1-1-2012

Calcium-Stimulated Adenylyl Cyclases are Critical Modulators of Fear Learning and Experience-Dependent Plasticity

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A dissertation presented to the

Graduate School of Arts and Sciences

of Washington University in

partial fulfillment of the

requirements for the degree

of Doctor of Philosophy

May 2012

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biology and Biomedical Sciences

(Neurosciences)

Washington University in St. Louis, 2012

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Stress can exacerbate psychiatric disease, often resulting in cognitive deficits. Consequently, a better understanding of what modulates stress-facilitated memory processing will help identify new targets for possible therapeutic intervention. Recent evidence suggests a role of the Ca^{2+}-stimulated adenylyl cyclases (AC), AC1 and AC8, in modulating fear memory. Ca^{2+}-stimulated AC activity couples neuronal activity and intracellular Ca^{2+} increases to the production of cAMP, and therefore, can very tightly regulate signal transduction after learning; yet, the details by which this occurs are not well understood. In this dissertation, I first investigated the temporal and regional importance of Ca^{2+}-stimulated AC activity during different stages of memory processing using the tetracycline-off system, which allowed me to produce AC8 Rescue mice with forebrain-specific inducible expression of AC8 on an AC1 and AC8 double knockout (DKO) background. The results showed that forebrain Ca^{2+}-stimulated AC activity was

necessary to modulate long-term memory on several learning paradigms, and more specifically, that it was necessary during memory consolidation and retention. This finding is further supported by an overall decrease in transcriptional changes in DKO mice across several time points after conditioned fear (CF) learning, but most strikingly, at periods when memory consolidation and retention should be occurring. Since transcriptional changes are often dictated by synaptic activity and AC1 and AC8 are both localized at the synapse, I examined synaptic activity in DKO mice. Initial analysis of synaptic protein abundance in hippocampal cell cultures revealed decreased SV2 levels in DKO mice, but this can be rescued by infection with an AC8 lentivirus. Moreover, DKO mice also display synaptic deficits after learning as measured by p-synapsin. The CA1 LTP results coincide with the above data as DKO mice, but not AC8 Rescue mice, show impaired LTP. Finally, WT mice show changes in CF memory strength that is dependent on prior environmental exposure, but DKO mice do not, suggesting that Ca^{2+}-stimulated AC activity modulates plasticity at the behavioral level as well. From these studies, I have observed a critical role for Ca^{2+}-stimulated AC activity in modulating the consolidation and retention of fear memory and experience-dependent plasticity.

ACKNOWLEDGEMENTS

My graduate career path has definitely been a unique one with many professional and personal hurdles. A variety of individuals have been there to support me through this process and I would like the opportunity to acknowledge these people. First, I would like to thank all the past and present Muglia lab members, because they truly are the ones that make the day-to-day routine manageable. Specifically, I would like to thank the two lab managers I have had the pleasure of working with, Sherri Vogt and Donte Smith. Both provided continual support to ensure that I had what I needed to conduct my experiments. Special thanks to Sherri for continually carrying about my success throughout my whole graduate career. I would also like to thank lab members Sandip Bhattacharyya, Alana Conti, Ben Kolber, Chrissy Ratajczak, and Liz Hammock for their continual openness to provide scientific or personal advice. Furthermore, Ben Kolber was really that lab member who was continually open to being bombarded by my unending number of questions and his patience was very much appreciated. I would also like to thank friend, Yarimar Carrasquillo, for her continual critique of my scientific work. I would like to acknowledge my present dissertation committee members, Timothy Holy, Valeria Cavalli, Theodore Cicero, Robert Gereau, Joshua Rubin and Gina Story as well as past member, Guojun Bu.

As my graduate career entailed some unique transitions, I would like to thank those in my personal life that kept me grounded. I very much appreciate my family for continuing to support whatever decision I made. I would also like to thank Sarah Deane (Deaner) for her continual support throughout this process. She is my rock and was continually there whenever I needed her. For this, I am forever grateful and look forward

to her joining me in my upcoming journey. I would also like to thank my fellow classmates, Mena Morales, Karen Lawrence, Jen Semrau and Cassandra VanDunk, for their ability to always keep me smiling.

Finally, I would like to thank my mentor, Lou Muglia. Lou was the first person I interacted with at Wash U. His passion for research, and more so, his passion for creating great scientists is what has made me truly grateful that he was my mentor. Lou was continually open to discussing scientific or personal issues. He truly went out of his way to ensure the best experience for me as a scientist. Also, I am thankful for his continual scientific guidance, but also, his ability to be open to whatever area of research I wanted to explore. The balance Lou created by both lending a helping hand as well as letting creativity flourish is what has molded my current success and will continue to positively affect me throughout my scientific career.

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CHAPTER 1

Introduction

Adaptation in the presence of physical or psychological stress determines the ability of one to cope effectively with a stress challenge. The inability to adapt appropriately to stress often precipitates psychiatric diseases or medical conditions (1, 2). Associated with many of these diseases are cognitive deficits (3-6). Therefore, understanding the molecular mechanisms that are involved in the response to stress and precipitate these cognitive deficits is essential for the design of effective therapeutic agents.

Many mediators of cognitive deficits have been implicated in mouse models, including the Ca^{2+}-stimulated adenylyl cyclase (AC) pathway being one. There are two Ca^{2+}-stimulated ACs: AC1 and AC8, which are both highly conserved making them likely modulators of cognitive deficits in humans as they are in mouse. Bipolar disorder is often associated with cognitive impairments. The human genome region, 8q24, has been linked to bipolar disorder (7), which is homologous to mouse chromosome 15, where AC8 is located (8). Moreover, avoidance behavior, a characteristic trait of mood disorders, was genetically mapped to mouse chromosome 15 through quantitative trait loci analysis. This behavior was significantly reduced by chronic infusion of the human mood stabilizer, carbamazepine, which acts via AC activity (9). Additionally, Alzheimer's disease, whose hallmark symptom is cognitive decline, has been linked with significant decreases in AC1 activity and a trend for a decrease in AC8 activity (10, 11). The human research above suggests a possible role for Ca^{2+}-stimulated AC activity in modulating cognitive impairment.

Ca^{2+}-stimulated AC signaling

There are 10 mammalian isoforms of AC, which have been cloned, with nine membrane bound forms and one soluble form (12). Of the 10 isoforms, two are Ca^{2+}-

stimulated, AC1 and AC8. Structurally, they both have similar membrane topology, which consists of two six-transmembrane domains (M1 and M2) and two cytoplasmic domains (C1 and C2) with the total size ranging from 120 to 140 kD (13) (Figure 1). Activation of AC1 and AC8 via Ca^{2+} occurs through Ca^{2+} binding to calmodulin (14, 15) (CaM). The Ca^{2+}/CaM complex activation of the ACs catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP). Both AC1 and AC8 are also positively regulated by Gsα receptors *in vitro* in membrane preps. *In vivo*, however, Gsα only activates AC1, not AC8, and this occurs only when AC1 is already stimulated by Ca^{2+} to create a synergistic effect (16, 17). Moreover, $G_i\alpha$ inhibits AC1 with no affect on AC8 (17). This process allows the Ca^{2+}-stimulated ACs to tightly couple neuronal activity and intracellular Ca^{2+} increases to the production of cAMP, which can modulate a multitude of diverse processes, such as, neurogenesis, synaptic plasticity, and memory.

