

# Absence of $\text{Ca}^{2+}$ -stimulated adenylyl cyclases leads to reduced synaptic plasticity and impaired experience-dependent fear memory

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$\text{Ca}^{2+}$ -stimulated adenylyl cyclase (AC) 1 and 8 are two genes that have been shown to play critical roles in fear memory. AC1 and AC8 couple neuronal activity and intracellular  $\text{Ca}^{2+}$  increases to the production of cyclic adenosine monophosphate and are localized synaptically, suggesting that  $\text{Ca}^{2+}$ -stimulated ACs may modulate synaptic plasticity. Here, we first established that  $\text{Ca}^{2+}$ -stimulated ACs modulate protein markers of synaptic activity at baseline and after learning. Primary hippocampal cell cultures showed that AC1/AC8 double-knockout (DKO) mice have reduced SV2, a synaptic vesicle protein, abundance along their dendritic processes, and this reduction can be rescued through lentivirus delivery of AC8 to the DKO cells. Additionally, phospho-synapsin, a protein implicated in the regulation of neurotransmitter release at the synapse, is decreased *in vivo* 1 h after conditioned fear (CF) training in DKO mice. Importantly, additional experiments showed that long-term potentiation deficits present in DKO mice are rescued by acutely replacing AC8 in the forebrain, further supporting the idea that  $\text{Ca}^{2+}$ -stimulated AC activity is a crucial modulator of synaptic plasticity. Previous studies have demonstrated that memory is continually modulated by gene–environment interactions. The last set of experiments evaluated the effects of knocking out AC1 and AC8 genes on experience-dependent changes in CF memory. We showed that the strength of CF memory in wild-type mice is determined by previous environment, minimal or enriched, whereas memory in DKO mice is unaffected. Thus, overall these results show that AC1 and AC8 modulate markers of synaptic activity and help integrate environmental information to modulate fear memory.

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## Introduction

The  $\text{Ca}^{2+}$ -stimulated adenylyl cyclase (AC) pathway couples neuronal activity and intracellular  $\text{Ca}^{2+}$  increases to the production of cyclic adenosine monophosphate. Through this activity-dependent increase in cyclic adenosine monophosphate, the  $\text{Ca}^{2+}$ -stimulated ACs, AC1 and AC8, are able to significantly modulate processes that are defined by marked changes in synaptic plasticity, memory, and long-term potentiation (LTP). This is evident on a variety of learning paradigms where AC1/AC8 double-knockout (DKO) mice showed memory impairments, such as passive avoidance,<sup>1</sup> conditioned fear (CF),<sup>1,2</sup> and novel object recognition.<sup>2,3</sup> Moreover, Schaeffer collateral-CA1 LTP is impaired in DKO mice,<sup>1,4</sup> while mossy fiber LTP is impaired in AC1<sup>5</sup> or AC8<sup>6</sup> single-knockout mice. AC1 and AC8 are both localized to the synapse as evident by a synaptosome fractions study<sup>7</sup> and colocalization of AC8 with synapsin and synaptophysin,<sup>6</sup> and therefore, it is not surprising that they modulate both memory processing and LTP.

Not only do genetic variations influence memory processing, but also the environmental context. A recent study

looking at the effects of environmental enrichment on CF memory and LTP showed that an enriched environment enhanced CF memory as well as LTP in a PKA (protein kinase A)-dependent manner.<sup>8</sup> PKA is activated by  $\text{Ca}^{2+}$ -stimulated AC-mediated binding of cyclic adenosine monophosphate,<sup>9</sup> and therefore, initial activation of  $\text{Ca}^{2+}$ -stimulated ACs may be necessary for environmental-dependent changes in fear memory. Synaptic plasticity is one of two major processes shown to modulate experience-dependent changes in memory.<sup>10</sup> For example, environmental enrichment increases the expression of synaptic proteins, such as synaptophysin and postsynaptic density-95<sup>11,12</sup> as well as overall synaptic strength as evident by increases in LTP.<sup>8,13–15</sup> Neurogenesis, the other major process shown to mediate experience-dependent changes in memory, is increased after environmental enrichment.<sup>16–18</sup>

Consequently, we first evaluate the roles of  $\text{Ca}^{2+}$ -stimulated AC activity on mediating synaptic plasticity and neurogenesis using both genetic and gene therapy techniques. More specifically, we focus on the role of AC8, using forebrain-inducible AC8 mice and an AC8 lentivirus. We then evaluate a possible novel gene–environment interaction by looking at

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whether an absence of  $\text{Ca}^{2+}$ -stimulated AC activity affects experience-dependent changes in CF memory. Our data demonstrates that  $\text{Ca}^{2+}$ -stimulated ACs are necessary modulators of synaptic plasticity, neurogenesis, and experience-dependent fear memory.

## Materials and methods

**Animals.** All mouse protocols were in accordance with the National Institutes of Health guidelines and were approved by the Animal Care and Use Committees of Washington University School of Medicine (St. Louis, MO; protocol approval #20080030) and Vanderbilt University (Nashville, TN; protocol approval #M08617). Mice were housed on a 12h/12h light/dark cycle with *ad libitum* access to rodent chow and water. DKO<sup>1,19</sup> and AC8 Rescue mice<sup>2</sup> were generated as previously described and were backcrossed > 10 generations to C57Bl/6 strain. Briefly, DKO mice have both AC1 and AC8 deleted globally, while AC8 Rescue mice have AC8 replaced in the forebrain of mice of a DKO background. Through the use of a tetracycline-inducible system, AC8 can be turned on or off by administration of doxycycline (200 mg doxycycline per 1 kg; Research Diets, New Brunswick, NJ, USA). Forebrain specificity of AC8 is driven by the CamKII $\alpha$  promoter. AC8 is not turned on until mice are weaned at 21 days. C57Bl/6 were used as control mice (wild type (WT)).

