,  $AC3^{-/-}$  mice ( $n = 11$ ) learned TDPA slower than  $AC3^{+/+}$  mice ( $n = 14$ ). The crossover latency for  $AC3^{+/+}$  and  $AC3^{-/-}$  mice during training for TDPA differed significantly on day 2 ( $p = 0.005$ ), day 3 ( $p = 0.028$ ), and day 4 ( $p = 0.034$ ) of training.  $**p < 0.01$ ;  $*p < 0.05$ . Data are represented as means  $\pm$  SEM. **B**, The percentage of  $AC3^{-/-}$  mice ( $n = 11$ ) reaching the cutoff value (300 s) for crossover latency during the course of TDPA training was lower than  $AC3^{+/+}$  mice ( $n = 14$ ). **C**, Long-term memory for TDPA is impaired in  $AC3^{-/-}$  mice. Long-term memory was tested 21 d after animals reached the cutoff value (300 s) for crossover latency during TDPA training.  $AC3^{-/-}$  mice ( $n = 10$ ) showed poorer retention of memory for TDPA training than  $AC3^{+/+}$  mice ( $n = 14$ ) ( $p = 0.001$ ).  $**p < 0.01$ . Data are represented as means  $\pm$  SEM. **D**, Short-term memory of  $AC3^{-/-}$  mice for TDPA training is impaired. The crossover latency (in seconds) of animals entering the dark chamber was monitored 10 min after a single training session for TDPA. There were significant difference between  $AC3^{+/+}$  mice ( $n = 8$ ) and  $AC3^{-/-}$  mice ( $n = 9$ ) in crossover latency (in seconds) during the memory retention test ( $p = 0.014$ ).  $*p < 0.05$ . Data are represented as means  $\pm$  SEM.



**Figure 4.**  $AC3^{-/-}$  mice fail to exhibit short-term memory for novel objects or extinction of contextual memory. **A**,  $AC3^{+/+}$  mice ( $n = 6$ ) but not  $AC3^{-/-}$  mice ( $n = 6$ ) showed extinction of contextual memory during 7 d of extinction training [(day 5 ( $p = 0.015$ ), day 6 ( $p = 0.023$ ), and day 7 ( $p = 0.00004$ )).  $**p < 0.01$ ;  $*p < 0.05$ . Data are represented as means  $\pm$  SEM. **B**,  $AC3^{+/+}$  mice ( $n = 8$ ) but not  $AC3^{-/-}$  mice ( $n = 7$ ) showed short-term memory for a novel object. During training, both  $AC3^{+/+}$  mice ( $n = 8$ ) and  $AC3^{-/-}$  mice ( $n = 7$ ) showed no preference for two identical objects (A1 and A2). However, 5 min after training,  $AC3^{+/+}$  mice, but not  $AC3^{-/-}$  mice, showed a significant preference for the novel object (**B**) ( $p = 6.8 \times 10^{-7}$ ).  $**p < 0.01$ . Data are represented as means  $\pm$  SEM.

antibodies against SSTR3 and AC3 showed strong colocalization of SSTR3 and AC3 in primary cilia (Fig. 2C). The presence of primary cilia in  $AC3^{-/-}$  mice indicates that AC3 is not required for the formation of primary cilia (Fig. 2E). Primary cilia stained with an antibody for SSTR3 appeared similar in  $AC3^{+/+}$  and  $AC3^{-/-}$  mice (Fig. 2B,E). The presence of AC3 and SSTR3 in the same cilia of hippocampal neurons suggests that receptor-mediated regulation of AC3 may play a role in signaling mechanisms in primary cilia.

### TDPA memory is deficient in $AC3^{-/-}$ mice

We previously reported that  $AC3^{-/-}$  mice show normal learning and memory for classic passive avoidance training (Wong et al., 2000). However, training for passive avoidance generates a very strong episodic memory, which is generated by shocking the mouse immediately after it crosses over into the dark compartment. During TDPA training, the crossover event and the shock are separated by 10 min. Consequently, TDPA learning and memory requires multiple training sessions over a period of days and allows comparison of learning curves between wild-type and transgenic mouse strains. Accordingly, TDPA allows detection of learning and memory differences that otherwise would not be detected by passive avoidance training.