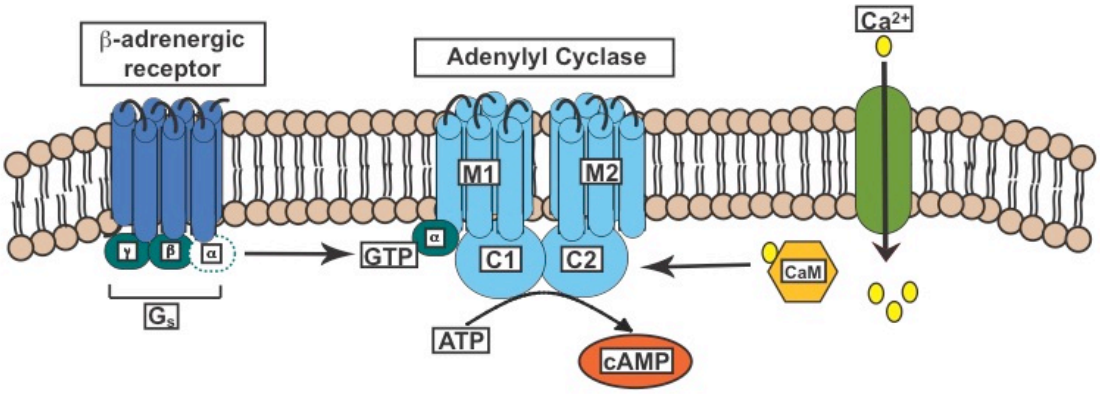

Figure 1. **Schematic of Ca^{2+}-stimulated adenylyl cyclase signaling.** The influx of Ca^{2+}binds to CaM, activating AC1 or AC8, which in turn catalyzes the conversion of ATP to cAMP. cAMP is a second messenger that activates a variety of signaling cascades. AC1 can also by synergistically activated by G protein-coupled receptors. Figure adapted from Ferguson & Storm, 2004, Physiology; AC = adenylyl cyclase, cAMP = cyclic adenosine monophosphate, CaM = calmodulin, M1 & M2 = transmembrane domains, C1 & C2 = cytoplasmic domains

Although AC1 and AC8 are structurally fairly similar, they differ in their spatial distribution and functional characteristics, suggesting Ca^{2+}-stimulated ACs possess diverse roles. In the adult brain, expression of AC1 is found in the cerebellum, olfactory bulb, cortex, dentate gyrus of the hippocampus, pineal gland and thalamus (18-22), while expression of AC8 mRNA is found in the cerebellum, olfactory bulb, cortex, thalamus, CA1 of the hippocampus, amygdala, and hypothalamus (14, 21, 23-25). The localization of AC8 to the hypothalamus suggests that it may play a key role in neuroendocrine processing, such as the processing of stress (24, 25). Moreover, both AC1 and AC8 mRNA are found in the amygdala of neonatal brains, supporting a developmental role of the ACs in the stress response (21). Synaptosome fraction analysis reveals further distinctions in the cellular localization. Both AC1 and AC8 expression is found in the extrasynaptic fraction, while only AC1 is found in the postsynaptic density and AC8 in the presynaptic active zone (22). Furthermore, AC1 and AC8 express different sensitivities to Ca^{2+} with AC1 traditionally thought to be approximately 5 times more sensitive; although, more recent *in vivo* findings are suggesting that these differences may not be so large (19, 26, 27). Collectively, the regional and functional characteristics of AC1 and AC8 suggest that they are likely activated by synaptic activity, and therefore, may in turn modulate synaptic function, such as the processing of memories.

Ca^{2+}-stimulated AC signaling: Long-term potentiation and long-term depression

Genetic mouse models with deletions of AC1 and/or AC8 have begun to help elucidate the functional roles these enzymes may play at the synapse. Long-term potentiation (LTP) and long-term depression (LTD) are activity-dependent changes in synaptic efficacy that reflect long-term molecular changes that occur at the synapse. LTP

and LTD are often viewed as a loose physiological correlates to learning and memory due to shared cellular and molecular events. Ca^{2+}-stimulated AC single knockout mice, AC1KO or AC8KO, show impairments in a variety of different types of LTP; however, they also display some intact physiological measures of LTP, which suggests that there may be some functional redundancy between the two ACs or the different types of LTP measured are not solely Ca^{2+}-stimulated AC-dependent. Both AC1KO and AC8KO mice demonstrate comparable levels of LTP at the Schaeffer collateral-CA1 synapse relative to WT mice; however, AC8KO mice show a slight reduction over time relative to AC1KO mice (27). This is not surprising considering AC8 expression is localized specifically to the CA1 of the hippocampus. Moreover, genetic deletion of both AC1 and AC8 (DKO) leads to a significantly dampened LTP response (27), while overexpression of AC1 (AC1 OE) enhances CA1 LTP (28).

Both AC1KO (29) and AC8KO (30) mice show impaired LTP in mossy fibers, which connects the dentate gyrus to the CA3 of the hippocampus. Again, this correlates well with the localization of the ACs as AC1 is localized to the dentate gyrus. AC8, while not abundant in the mossy fiber tract, is found specifically localized to the presynaptic zone. Mossy fiber LTP has been shown to be dependent on presynaptic Ca^{2+} and cAMP-dependent protein kinase (PKA) activity, and this may allow AC8 to influence mossy fiber LTP more readily despite its low expression in this region (31). Forskolin treatment to AC1KO (29) or DKO (27) mice rescued both mossy fiber LTP and CA1 LTP, respectively, suggesting no deficits in downstream signaling arise from knocking out one or both Ca^{2+}-stimulated ACs.

Finally, LTD in the CA1 of the hippocampus is impaired in AC8KO mice (25), and consequently, DKO mice as well (32). LTD is often measured after stress exposure as it is consistently enhanced with exposure to a physical or psychological stressor (33, 34). The impairment in LTD of AC8KO mice again supports the theory that specifically AC8 may play a role in modulating stress-induced changes, particularly stress-induced learning changes.

Ca^{2+}-stimulated AC signaling: Learning and memory

LTP and LTD are electrophysiological models for long-lasting changes that occur at the synapse. Consequently, the above studies provide insights into the molecular and cellular events contributing to the learning and memory changes seen in Ca^{2+}-stimulated AC genetic models to be discussed below. The original Ca^{2+}-stimulated AC genetic models were *Drosophila* mutants—all of which demonstrate some form of memory-like deficit: *Dunce*, encodes a cAMP phosphodiesterase (35); *DCO*, encodes a cAMP-dependent protein kinase (36); *amnesiac*, encodes a putative AC-activating peptide (37); and *rutabaga*, encodes a Ca^{2+}-stimulated AC (38). More recently, murine transgenic models have been generated that highlight the importance of Ca^{2+}-stimulated AC activity in modulating learning and memory. DKO mice demonstrate memory impairments on stress-facilitated learning, such as the conditioned fear (CF) and passive avoidance paradigms, but AC1KO or AC8KO mice show intact memory (27). The results suggest some functional redundancy of AC1 and AC8. Despite the ability of AC1 or AC8 to compensate for the absence of the other on the CF or passive avoidance tasks, Ca^{2+}-stimulated AC single knockout mice show contrasting memory results on the Morris water maze, suggesting that each AC isoform also has a unique role. For example,

AC1KO mice display impairment in reference memory on the Morris water maze (39), while AC8KO mice show intact memory (40).

Ca^{2+}-stimulated AC single and double knockout mice show memory impairments on a variety of other learning paradigm, displaying their diverse and necessary role in modulating memory processing. AC8KO mice demonstrate memory deficits on a novel objection recognition task (40), and as expected, so do DKO mice (28). Moreover, DKO mice display an impairment on the Morris water maze, but the impairment could be attenuated by overtraining and a shorter intertrial interval (32). Moreover, if mice are then asked to suppress their old memory and acquire a new memory using a reversal task on the Morris water maze, both AC8KO (40) and DKO (32) mice now show impairments. Conversely, overexpression of Ca^{2+}-stimulated AC activity can enhance memory. AC1 OE mice have both enhanced novel object (28) and social recognition memory (41). Over time, though, overexpression of Ca^{2+}-stimulated AC activity appears to be deleterious as aged AC1 OE mice display impairment in consolidation of spatial memory as measured by the Barnes maze memory test (41). Thus, the above data support the ability of Ca^{2+}-stimulated AC activity to tightly regulate memory processing, and subtle changes in activity levels, can impair or enhance memory.