**Electrophysiology.** LTP in the Schaffer collateral afferent fibers of the hippocampal CA1 region was induced as previously described<sup>1</sup> with minor modifications to the slice preparation as detailed in the Supplementary Materials and Methods.

**Lentivirus production.** The full-length mouse AC8 cDNA was cloned into a lentivirus vector by Applied Biological Materials, Inc (Richmond, BC, Canada). See Supplementary Material and Methods online for cloning details. Figure 2 outlines the lentivirus construct as well as displays *in vivo* expression of AC8 after infection of LV-Adcy8 into the hippocampus.

**Cell culture and infection.** Hippocampal low-density cultures were prepared as previously described.<sup>20,21</sup> Slight modifications were made to adjust for mouse neurons. Cells were extracted from P0 or P1 mice and fixed at DIV10 or 9, respectively. Hippocampal tissue was dissociated using a final concentration of 0.25% ethylenediaminetetraacetic acid-free trypsin. Cells were plated at 500 000 cells per 60 mm dish containing 5 poly-L-lysine coated coverslips. Cells were kept in culture media containing Neurobasal Media, 2% B27, 1% L-glutamine, and 0.1% Insulin-Transferrin-Selenium (Invitrogen, Carlsbad, CA, USA). Heat-inactivated fetal bovine serum was added to the media at the following concentrations to help promote cell survival: DIV1 2%, DIV3 1%, and DIV5 0.5%. Ara-C was added on DIV3 as previously described to minimize glia growth and subsequently added on DIV5 at half the concentration if glia overgrowth was occurring. Several DKO cultures were infected for 48 h on DIV1 with 2  $\mu\text{l}$  of LV-Adcy8 (titer  $3.1 \times 10^8$  IFU  $\text{ml}^{-1}$ ) per 3 ml media per 60 mm dish and fixed at DIV 9 or 10 as described above.

**Immunohistochemistry.** See Supplementary Material and Methods online for details.

**Western blot analysis.** Tissue extraction and protein analysis was conducted as previously described.<sup>2</sup> See Supplementary Materials and Methods online for details.

**Environmental enrichment.** Male mice aged 2–5 months were used for all behavioral experiments. All mice were on a C57Bl/6 inbred background and housed 2–5 mice/cage. Behavior was conducted on WT and DKO mice reared in two different colonies at the same university. Mice were reared in one of the two housing conditions: (1) *corn*—corn bedding with paper inserts that mice were able to tear up for added bedding, or a slightly more natural environment; and (2) *carefresh*—CareFRESH Natural bedding. Mice were born and kept in their reared environment until 2 weeks before CF testing at which point mice were subjected to one of the two experimental environments: (1) *minimal*—corn bedding only; or (2) *enriched*—carefresh bedding, nestlet, and enrichment hut.

Mice were split into two experiments based on their initial housing conditions. Experiment 1 is mice reared on corn bedding and experiment 2 is mice reared on carefresh bedding. Both experiments have four groups for a total of eight groups all together: WT-minimal, WT-enriched, DKO-minimal, and DKO-enriched. Refer to Figure 6a for a schematic representation.

**CF.** Contextual CF training began 2 weeks after exposure to the experimental environment. CF training and analysis occurred as previously described.<sup>2</sup> Briefly, mice were subjected to a 2 s  $\times$  0.7 mA shock during training and then tested 1 week after training.

**Data analysis.** Results are expressed as the mean  $\pm$  s.e.m. Student's *t*-test was used to compare pairs of means. In cases with multiple conditions, a two-way analysis of variance was used followed by Bonferroni *post hoc* tests when appropriate. A one-way analysis of variance was used for single-condition analysis followed by Tukey's Multiple Comparison *post hoc* tests when appropriate. A *P*-value of  $\leq 0.05$  was considered statistically significant. All statistical comparisons were done with Prism 4 software (GraphPad, La Jolla, CA, USA).