TDPA training revealed a number of significant differences between  $AC3^{-/-}$  mice and their  $AC3^{+/+}$  littermates (Fig. 3). Although  $AC3^{-/-}$  mice reached the same level of training as wild-type mice after 6 d of training, their initial rate of learning was significantly slower than  $AC3^{+/+}$  mice (Fig. 3A). For example, after one training session, the crossover latency for  $AC3^{+/+}$  mice was  $176.4 \pm 30.9$  s, but only  $59.1 \pm 21.8$  s for  $AC3^{-/-}$  mice (Fig. 3A). After two training sessions, 85.7% of  $AC3^{+/+}$  mice reached the cutoff time limit of 300 s, but only 36.4% of  $AC3^{-/-}$  mice (4 of 11) exhibited an escape latency of 300 s (Fig. 3B). All of the  $AC3^{+/+}$  mice but only 60% of the  $AC3^{-/-}$  reached a crossover latency of 300 s after 4 d of training (Fig. 3B). We also tested the long-term memory of  $AC3^{-/-}$  mice for TDPA learning 21 d after they reached a maximum crossover latency of 300 s. Although  $AC3^{+/+}$  mice maintained their memory for TDPA training during this period,  $AC3^{-/-}$  mice showed considerable memory loss after 21 d ( $AC3^{+/+}$  mice,  $298.3 \pm 1.2$  s;  $AC3^{-/-}$  mice,  $119.3 \pm 37.7$  s) (Fig. 3C).

Since short-term and long-term memory are independently processed in the hippocampus and the underlying mechanisms involve separated signaling pathways (Satoh et al., 2007; Cowan, 2008), we also tested short-term memory of  $AC3^{-/-}$  and  $AC3^{+/+}$  for TDPA after a single training session (Fig. 3D). There was a significant difference in crossover latency between  $AC3^{+/+}$  and  $AC3^{-/-}$  mice when they were tested 10 min after training ( $253 \pm 33.6$  s for  $AC3^{+/+}$  mice,  $112.2 \pm 38.6$  s for  $AC3^{-/-}$  mice;  $p = 0.014$ ). A majority of the  $AC3^{+/+}$  mice (62.5%) reached the 300 s cutoff latency (5 of 8). However, only 22.2% of  $AC3^{-/-}$

mice exhibited an escape latency of 300 s (2 of 9). These data indicate that AC3 is critical for both short- and long-term TDPA memory.

#### AC3<sup>-/-</sup> mice do not exhibit extinction of contextual fear memory

AC3<sup>-/-</sup> mice exhibited no impairment of memory for contextual fear conditioning, another form of hippocampus-dependent memory (Fig. 4A). AC3<sup>+/+</sup> mice and AC3<sup>-/-</sup> mice showed indistinguishable freezing behavior when returned to the training context 24 h after training for context (40.6 ± 6.2% for AC3<sup>+/+</sup> mice and 46.7 ± 8.6% for AC3<sup>-/-</sup> mice; *p* = 0.6). However, AC3<sup>+/+</sup> mice, but not AC3<sup>-/-</sup>, exhibited memory extinction for contextual fear conditioning when they were brought back into the training box without shock on consecutive days (Fig. 4A). Indeed, it appears that the AC3<sup>-/-</sup> mice continued to consolidate contextual memory during testing for extinction with the freezing time increasing from 45% to 70% over the course of extinction training. Apparently, AC3<sup>-/-</sup> mice were unable to learn that the context is no longer associated with a shock during extinction training. This suggests that cAMP production by AC3 in the cilia of neurons is necessary for extinction of contextual memory, which is another form of learning.

#### AC3<sup>-/-</sup> mice fail to exhibit short-term memory for novel object recognition

When exposed to a familiar object alongside a novel object, rodents prefer to explore the new object, indicating that the animals have a memory for the familiar object. We examined novel object recognition because AC3 and SSTR3 colocalize to the same cilia in hippocampal neurons (Fig. 2C) and SSTR3 is critical for object recognition memory (Einstein et al., 2010). During the initial training, AC3<sup>+/+</sup> and AC3<sup>-/-</sup> mice spent the same amount of time examining the two identical objects, A1 and A2. When tested 5 min later with the replacement of object A2 with a new object B, AC3<sup>+/+</sup> mice showed a strong preference for the new object B while AC3<sup>-/-</sup> mice showed no preference for the new object (Fig. 4B). Similar behaviors were observed when tested with another cohort of AC3<sup>-/-</sup> mice tested 20 min after training (data not shown). This indicates that AC3<sup>-/-</sup> mice fail to distinguish novel from familiar objects or that they have no short-term memory for novel object recognition.