Although the Ca^{2+}-stimulated ACs have been clearly implicated in modulating memory processing, it should be noted that AC8 appears to play a role in modulating anxiety, which could contribute to the memory results found above. AC8KO mice show normal levels of anxiety as measured on the open field and elevated plus maze; however, AC8KO mice show reduced anxiety when subjected to repeated stress (25, 42). This reduced response to stress may contribute to the CF or passive avoidance deficits seen in

DKO mice as they may process the stress of a shock differently than WT mice. Pain could be another potent contributor to memory differences seen in WT and DKO mice on these task as the shock may be differentially perceived by both lines. Both AC1 and AC8 are found in the anterior cingulate cortex, an area known to mediate the emotional component of pain. While DKO mice show normal levels of acute pain, they show reduced responses to formalin and complete Freund's adjuvant; although, this seems to be largely mediated by AC1, and not AC8, based on single knockout results (43). The pain differences, however, are most likely not contributing to changes in memory as the application of a shock is more similar to an acute pain response than an inflammatory stimulus response. Collectively, the memory results detailed above cannot be interpreted without considering the effect Ca^{2+}-stimulated AC activity has on anxiety and pain.

Ca^{2+}-stimulated AC signaling: Downstream targets after learning

Although Ca^{2+}-stimulated AC activity has been implicated in modulating memory processing, the downstream mechanism by which this occurs has still not been well elucidated. The most well classified cAMP effector is PKA. The holoenzyme is composed of four subunits: two regulatory subunits and two catalytic subunits. PKA remains in an inactive state until cAMP binds to the PKA regulatory subunits, which results in the dissociation of the PKA catalytic subunits, resulting in subsequent phosphorylation of PKA substrates (44). In response to learning, PKA has been implicated in readily phosphorylating the mitogen-activate protein kinase/extracellular signal-regulate kinase (MAPK/ERK) pathway, which in turn, causes the translocation of MAPK into the nucleus and subsequent activation of the cAMP response element binding protein (CREB). More recently, phosphorylated (p) levels of ERK were shown to

increase 30 min after CF training in WT, but not DKO, mice. Moreover, PKA, MAPK and CREB were all found to coactivate in a subset of hippocampal CA1 pyramidal neurons after CF training, and this was significantly impaired in DKO mice (45). As a preliminary analysis, I sought to recapitulate this finding in the hippocampus of DKO mice as well as look at a novel region, the amygdala, because both regions are known to be critical for modulating CF learning (46) (Figure 2). I found no significant differences in protein levels between WT and DKO mice in either brain region. It should be noted that I looked at 1 h and not 30 min, because an initial study we conducted in WT mice showed the largest rise in pERK occurred 1 h after CF training, which coincides with previous studies (47, 48). Although the previous data and my results may both be valid due to variations in protocols, it appears that activation of the MAPK/ERK pathway may not be the most significant downstream mediator differentially influenced after CF memory by the absence of Ca^{2+}-stimulated AC activity.

Additional data suggests that epigenetic changes, which can have profound long-lasting influences on memory, occur in the dentate gyrus granule neurons through NMDA receptor activation after training on a forced swim learning paradigm or exposure to a novel environment (49, 50). DKO mice show impairments on the forced swim learning paradigm (discussed in Chapter 2) as well as variations in response to stress exposure as described above. Moreover, the data highly suggest that AC1 and AC8 are activated by the influx of Ca^{2+} through NMDA receptors. Therefore, the Ca^{2+}-stimulated ACs may be downstream targets that regulate the epigenetic changes seen after learning or stress exposure. The specific epigenetic mechanism found to increase is the

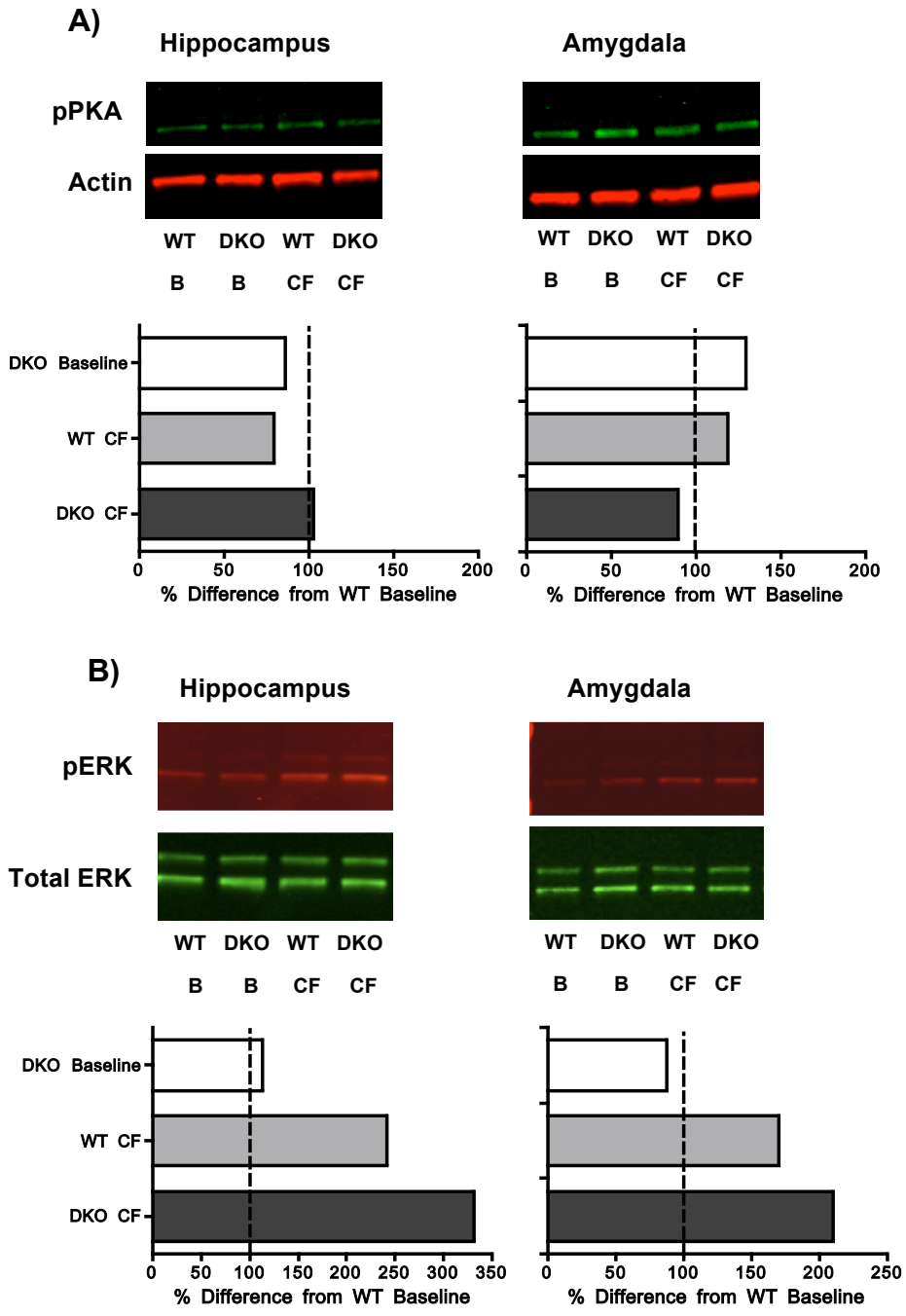

Figure 2. DKO mice show no differences in pPKA or pERK after CF training. DKO protein levels of **A)** pPKA and **B)** pERK are not significantly different than WT protein levels 1 h after CF training in the hippocampus or amygdala as detected by Western blot analysis in nuclear preps. Each lane represents 10 pooled samples. WT = wildtype, DKO = AC1/8 double knockout, B = baseline, CF = conditioned fear