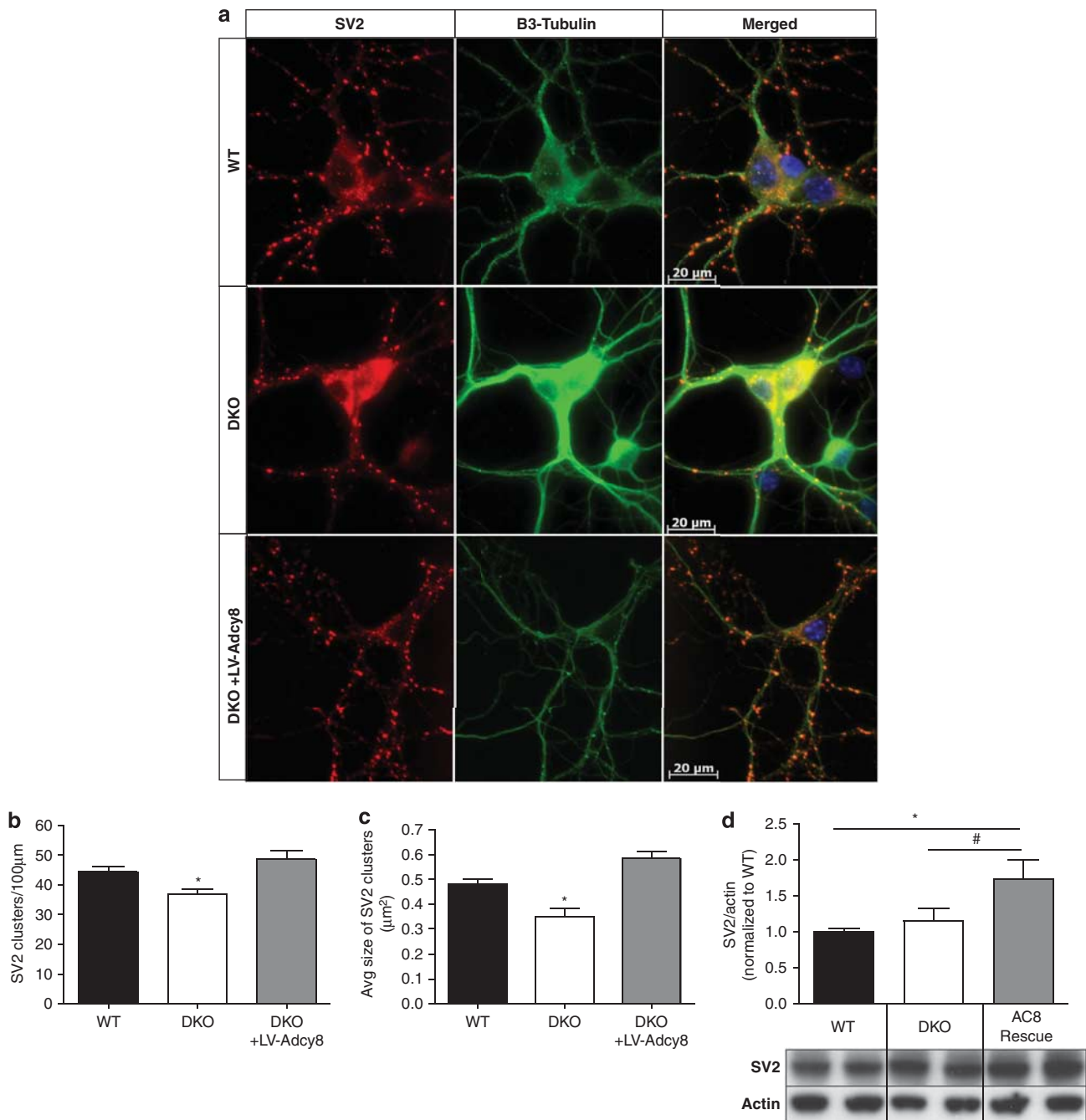
## Results

**AC8 activity modulates the synaptic vesicle protein 2 (SV2) but not neurogenesis.** The expression of SV2 is modulated by changes in synaptic activity.<sup>22</sup> To examine if  $\text{Ca}^{2+}$ -stimulated AC activity modulates the abundance and distribution of SV2 along the dendritic processes, we first measured the number of SV2 clusters as well as the average SV2 cluster size *in vitro*, in hippocampal cultures from WT and DKO mice. These experiments revealed a significant decrease in the average number of SV2 clusters in DKO (36.8 clusters per 100  $\mu\text{m}$ ), relative to WT (44.3 clusters per 100  $\mu\text{m}$ ), neurons along the processes (Figure 1b, *t*-test, *P* = 0.01). Additionally, the average size of the SV2 clusters

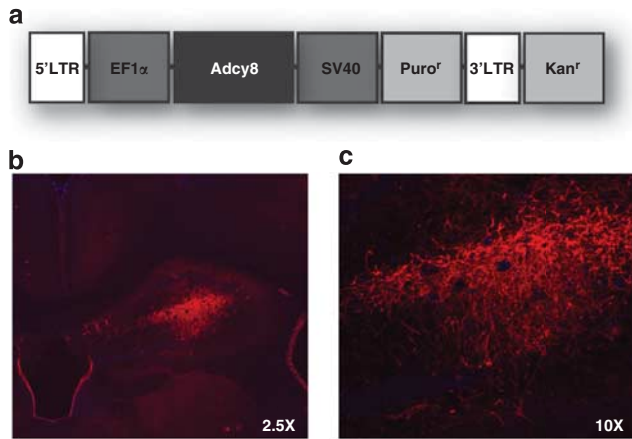
was significantly decreased in DKO ( $0.349 \mu\text{m}^2$ ), relative to WT ( $0.484 \mu\text{m}^2$ ), neurons (Figure 1c, *t*-test,  $P=0.01$ ).

To assess whether  $\text{Ca}^{2+}$ -stimulated AC activity is sufficient to modulate the changes in SV2 levels, we reintroduced AC8 into DKO hippocampal cultures. To do this, we first

generated a lentivirus containing the full-length mouse AC8 cDNA (LV-Adcy8; Figure 2a). The currently available AC8 antibody does not allow for specific staining *in vitro*, so in order to show the effectiveness of the lentivirus, we did *in vivo* injections of LV-Adcy8 into the hippocampus of several DKO



**Figure 1** Synaptic vesicle protein 2 (SV2) dendritic expression is altered in double-knockout (DKO) hippocampal cultures, but not in DKO adult, hippocampal whole-cell preparations. (a) Representative images of DIV9-10 hippocampal neurons in primary cultures derived from P0 or P1 pups. SV2 is labeled with Alexa Fluor 555.  $\beta$ 3-Tubulin, a neuronal marker, is labeled with Alexa Fluor 488. Dapi is used to visualize nuclei. The top row of images represents wild-type (WT) cultures, the middle row represents DKO cultures, and the bottom row represents DKO cultures infected with LV-Adcy8 at DIV1 for 48 h. SV2 expression along the dendritic projections is reduced in DKO cultures, but not DKO cultures infected with LV-Adcy8 as measured by (b) the number of clusters and (c) the average size of a cluster along the dendrites. (d) SV2 protein levels in DKO mice are similar to WT levels in adult, hippocampal micropunches, while AC8 (adenylyl cyclase 8) Rescue mice show a trend towards an increase ( $n=4/\text{group}$ ). \*WT vs AC8 Rescue  $P<0.05$ , # DKO vs AC8 Rescue  $P=0.1$ .