## Discussion

Studies using transgenic animals have established that the cAMP signaling is essential for learning and memory (Wu et al., 1995; Abel et al., 1997; Wong et al., 1999; Pineda et al., 2004). To date, clones for 10 adenylyl cyclases have been isolated, each with unique regulatory properties. Although almost every isoform of adenylyl cyclase is expressed in the hippocampus (Visel et al., 2006), only the calmodulin-stimulated adenylyl cyclases have been shown to be critical for hippocampus-dependent long-term memory (Wu et al., 1995; Wong et al., 1999). With the exception of AC5, which is critical for striatum-dependent learning (Kheirbek et al., 2008; Kheirbek et al., 2009), the contribution of other adenylyl cyclases to learning and memory is largely unknown. Moreover, the importance of adenylyl cyclase activity in neuronal cilia for learning and memory or memory extinction has not been addressed. Since AC3 is expressed exclusively in primary cilia throughout the brain (Bishop et al., 2007), it was important to investigate the contribution of AC3 to various types of learning and memory.

In this study, we discovered that AC3<sup>-/-</sup> mice exhibit defects in several forms of learning and memory. Although AC3<sup>-/-</sup> mice exhibit normal learning and memory for passive avoidance as well as contextual fear conditioning, their learning curve for TDPA is significantly slower than wild-type mice. Furthermore, both short-term and long-term memory for TDPA are impaired in AC3<sup>-/-</sup> mice. Moreover, AC3<sup>-/-</sup> mice also failed to exhibit short-term memory for novel objects or extinction of contextual memory. Apparently, AC3<sup>-/-</sup> mice are able to recognize contexts since they exhibit classical contextual and passive avoidance memory. It appears that the more challenging memory tasks depend on AC3 activity. Since extinction of contextual memory and TDPA learning requires multiple training sessions, these data indicate that AC3 is required for more difficult forms of memory requiring repeated training sessions. Nevertheless, the fact that long-term memory for TDPA is poorer in AC3<sup>-/-</sup> mice is consistent with the possibility that cAMP signals generated in primary cilia by AC3 may support translational or transcriptional events required for formation or maintenance of long-term memory. Acquisition and extinction of contextual memory both depend on cAMP signaling. However, the data in this paper indicate that cAMP generated in primary cilia is critical for extinction of contextual memory but not crucial for acquisition of contextual memory. Collectively, these observations emphasize the importance of different intracellular cAMP pools for specific learning and memory tasks.

It is interesting that AC3<sup>-/-</sup> mice and SSTR3<sup>-/-</sup> mice both are deficient on novel object memory, suggesting that cAMP signals in primary cilia are critical for novel object memory. However, in contrast to AC3<sup>-/-</sup> mice, SSTR3<sup>-/-</sup> mice showed no decrease in object recognition memory 5 min after training but did so 60 min after training (Einstein et al., 2010). This difference between the two transgenic strains may reflect the fact that there are multiple G-protein receptors in the primary cilia of CNS neurons that can couple to AC3. For example, the 5-hydroxytryptamine-6 receptor (5-HT<sub>6</sub>) couples to adenylyl cyclase (Sebben et al., 1994; Unsworth and Molinoff, 1994) and is expressed in primary cilia of CNS neurons (Brailov et al., 2000). In addition, MCHR1 is also located in primary cilia of CNS neurons (Berbari et al., 2008). Both of these G-protein receptors are implicated in learning and memory.

In summary, AC3<sup>-/-</sup> mice show impaired learning and memory for TDPA training, and no short-term memory for novel objects or extinction of contextual memory. Since neuronal AC3 is expressed exclusively in primary cilia, these data indicate that cAMP signals generated in the primary cilia by AC3 are crucial for some forms of learning and memory.

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