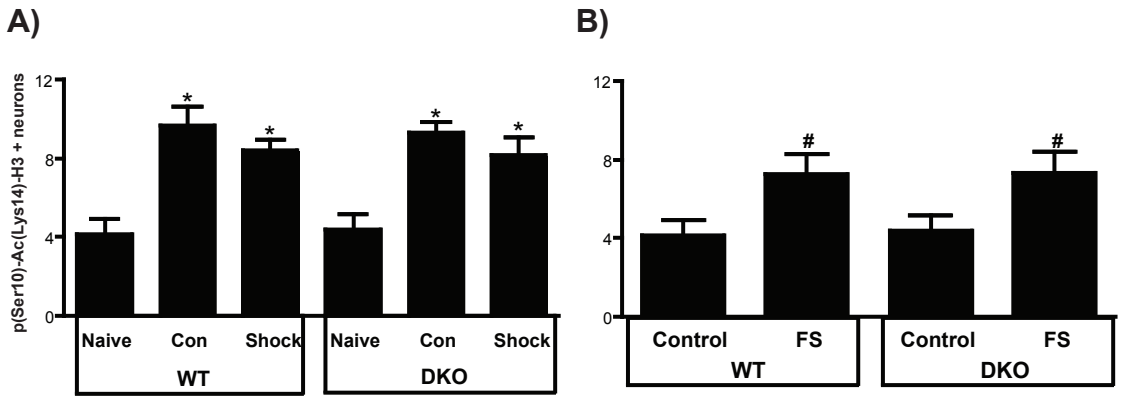

Figure 3. DKO mice display no differences in phospho-acetylation of histone H3 expression after CF or forced swim learning. The number of positive p(Ser10)-Ac(Lys14)-H3 neurons in the dentate gyrus is similar between WT and DKO mice after **A)** CF and **B)** forced swim training. * $p < 0.05$ relative to respective control, # $p < 0.06$ relative to respective control; Con = context, Shock = context + shock, FS = forced swim; data gathered in collaboration with H.J.M. Reul

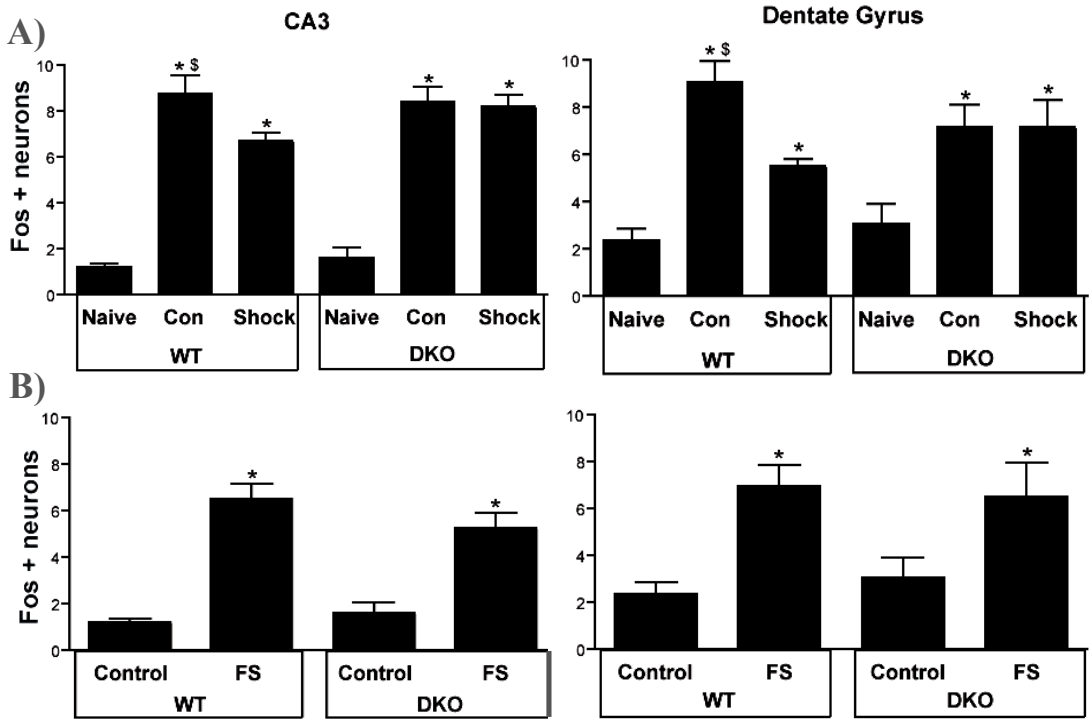

Figure 4. DKO mice display no differences in c-fos expression after CF or forced swim learning. The number of positive c-fos neurons in the CA3 and dentate gyrus is similar between WT and DKO mice after **A)** CF and **B)** forced swim training. * $p < 0.05$ relative to respective control, $ $p < 0.05$ relative to respective Con group; Con = context, Shock = context + shock, FS = forced swim; data gathered in collaboration with H.J.M. Reul

phosphorylation (at Serine-10) and phospho-acetylation (at Serine-10/Lysine-14) of histone H3 (p(Ser10)-Ac(Lys14)-H3). Additionally, c-fos, an immediate early gene, was shown to be upregulated and colocalize with p(Ser10)-Ac(Lys14)-H3 positive neurons. Therefore, I looked at p(Ser10)-Ac(Lys14)-H3 and c-fos levels after both exposure to a novel context (con) as well as a novel context plus a shock (shock) in an effort to simulate the CF training procedure (Figure 3A and 4A). I also looked at p(Ser10)-Ac(Lys14)-H3 and c-fos after forced swim training (Figure 3B and 4B). The results demonstrate that WT and DKO mice show a similar number of positive p(Ser10)-Ac(Lys14)-H3 and c-fos neurons. This first suggests that epigenetic changes, or at least specific changes in p(Ser10)-Ac(Lys14)-H3, are likely not being targeted downstream of the Ca^{2+}-stimulated ACs to regulate the differences in learning between WT and DKO mice. Moreover, the lack of difference in c-fos, which is an immediate-early gene known to increase rapidly and transiently in response to numerous stimuli, suggests that there may be other compensatory mechanisms that are able to initially compensate for the lack of Ca^{2+}-stimulated AC activity.

Herein, I dissect the importance of Ca^{2+}-stimulated AC activity on learning and memory. Through use of a tetracycline-inducible transgenic mouse model, I am able to turn AC8 on or off in the forebrain of mice on a DKO background (AC8 Rescue mice). It should be noted that the AC8 transgene used in the AC8 Rescue mice is not the full length (refer to Chapter 2, Figure 1B), but rather, a normally occurring splice variant. There are three isoforms of the mouse AC8 gene with exons 8 and 11 corresponding to the regions deleted in the previously characterized splice variants C and B, respectively,

in the rat (14, 24). The AC8 Rescue mice are missing exon 11, which contains a glycosylation site. RT-PCR analysis of isolated mouse brain regions demonstrate that all three isoforms are distributed similarly (24) and that the EC_{50} for activation of splice variant C by Ca^{2+}/CaM is similar to the full length AC8 gene (14); therefore, it appears as if the splice variant present in AC8 Rescue mice functions similarly to the full length AC8 gene.