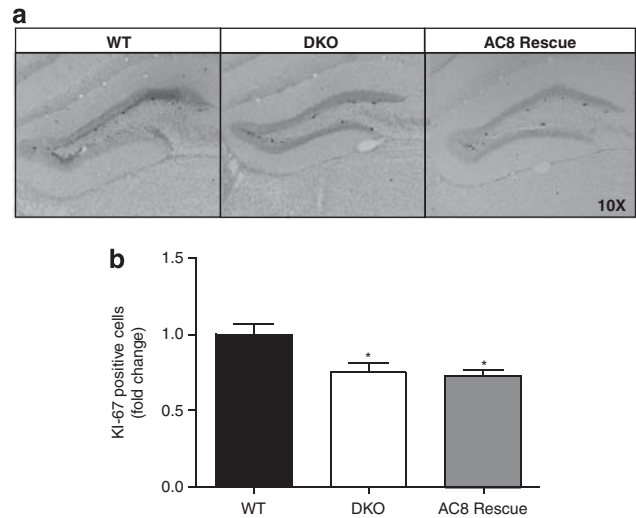


**Figure 2** AC8 (adenylyl cyclase 8) lentivirus expression. (a) A schematic of the major components of the LV-Adcy8 plasmid. A hippocampal image of LV-Adcy8 infection at (b)  $\times 2.5$  and (c)  $\times 10$ . The CY3 staining represents positive AC8 expression, while the dapi counterstain is used to visualize nuclei. EF1 $\alpha$ , elongation factor; Kan<sup>r</sup> = kanamycin resistant; LTR, long terminal repeat; Puro<sup>r</sup>, puromycin resistant; SV40, Simian virus 40.

mice and looked at AC8 distribution between 1 and 2 week after injection (Figures 2b and c). Figure 2 shows abundant AC8 distribution, and specifically, Figure 2c shows AC8 within the cell body as well as along axons and dendrites. Analyses of the number of SV2 clusters and SV2 cluster size after the infection of DKO neurons with LV-Adcy8 revealed that SV2 expression along the processes is similar in WT and DKO with LV-Adcy8 as shown in Figure 1a and quantified in Figure 1b (DKO vs DKO +LV-Adcy8, 48.8 clusters per 100  $\mu\text{m}$ ,  $P < 0.05$ ) and Figure 1c (DKO vs DKO +LV-Adcy8, 0.586  $\mu\text{m}^2$ ,  $P < 0.001$ ). LV-Adcy8 infections of DKO cultures show a trend towards increasing the number and size of SV2 clusters above WT levels, which may result from somewhat supraphysiological expression of AC8 in this system.

SV2 protein levels were then measured using hippocampal micropunches (Figure 1d). A one-way analysis of variance showed a main effect of genotype ( $P = 0.05$ ). AC8 Rescue mice showed a significant increase in SV2 protein levels compared with WT mice ( $t$ -test,  $P < 0.05$ ), while a trend persists for an increase compared with DKO mice ( $t$ -test,  $P = 0.1$ ). This finding implies that acute forebrain AC8 activity after development can modulate SV2 abundance, providing *in vivo* evidence for the direct modulation of a synaptic marker by  $\text{Ca}^{2+}$ -stimulated AC activity.

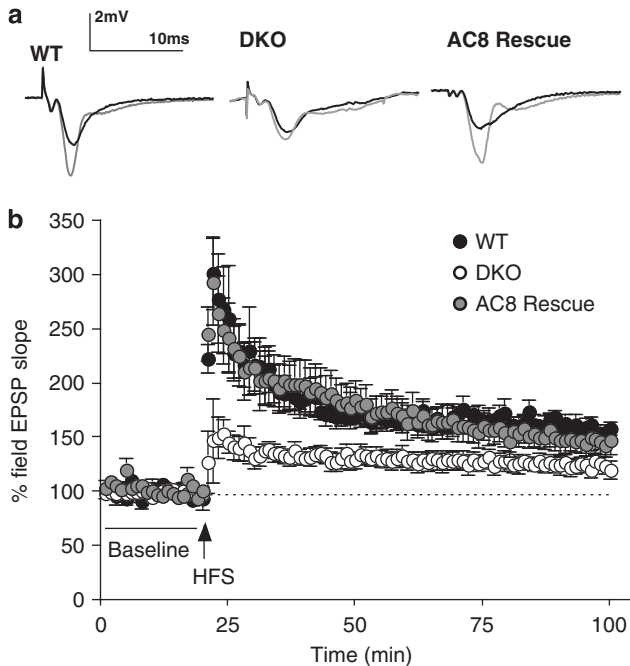
Alterations in neurogenesis in the adult dentate gyrus have been implicated in memory processing, with memory deficits often reflective of a decrease or total absence of neurogenesis.<sup>23–26</sup> Because of the localization of  $\text{Ca}^{2+}$ -stimulated AC activity to the dentate gyrus<sup>27</sup> and the CF memory deficits in DKO mice,<sup>1,2</sup> we hypothesized that neurogenesis would be impaired in these mice. Using mice 2–5 months old, we found that DKO mice displayed a small, but significant, decrease in neurogenesis compared with WT mice as measured by Ki-67 staining ( $P < 0.01$ ; Figures 3a and b). It has previously been shown that cell densities in the dentate gyrus are similar between WT and DKO mice;<sup>1</sup> thus, the decrease in Ki-67 staining reflects a specific decrease in absolute and fractional