Using DKO and AC8 Rescue mice, DKO and AC8 Rescue mice, I examined the importance of Ca^{2+}-stimulated AC activity on memory processing, particularly focusing on fear learning using the CF paradigm. I was able to analyze the necessity of this activity at different time points after learning, coinciding with different stages of memory processing (Chapter 2). Moreover, I looked at one mechanism by which Ca^{2+}-stimulated AC activity may influence more long-term memory changes by analyzing global gene expression via microarray analysis (Chapter 3). Because transcription is a necessary process for long-term synaptic plasticity changes, we also examined Ca^{2+}-stimulated AC activity's role on synaptic plasticity. Finally, we analyzed whether Ca^{2+}-stimulated AC activity contributes to synaptic plasticity changes at the behavioral level by looking at experience-dependent fear memory (Chapter 4). The work in these chapters presents an important analysis of the Ca^{2+}-stimulated AC pathway and how it modulates learning and memory.

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CHAPTER 2

The regional and temporal role of Ca^{2+}-stimulated adenylyl cyclase activity during learning and memory

(as partially published in Wieczorek et al. *PLoS One*. 2010 Oct 14; 5(10):e13385)

INTRODUCTION

Ca^{2+}-stimulated adenylyl cyclases (AC), AC1 and AC8, link activity-dependent increases in intracellular Ca^{2+} to the production of cAMP, which allows them to play a critical role in long-term memory. Seminal research in invertebrate models has shown a role of Ca^{2+}-stimulated AC activity in modulating learning and memory. *Drosophila* mutants having mutations in this signaling pathway all show some form of memory-like deficit: *Dunce*, encodes a cAMP phosphodiesterase (1); *DCO*, encodes a cAMP-dependent protein kinase (2); *amnesiac*, encodes a putative AC-activating peptide (3); and *rutabaga*, encodes a Ca^{2+}-stimulated AC (4). Additionally, cAMP-dependent protein kinase (PKA) contributes to short- and long-term synaptic changes in mechanosensory neurons of *Aplysia* (5).

More recently, research has shown a role for Ca^{2+}-stimulated AC activity specifically in stress-facilitated learning and memory. The generation of transgenic mouse models that have alterations in Ca^{2+}-stimulated AC activity was pivotal to this finding (6, 7). Double knockout (DKO) mice, which lack both AC1 and AC8, show behavioral deficits on a variety of stress-facilitated learning paradigms, such as passive avoidance and conditioned fear (CF) (6). However, AC1 (AC1KO) and AC8 (AC8KO) single knockout mice show intact learning on passive avoidance and CF (6), which implies that loss of just one Ca^{2+}-stimulated AC in these paradigms is not sufficient for memory loss. Conversely, memory can be enhanced by overexpressing Ca^{2+}-stimulated AC activity as mice overexpressing AC1 in the forebrain show slower rates of extinction for contextual CF (8). Additionally, previous literature has shown that AC8KO mice show reduced stress-facilitated anxiety (7, 9). The data implies that AC1 and AC8 may both have

converging and distinct roles regulating neuronal activity. Therefore, we propose to look at Ca^{2+}-stimulated AC activity by modulating overall expression levels, but have to note that modulation of one particular Ca^{2+}-stimulated AC activity may not necessarily reflect all Ca^{2+}-stimulated AC activity as AC8 may differentially modulate stress-facilitated memory as compared to AC1.

We examine the temporal and regional importance of Ca^{2+}-stimulated AC activity on learning and memory, particularly stress-facilitated memory. We have generated a unique line of mice that uses a tetracycline-inducible system to rescue forebrain AC8 expression in mice on a DKO background (AC8 Rescue mice). This allows us to assess forebrain-specific Ca^{2+}-stimulated AC activity. Additionally, the ability to turn AC8 on or off allows us to establish its role at different stages of memory. The results show that forebrain Ca^{2+}-stimulated AC activity is necessary during stress-facilitated memory consolidation and retention.

MATERIALS AND METHODS

Animals. All mouse protocols were in accordance with 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). Mice were housed on a 12 h/12 h light/dark cycle with *ad libitum* access to rodent chow and water. For control of the inducible tetracycline-off system, mice were either fed doxycycline chow (200 mg doxycycline/1 kg; Research Diets) to repress transgene expression or fed normal rodent chow to permit transgene expression.

DKO (10, 11), AC1KO (11) and AC8KO (7) mice were generated as previously described. To produce forebrain-specific, inducible AC8 expression mice (AC8 Rescue) on a DKO background, a tetracycline-off system was used to allow for temporal control over AC8 cDNA expression. The tetracycline-off system is based on the interaction of a tetracycline transactivator (tTA) with a tetracycline-responsive element (tetop) (12-14). In the presence of tetracycline or doxycycline, tTA loses its ability to bind tetop and expression is turned off. In our system, we have inserted AC8 cDNA under the control of a tetracycline-responsive CMV minimal promoter, generating tetop-AC8 mice. The linearized sequence was microinjected in C57Bl/6 oocytes and founder lines were identified. To confirm that the founder lines were capable of inducible AC8 cDNA expression, they were mated with tTAluc mice (15), which express tTA in many tissues with no detectable endogenous AC8 expression, allowing us to establish that the transgene was expressed (data not shown). Once inducible expression of the AC8 cDNA was established, mice were mated with CaMKII-tTA mice (CaMKII-tTA mice from Jackson Laboratories) (16), which have tTA under control of the CaMKII forebrain-

specific promoter. CaMKII-tTA and tetop-AC8 mice were then mated to DKO mice to generate AC8 Rescue mice. Each separate line was maintained on an inbred C57BL/6 background. All AC8 Rescue matings were on doxycycline and pups were kept on doxycycline until weaned at 21 days to keep AC8 off during development, which allows us to truly assess the learning changes that result from acutely replacing Ca^{2+}-stimulated AC activity in the forebrain of developed DKO mice. DKO mice used in the present studies were mice positive for the tetop-AC8 transgene or CaMKII-tTA transgene alone or wild-type littermates of the AC8 Rescue mice. C57Bl/6 were used as non-littermate control mice (WT).

Immunohistochemistry. Tissue slices were stained for AC8 as described previously (17). Briefly, mice were anesthetized and transcardially perfused with 4% paraformaldehyde. Frozen tissue was cut at 30 μm and sections were incubated in goat anti-AC8 antibody (1:400, Santa Cruz Biotechnology) overnight at 4°C. Sections were then incubated with a biotinylated rabbit anti-goat secondary antibody (Vector Labs) at 1:800 for 1 hr. Biotin was detected with diaminobenzidine and sections were slide mounted with DAPI, a nuclear stain. All images were obtained using matched settings between genotypes on an Olympus BX60 fluorescent microscope equipped with Axiovision software. Images were prepared using Adobe Photoshop.

Western blot analysis. AC8 protein levels were assessed in mice at various time points after doxycycline treatment as described previously (17). Briefly, mice were killed by CO_2 inhalation and the brains rapidly removed. Subregions were dissected on ice, snap frozen in liquid nitrogen and stored at −80°C. Frozen tissues were homogenized with a lysis buffer containing a protease inhibitor and phosphatase cocktail and protein

concentration was determined by the BCA assay (Pierce Biotechnology). For AC8 detection, 20μg of membrane extract from each region was separated by 4–12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with goat anti-AC8 antibody (1:500, Santa Cruz Biotechnology) overnight at 4°C. Equal protein loading conditions were verified by immunodetection for mouse anti-binding protein (BiP) in all samples. Signals were detected using an anti-goat HRP-conjugated secondary antibody, and visualized using chemiluminescence (SuperSignal WestDura; Pierce Biotechnology).

Adenylyl cyclase assay. Brain regions were excised from WT, DKO, and AC8 Rescue mice and assayed for AC activity as previously described (18). Free Ca^{2+} concentrations were calculated using the Bound and Determined computer algorithm (19). AC activity levels are the means of triplicate samples. Protein concentration in the cell membranes was determined as previously described (20).

Behavioral analysis. An observer blinded to genotype performed all behavioral analysis. Behavioral experiments were conducted 2-5 hr after lights on. Male mice ages 2-4 mo were used for all behavioral experiments, except for the first CF experiment, where male and female mice ages 2-8 mo were used. All mice were on a C57Bl/6 inbred background.

Conditioned Fear. Cognitive skills were evaluated using a test of Pavlovian fear conditioning. The CF chamber was a standard grid box (20.3 x 15.9 x 10.0 cm; Med Associates) with a small vial of coconut oil, which served as an olfactory cue. During CF training, mice were put in the chamber for 2 min to assess basal (pretraining) freezing levels, followed by 28 sec of white noise and a 2 sec 0.7 mA foot shock (white noise was used for cued CF but data is not shown). Subsequently, postshock (posttraining)

exploration was monitored for an additional 2 min. Freezing was monitored in 5 sec bins. Graphs express the percent freezing over the course of a testing period. 1 wk, 1 mo and 1.5 mo after training, mice were put back in the original training chamber to assess contextual CF memory. Freezing was monitored as before for 5 min. Doxycycline was given or removed for at least 2 wk, in most cases a month, in between testing to allow for AC8 expression to be turned on or off effectively.

2 Day Forced Swim. Forced swim was used as a test of stress-facilitated learning as described previously (21, 22). Briefly, mice were placed in a 2 L beaker filled 1.5 L high with room temperature (25°C) water for 10 min (Day 1). 24 h later (Day 2), mice were placed in a beaker with the same conditions for 5 min. Testing sessions were video recorded and immobility behavior was scored by assessing the time during which the animal is floating in a relatively immobile position with no limb movement apart from an occasional leg movement to maintain the head above the water. Behavior was scored during the first 5 min of testing on Day 1 and Day 2.

Elevated Plus Maze. Similar procedures as previously described (7) were followed to measure anxiety on the elevated plus maze (EPM), but briefly, mice were placed in a dimly lit room (12 lux) and allowed to roam freely on the EPM for 5 min/day for 3 days. The Anymaze capture system (Stoelting) was used to record and analyze the performance of mice. The percent time spent in the open arm was measured as open arm time/(open arm time + closed arm time).

Open Field. Anxiety and locomotion was assessed using an open field apparatus. The open field apparatus consisted of a Plexiglass box (75 x 75 x 30 cm). Each mouse was placed in the corner of an open field. Each trial lasted for 5 min with 1 trial per

mouse. The maze was rinsed with 70% ethanol between sessions. Anxiety was assessed by the time spent in the center squares (15 x 15 cm/square) versus the peripheral squares. Locomotion was assessed by the total number of grid crossings within the 5 min trial period.

Pain Threshold. Mice were placed in a CF chamber (as detailed above). An ascending series of mild foot shocks was delivered through the grid floor of the CF chamber. The shocks were 1 sec in duration and intensities as follows: 0.08, 0.1, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, and 0.7 mA. There was an interstimulus interval of 30 sec and the intensities at which the mouse a) flinched, b) ran/jumped, and c) vocalized were recorded. The session was terminated immediately following the first vocalization.

Novel Object Recognition. The mice were habituated to the testing chamber for 4 hr before training. Following habituation, two different shaped blocks were presented during a 10 min training session. Testing occurred 90 min later with mice being presented with one familiar object from training and one novel object for 5 min. Memory was assessed by measuring object preference using the discrimination ratio (novel object interaction/total object interaction). A mouse was classified as interacting with an object if it approached or sniffed the object. Trials were videotaped (Sony mini-DV camera) and scored off-line.

Data analysis. Results are expressed as the mean ± SEM. Student's t-test was used to compare pairs of means. In cases with multiple conditions, a two-way ANOVA was used followed by Bonferroni *post hoc* tests when appropriate. A one-way ANOVA was used for single condition analysis followed by Tukey's Multiple Comparison *post hoc* tests

when appropriate. For forced swim and novel object recognition, a one sample t-test was run to assess whether the percent immobility difference from Day 1 to Day 2 was different from 0 and whether the percent interaction with an object was different from chance (50%), respectively. A p-value of ≤ 0.05 was considered statistically significant. All statistical comparisons were done with Prism 4 software (GraphPad).

RESULTS

Generation of AC8 Rescue mice

To functionally test the temporal role of AC activity during CF learning, we restored Ca^{2+}-stimulated AC function at different time points during behavioral testing with a transgenic model of AC8 expression. We generated a system where AC8 is expressed in the forebrain of mice on a DKO background (AC8 Rescue mice). We used a doxycycline-regulated system (e.g. tetracycline-off system) to restore AC8 expression in a temporally regulated fashion. Figure 1A shows the protein distribution of AC8 within WT and DKO mice. Consistent with previous reports, AC8 is localized to the cortex, thalamus, hippocampus, and cerebellum (7). AC8 Rescue mice display AC8 expression within forebrain specific regions, but not within the thalamus or hindbrain. We find that AC8 expression begins to turn on two weeks after taking mice off doxycycline, and conversely, AC8 expression is turned off completely after 2 wk on doxycycline (Figure 1B). We measured Ca^{2+}-stimulated AC activity in the cortex (Figure 1C) and hippocampus (Figure 1D) to determine the magnitude of physiological replacement in AC8 Rescue mice. Although expression of AC8 protein in the AC8 Rescue mice appears higher than what is present endogenously within the WT mice (Figure 1A), we found that that overall Ca^{2+}-stimulated AC activity was recovered to approximately 50% and 30% of WT levels in the cortex (Figure 1C) and hippocampus (Figure 1D), respectively. As shown previously (10), DKO mice have no measurable Ca^{2+}-stimulated AC activity. Importantly, AC8 Rescue mice on doxycycline show no Ca^{2+}-stimulated AC activity, confirming that doxycycline efficiently represses AC8 transgene expression.