**Figure 3** Neurogenesis is impaired in double-knockout (DKO) mice, but not restored in adenylyl cyclase 8 (AC8) Rescue mice. (a) Representative  $\times 10$  images of Ki-67 staining in the dentate gyrus of wild-type (WT), DKO, and AC8 Rescue mice. (b) The number of Ki-67 positive cells in the dentate gyrus was measured using size-matched hippocampal slices. Ki-67 staining is decreased in DKO mice and not rescued by replacing forebrain AC8 in AC8 Rescue mice. \* $P < 0.05$ ; WT  $n = 5$ , 10 bilateral sections; DKO  $n = 6$ , 12 bilateral sections; AC8 Rescue  $n = 4$ , 8 bilateral sections.

cell proliferation. This decrease, however, was not restored in AC8 Rescue mice (AC8 Rescue vs DKO  $P > 0.05$ ), which have intact CF memory,<sup>2</sup> suggesting that reductions in neurogenesis do not underlie the memory impairments seen in DKO mice.

**Forebrain AC8 activity is sufficient to rescue CA1 LTP deficits in DKO mice.** The above data, which shows the ability of AC8 activity to modulate SV2 dendritic abundance or localization, suggest that synaptic activity may be modulated by AC8 as well. Previous studies have found that DKO mice have deficits in LTP within the CA1 region of the hippocampus, but these deficits can be rescued by unilateral acute administration of forskolin to the CA1.<sup>1</sup> These results imply that acutely increasing intracellular levels of cyclic adenosine monophosphate through non  $\text{Ca}^{2+}$ -stimulated AC activation in the CA1 of DKO mice is sufficient to restore CA1 LTP. We assessed whether acute restoration of forebrain AC8 is sufficient to restore LTP deficits as AC1 and AC8 single-knockout mice show initially similar levels of CA1 LTP relative to WT mice.<sup>1</sup> We found that activation of only AC8 in the adult forebrain is sufficient to rescue CA1 LTP as measured in the AC8 Rescue mice (Figures 4a and b). Our results showed that DKO mice fail to show strong potentiation after high-field stimulation (HFS) as measured by the % field EPSP (excitatory post-synaptic potential) slope (DKO 128 vs WT 224% at 1 min post-HFS,  $P < 0.05$ ); however, activation of AC8 activity in the adult DKO forebrain was sufficient to rescue this lack of potentiation (AC8 Rescue 246% vs DKO at 1 min post-HFS,  $P < 0.01$ ; AC8 Rescue vs WT,  $P > 0.05$ ). Furthermore, the data show that LTP in AC8 Rescue mice shows a slightly

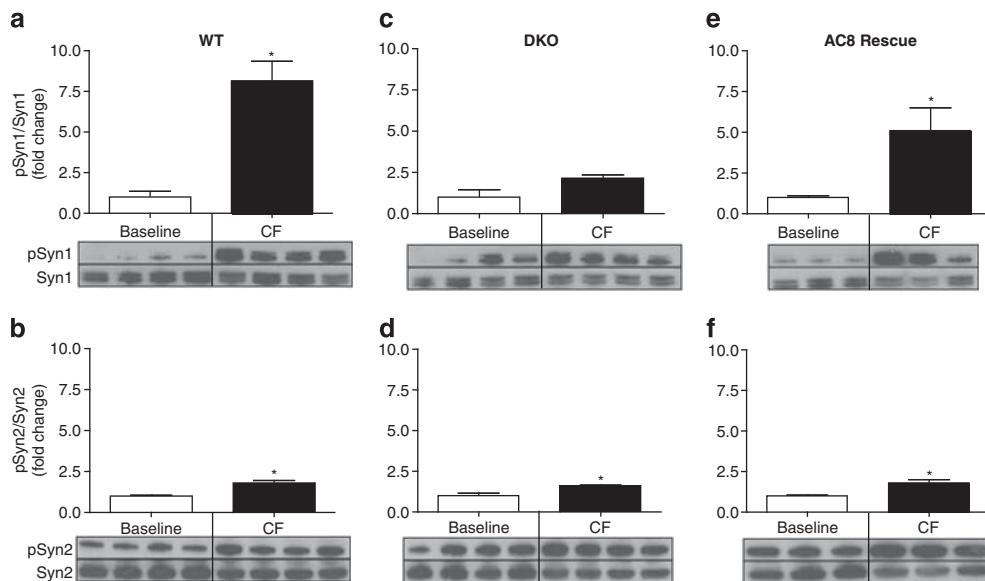


**Figure 4** Adenylyl cyclase 8 (AC8) in the forebrain is sufficient for CA1 LTP (long-term potentiation). (a) Averaged representative traces of pre-tetanus (black) and 80 min post-tetanus (red), four tetanic trains of 100 Hz (200 ms duration, 6 s interpulse interval). Potentiation was measured by % field EPSP (excitatory post-synaptic potential) slope relative to baseline. (b) EPSP responses at 1 min post-HFS (high-field stimulation) demonstrate that double-knockout (DKO) mice (open circles,  $n=7$ ) have dampened potentiation relative to WT mice as measured by % field EPSP slope (closed circles,  $n=7$ ; % EPSP slope in DKO vs WT 1 min post-HFS,  $P<0.05$ ). However, replacing AC8 within the forebrain is sufficient to restore initial LTP deficits as seen in the AC8 Rescue mice (grey circles,  $n=7$ ; % EPSP slope in AC8 Rescue vs WT 1 min post-HFS,  $P>0.05$ ). Scale bar 2 mV, 10 ms.

faster rate of decay over time. The field EPSP slope by 80 min post-HFS is still significantly different between WT and DKO mice (WT 159 vs DKO 127%,  $P<0.01$ ), but no longer significant between AC8 Rescue and WT or DKO mice (AC8 144%,  $P>0.05$ ).

**AC8 activity modulates synapsin I phosphorylation after CF learning.**

Phosphorylation of the presynaptic proteins, synapsin I and II, controls the availability of synaptic vesicles for release and thereby controls the efficiency of neurotransmitter release, which is crucial for the modulation of synaptic plasticity.<sup>28,29</sup> Previous data have shown an increase in hippocampal phosphorylation of synapsin I after CF.<sup>30</sup> Therefore, we investigated whether the impairments in fear memory<sup>1,2</sup> and LTP (Figure 4)<sup>1</sup> seen in DKO mice are correlated with CF-induced changes in the phosphorylation of synapsin. Hippocampal micropunches were taken from mice at baseline and 1 h after CF training; phosphorylated synapsin (p-synapsin) I and II were quantified. Consistent with previous reports,<sup>30</sup> WT mice showed a significant increase in p-synapsin I after CF learning ( $P=0.001$ ; Figure 5a). Although baseline p-synapsin I levels were indistinguishable in WT and DKO mice (Figures 5a and c), DKO mice did not show statistically significant increases in p-synapsin levels after CF learning ( $P=0.06$ ; Figure 5c). Replacing AC8 in the adult forebrain of DKO mice rescued this alteration as AC8 Rescue mice showed a significant increase in p-synapsin I levels after CF learning ( $P<0.05$ ; Figure 5e). However, CF-induced increases in p-synapsin I levels in AC8 rescue mice were significantly less than those in WT mice ( $P<0.05$ ; Figure 5e). These data suggest that CF-induced increases in p-synapsin I levels are also regulated by mechanisms independent of AC8, probably



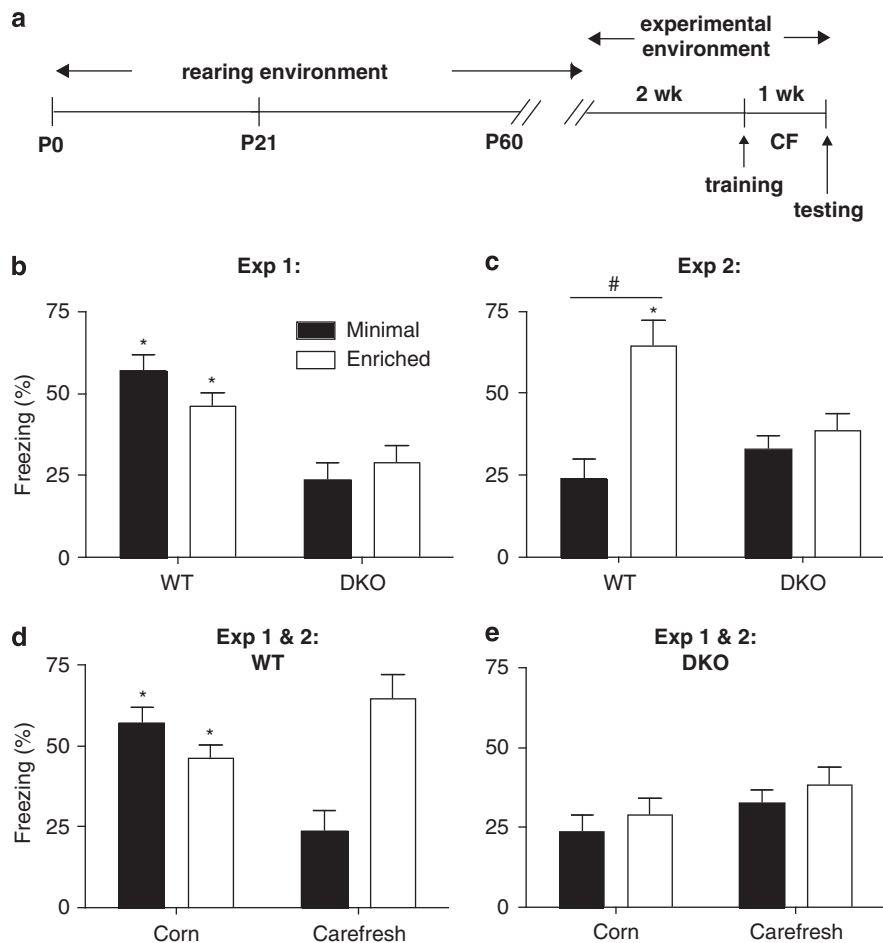
**Figure 5** Double-knockout (DKO) mice show a reduction in p-synapsin I (pSyn1) 1 h after CF training. (a, c, e) p-Synapsin I levels measured at baseline and 1 h after conditioned fear (CF) training in whole-cell membrane preparations from the hippocampus. Wild-type (WT) and adenylyl cyclase 8 (AC8) Rescue mice show an eightfold and fivefold increase after CF training, respectively. DKO mice fail to show an increase. (b, d, f) p-Synapsin II (pSyn2) levels measured at baseline and 1 h after CF training. All genotypes show a similar increase.  $*P<0.05$ ; WT  $n=4$ /condition, DKO  $n=4$ /condition, AC8 Rescue  $n=3$ /condition.