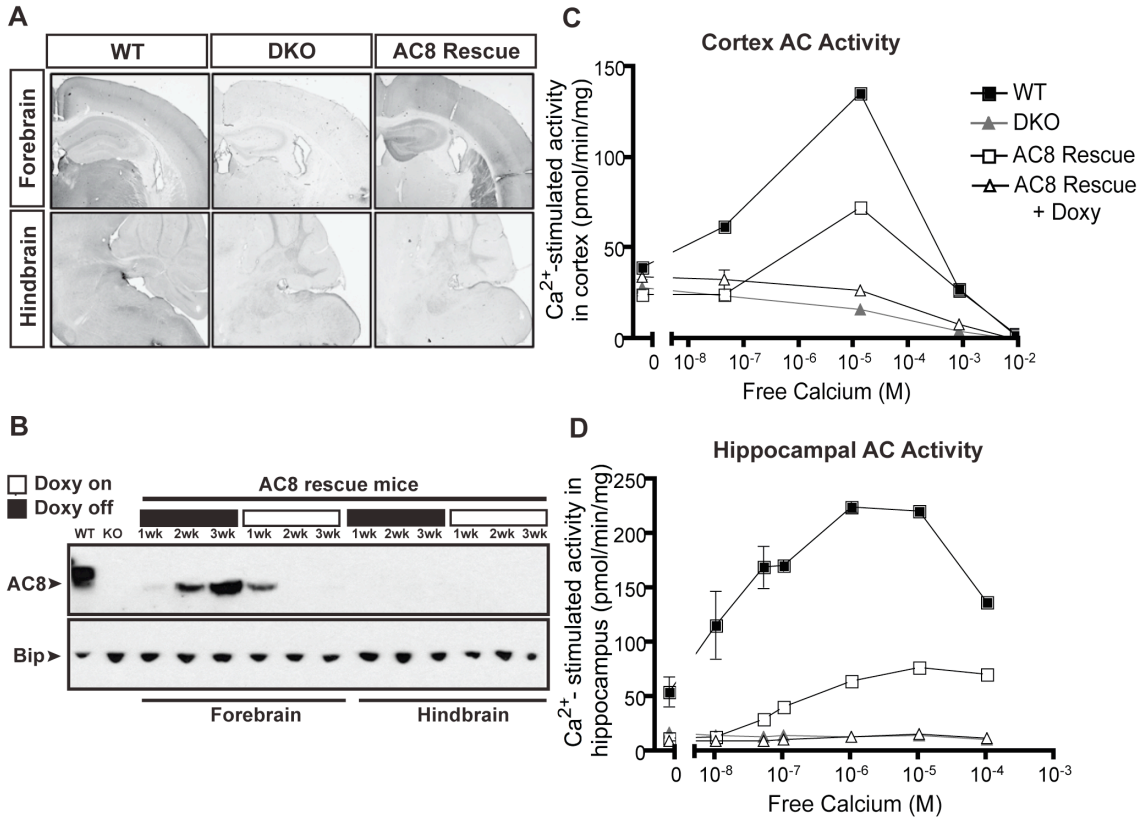

Figure 1. Generation of inducible forebrain-specific AC8 mice on a DKO background.
(A) Immunohistochemistry results confirm complete absence of AC8 protein levels in DKO mice, while AC8 Rescue mice have AC8 replaced within forebrain-specific regions but not in the hindbrain or thalamus. **(B)** Using doxycycline to manipulate AC8 expression within the forebrain, we observed AC8 expression after 2 wk off doxycycline. In contrast, expression begins to rapidly turn off within one week off doxycycline treatment and is undetectable by 2wk. Hindbrain results reveal no AC8 expression with or without doxycycline treatment. Bip is used as a loading control. The adenylyl cyclase assays in the **(C)** cortex (n = 3/genotype) and **(D)** hippocampus (n = 3/genotype) reveal no Ca^{2+}-stimulated activity in DKO mice or AC8 Rescue mice on doxycycline, while activity is rescued to approximately 50% **(C)** and 30% **(D)** of WT levels in AC8 Rescue mice off doxycycline.

Ca^{2+}-stimulated AC activity role in memory processing

After confirming that our AC8 Rescue system was able to restore AC8 expression and activity to the forebrain, we turned AC8 activity on and off at different time points throughout CF testing. We found that acutely restoring AC8 continuously during CF training and testing was sufficient to rescue memory deficits seen in DKO mice (Figure 2B). However, when the AC8 transgene was repressed during training only, memory is impaired (Figure 2C), suggesting that Ca^{2+}-stimulated AC activity is necessary during memory consolidation. Additionally, when AC8 was kept on during training but turned off during the retention period, memory was again impaired at 1 mo (Figure 2D).

To investigate if Ca^{2+}-stimulated AC activity was needed just for retrieval, rather than retention, we turned AC8 expression on again and tested mice two weeks later (1.5 mo after training). Suggesting that Ca^{2+}-stimulated AC activity is necessary during memory retention, we found that AC8 expression for 2 wks after the 1 mo testing session was unable to rescue CF memory at 1.5 mo (Figure 2D).

To assess whether these stress-facilitated learning deficits in DKO mice on the CF paradigm can be seen on other stress-facilitated learning paradigms, we assessed immobility on a two day forced swim learning paradigm. Traditionally, the forced swim paradigm is a one day test used to assess despair with immobility directly correlating with despair. As Figure 3A and 3B indicate, there are no differences in latency to immobility or immobility between WT, DKO and AC8 Rescue mice on Day 1, which suggests similar levels of despair. However, when the test is turned into a two day paradigm, it is postulated that the re-test on Day 2 is a measure of a learned, adaptive behavior as the mouse learns the situation is inescapable, and therefore, conserves its energy by

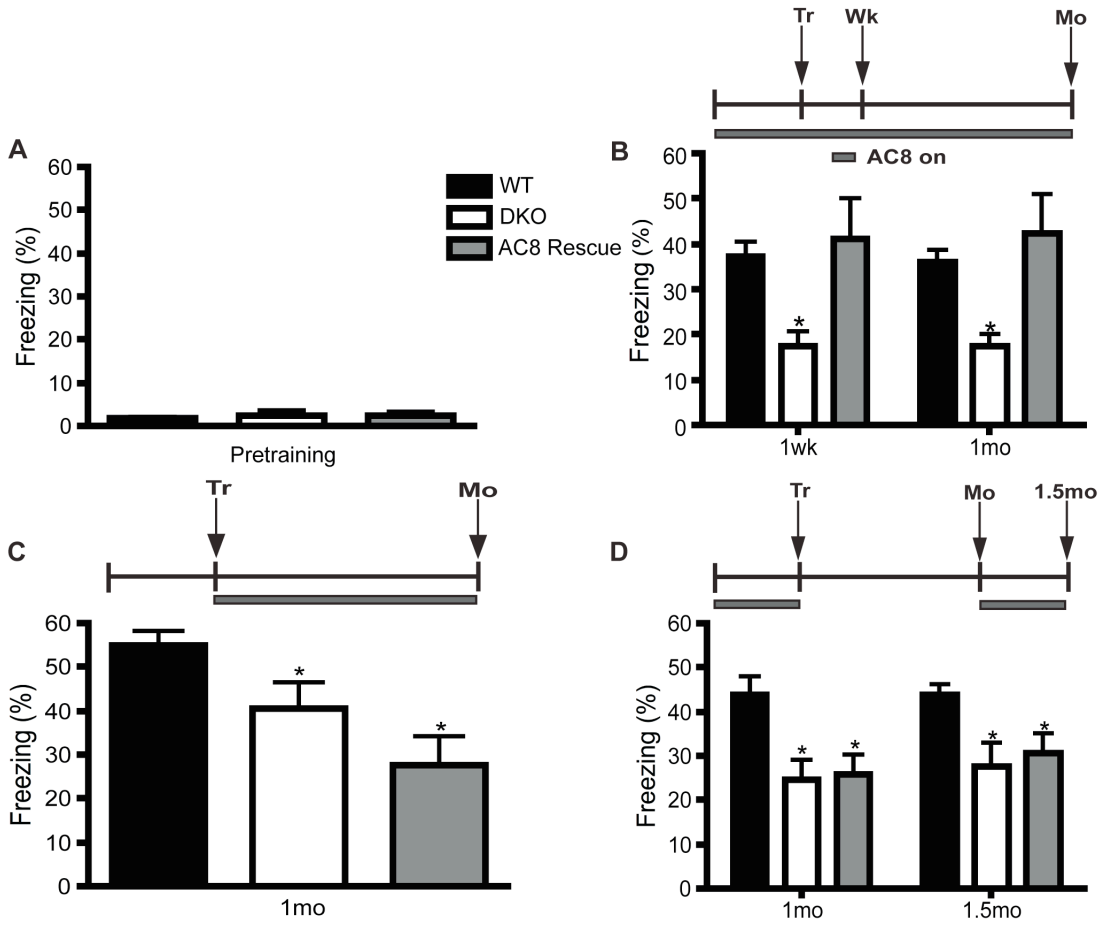

Figure 2. Ca^{2+}-stimulated AC activity is necessary for memory consolidation and retention. Memory changes are assessed by the overall freezing percentage. **(A)** There are no baseline freezing changes between the genotypes (no differences in baseline freezing levels between graphs B, C, or D, so subjects were combined; $p > 0.05$). **(B)** DKO mice show memory deficits at 1 wk and 1 mo compared to WT mice. AC8 Rescue mice reveal that replacing AC8 expression within the forebrain of DKO mice throughout training, retention and testing is sufficient to rescue CF memory deficits (n = 9-11/genotype, $*p \leq 0.05$ AC8 vs DKO). Turning AC8 off during **(C)** CF training (n = 8-10, $*p \leq 0.05$, AC8 and DKO vs WT) or **(D)** during the retention period (n = 9-11, $*p \leq 0.01$, AC8 and DKO vs WT) prevents restoration of the memory deficits. Furthermore, AC8 Rescue mice still show memory deficits after turning AC8 back on for 2 wks after having it off since training (n = 9-11, $*p \leq 0.05$, AC8 and DKO vs WT). AC8 on = doxycycline off; Symbols: Tr, training; Wk, week; Mo, month