AC1. p-Synapsin II level was increased after CF training in all genotypes (WT  $P < 0.01$ , DKO  $P = 0.01$ , AC8 Rescue  $P = 0.01$ ; Figures 5b, d and f), and the magnitude of change was similar between genotypes, suggesting that AC1 and AC8 do not regulate CF-induced increases in p-synapsin II levels.

**Fear memory in DKO mice is unaffected by environmental changes.** The above data show the genetic influence of  $Ca^{2+}$ -stimulated AC activity on synaptic plasticity. Environmental context has also been linked to alterations in fear memory<sup>8,31,32</sup> and synaptic plasticity.<sup>10</sup> Moreover, gene-environment interactions have been widely implicated in influencing memory in a variety of paradigms.<sup>10,33,34</sup> Therefore, we investigated the gene-environment influence on fear memory in DKO mice by manipulating their environment before CF testing.

Mice were reared and housed in either corn bedding (experiment 1) or carefresh bedding (experiment 2) until at least 2 months of age. Then, mice were subjected to a minimal

or enriched experimental environment for 2 weeks before CF training. One week later, mice were tested (Figure 6a). Freezing levels were assessed to analyze whether environmental exposure affected CF memory. Freezing behavior pre-shock and immediately post-shock was not significantly different regardless of genotype or environment (data not shown). For experiment 1, WT mice showed an increase in freezing behavior relative to DKO mice, which supports previous findings; moreover, this occurs regardless of the experimental environment (WT-minimal vs DKO-minimal  $P < 0.001$ , WT-enriched vs DKO-enriched  $P < 0.05$ ; Figure 6b). Interestingly, an experiment conducted in WT mice housed under different conditions (experiment 2) revealed dramatic differences in freezing levels based on the experimental environment. Only WT mice exposed to the enriched environment displayed a significant increase from DKO mice (Figure 6c). This was true for WT-enriched vs DKO-minimal ( $P < 0.002$ ) or WT-enriched vs DKO-enriched ( $P < 0.01$ ). In both experiments, DKO mice showed consistent freezing levels regardless of environmental exposure.



**Figure 6** Experience-dependent fear memory is unaltered in double-knockout (DKO) mice. (a) Mice reared in two different housing conditions, corn or carefresh, were experimentally exposed to a minimal or enriched environment for 2 weeks before conditioned fear (CF) training as shown in the schematic representation. (b) Experiment 1: Wild-type (WT) mice (reared in corn bedding) show enhanced freezing compared with DKO mice no matter the experimental exposure. (c) Experiment 2: WT mice (reared in carefresh bedding) show decreased freezing when exposed to a minimal environment, WT-minimal, compared with WT mice exposed to an enriched environment, WT-enriched. The interaction between the reared and experimental environment significantly influences freezing behavior in (d) WT mice, but not (e) DKO mice. \* $P < 0.05$  from respective DKO group (Figure b and c) or respective carefresh group (Figure d), #  $P < 0.001$  WT-minimal vs WT-enriched; Experiment 1: WT-minimal  $n = 13$ , DKO-minimal  $n = 11$ , WT-enriched  $n = 13$ , DKO-enriched  $n = 11$ ; Experiment 2: WT-minimal  $n = 7$ , DKO-minimal  $n = 16$ , WT-enriched  $n = 9$ , DKO-enriched  $n = 13$ .

For Figures 6d and e, we combined both the WT (Figure 6d) and DKO (Figure 6e) results from experiment 1 and 2 to see if there are any interactions between the reared and experimental environments. Figure 6d shows a strong statistical interaction between reared environment and experimental environment on the memory of WT mice (as measured by freezing behavior,  $P < 0.0001$ ), but this interaction is absent in DKO mice (Figure 6e,  $P = 0.97$ ). Collectively, the data demonstrate that fear memory is highly influenced by the environment in WT mice, whereas DKO mice show a lack of this behavioral plasticity, regardless of environmental exposure.

## Discussion

Overall, the present study examines how  $\text{Ca}^{2+}$ -stimulated AC activity affects markers of synaptic activity as measured at the molecular, physiological, and behavioral levels. We not only demonstrate that  $\text{Ca}^{2+}$ -stimulated ACs modulate synaptic plasticity and neurogenesis, but we also highlight a novel gene–environment interaction as an absence of the  $\text{Ca}^{2+}$ -stimulated ACs leads to an impairment in experience-dependent fear memory. The first evidence we provide for  $\text{Ca}^{2+}$ -stimulated AC activity's influence on synaptic activity was done by measuring expression of synaptic marker SV2. Owing to the regional localization of the  $\text{Ca}^{2+}$ -stimulated ACs to the synapses,<sup>7</sup> we examined SV2 expression along the dendritic processes. We found that  $\text{Ca}^{2+}$ -stimulated AC directly affects SV2 levels along the processes. Interesting, though, SV2 levels in the cell bodies appear slightly increased in DKO cultures. This observation suggests that the SV2 deficits along the dendritic processes in DKO mice may result from a deficit in SV2 protein or vesicle trafficking to the synapses. Further support for this notion comes from our finding that SV2 levels in adult DKO whole-cell hippocampal preparations are not reduced. This result suggests that there is no reduction of SV2 production, but rather, a difference in its localization.

Additionally, LTP, a process known to rely on a large recruitment of synaptic activity, was directly modulated by  $\text{Ca}^{2+}$ -stimulated AC activity. DKO mice show deficits in potentiation after HFS in accordance with previous studies,<sup>1,4</sup> and rescuing AC8 within the adult forebrain is sufficient to restore these deficits, which is consistent with AC single-knockout studies.<sup>1</sup> The results also suggest that no irreversible downstream deficits result from a loss of  $\text{Ca}^{2+}$ -stimulated AC activity during development. AC8 Rescue mice show a slightly faster rate of LTP decay as they no longer show a significant difference from DKO mice at 80 min post-HFS. This is not surprising as previous results showed a small, but subtle, decrease in LTP over time in AC8KO mice.<sup>1</sup> This finding may reflect that AC8 is over five times less sensitive to  $\text{Ca}^{2+}$  than AC1<sup>2</sup> and hippocampal  $\text{Ca}^{2+}$ -stimulated AC activity in AC8 Rescue mice is 50% to that of WT mice.<sup>35</sup>

Along with the induction of LTP comes an increase in a variety of synaptic markers, such as p-synapsin I.<sup>36</sup> The regional localization of synapsin I, along with AC8, to the presynaptic terminal<sup>37,38</sup> suggests that synapsin activity is downstream of AC8. Therefore, to further assess the function

of  $\text{Ca}^{2+}$ -stimulated AC activity in learning-induced plasticity, we assessed whether p-synapsin levels after CF learning were differentially regulated in DKO and AC8 Rescue mice. DKO mice were unable to significantly increase p-synapsin I levels 1 h after CF learning as seen in WT mice; however, AC8 rescue mice showed a significant, but not as robust, increase, providing more evidence for the role of  $\text{Ca}^{2+}$ -stimulated AC activity in modulating learning-induced changes in synaptic activity.

Not only do our results show that  $\text{Ca}^{2+}$ -stimulated AC activity modulates synaptic activity, but we also show that  $\text{Ca}^{2+}$ -stimulated AC activity modulates neurogenesis as DKO mice show a small, but significant, reduction in neurogenesis. However, this reduction is not attenuated in AC8 Rescue mice despite intact fear learning in the AC8 Rescue mice. Neurogenesis has been implicated in modulating CF learning,<sup>23,24</sup> but more recent evidence that uses a diphtheria toxin-based strategy to selectively remove new neurons before or after CF learning suggests that memory is impaired only when new neurons are removed after CF training.<sup>39</sup> Therefore, AC8 Rescue mice are likely able to compensate for the lack of neurogenesis through activation of other mature dentate granule cells during CF training. Moreover, endogenous AC1 is expressed significantly more in the dentate gyrus than AC8<sup>27</sup> that would suggest the modulation of neurogenesis by  $\text{Ca}^{2+}$ -stimulated AC activity is predominately controlled by AC1.

Overall, the changes in synaptic marker abundance and LTP impairments in DKO mice suggest a crucial role for the ACs in modulating synaptic plasticity. To evaluate whether the ACs contribute to synaptic plasticity at the behavioral level, DKO mice were exposed to minimal or enriched conditions. These experiments revealed that, consistent with previous reports, DKO mice have impaired CF memory.<sup>1,2</sup> Previous findings demonstrated that the freezing deficits result from an impairment in memory, but not learning, as DKO mice show an initial ability to learn that CF task with intact memory at 24 h but not 8 days.<sup>1</sup> Furthermore, exposure to an enriched environment did not alter freezing levels in DKO mice independent of the original housing conditions. Housing conditions, however, did significantly affect memory in WT mice. This coincides with a previous experiment showing variable behavioral results among several inbred mouse strains when tested at multiple universities despite standardization of testing protocols.<sup>40</sup> The genetic similarities, but environmental differences, suggested that these mice display a very plastic behavioral phenotype that is modulated by environmental influences. Our results support this finding as we demonstrate that memory in WT mice is very plastic and highly influenced by experience, and in addition, we show the novel finding that an absence of  $\text{Ca}^{2+}$ -stimulated AC activity prevents this behavioral plasticity.

Collectively,  $\text{Ca}^{2+}$ -stimulated AC activity modulates synaptic activity under baseline and learning conditions. Moreover, the lack of experience-dependent fear memory in DKO mice suggests an inability to adapt to changes in the environment without the presence of  $\text{Ca}^{2+}$ -stimulated AC activity. Overall, the data suggest that experience-dependent fear memory may be driven by  $\text{Ca}^{2+}$ -stimulated AC-induced changes in synaptic plasticity. Future studies will be needed to

further establish the direct causal interactions linking AC action to these plasticity changes.  $Ca^{2+}$ -stimulated ACs have been implicated in the pathology of several psychiatric diseases associated with cognitive decline, such as, Alzheimer's disease, which shows a marked decrease in  $Ca^{2+}$ -stimulated AC activity,<sup>41,42</sup> and bipolar disorder,<sup>43,44</sup> where genetic linkage of AC8 has been associated with the disease. Moreover, environmental enrichment techniques have been proven to increase cognition in Alzheimer patients, but not to the extent that it does in healthy individuals.<sup>45,46</sup> Thus, this provides an interesting follow-up study to try to understand if targeting  $Ca^{2+}$ -stimulated AC activity can further enhance environmental enrichment memory changes in psychiatric patients. If so, targeting  $Ca^{2+}$ -stimulated ACs combined with environmental enrichment therapy could provide a very powerful therapeutic treatment for psychiatric patients with cognitive deficits.

**Conflict of interest**

The authors declare no conflict of interest.

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