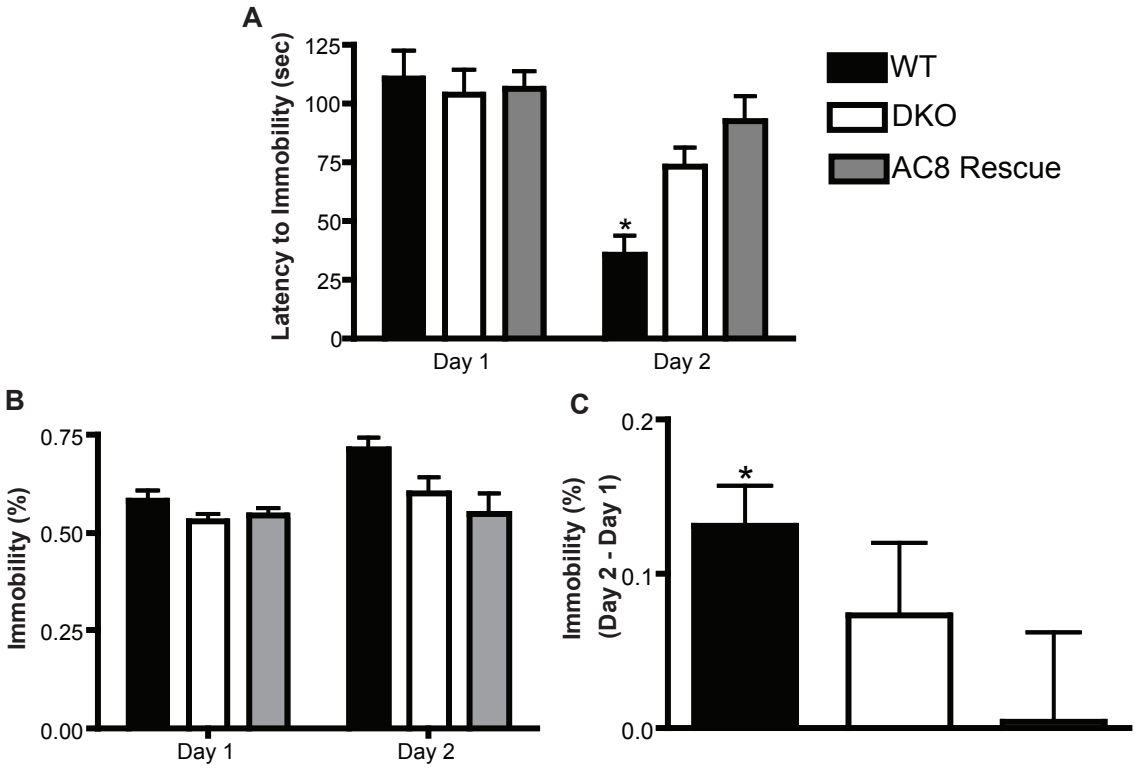

Figure 3. Forebrain expression of AC8 activity is not sufficient to rescue forced swim memory deficits in DKO mice. (A) WT mice display a faster latency to immobility on Day 2. **(B)** There is a significant main effect of genotype and day with WT mice showing a trend towards increased immobility relative to DKO and AC8 Rescue mice on Day 2. **(C)** Furthermore, the percent difference in immobility between Day 2 and Day 1 is significant for WT mice; whereas, DKO and AC8 Rescue mice show no significant difference between days (n = 9/genotype, *1-sample t-test immobility percentage is greater than 0). *$p \leq 0.05$

remaining immobile (22, 23). The view that this re-test measures a learned behavior is further supported by an increase in phospho-acteylation of histone H3 in the dentate gyrus, which is known to regulate transcriptional changes (21). The data suggests that DKO mice fail to learn as exemplified by the lack of difference in immobility between Day 1 and Day 2; whereas, WT mice show a significant decrease in latency to immobility on Day 2 (Figure 3A) and a significant difference in immobility between Day 1 and Day 2 (Figure 3C). Although, the DKO mice once again show deficits in stress-facilitated learning as measured on the forced swim learning paradigm, acutely restoring forebrain AC8 is not sufficient to restore these deficits as indicated by AC8 Rescue mice showing similar results as the DKO mice.

Although acute forebrain AC8 expression was unable to restore memory deficits on the forced swim learning paradigm, we were able to restore memory deficits on a relatively unaversive learning paradigm, novel object recognition. Consistent with previous reports (8), DKO mice show no bias for the novel object during the testing trial (90 min after training) (Figure 4). Restoration of AC8 expression in the forebrain of DKO mice elicits a response comparable to WT mice.

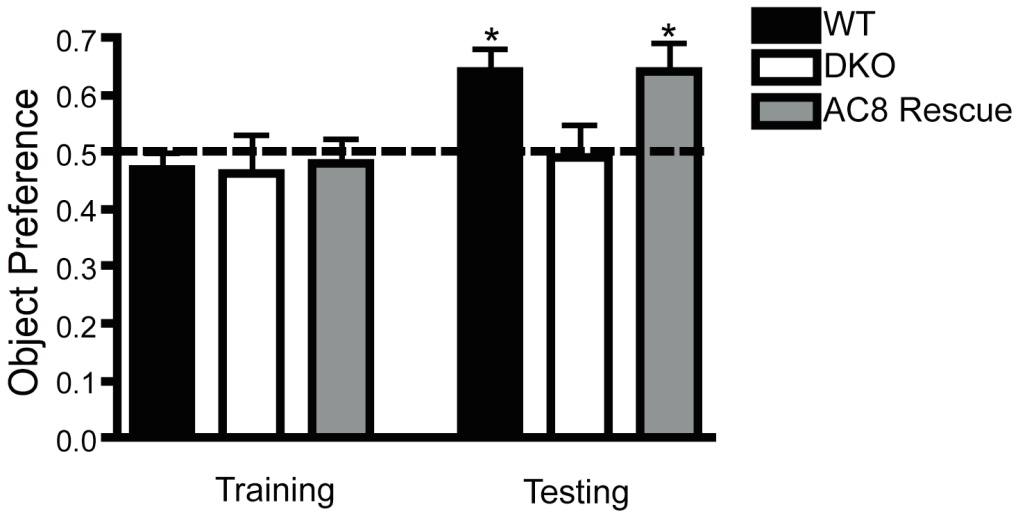

Figure 4. Ca^{2+}-stimulated AC activity is necessary for intact novel object recognition. The hash mark labels the 50% mark, which represents no preference for an object (object preference = novel object interaction/total object interaction). DKO mice show no preference for the novel object when tested 90 min after training (object preference is not significantly different than 50%, $p > 0.05$); however, replacing AC8 in the forebrain of DKO mice is sufficient to maintain memory. n = 9-10/genotype, *1-sample t-test object preference is greater than 50%, $p \leq 0.05$

Ca^{2+}-stimulated AC activity's effect on anxiety, locomotion and pain

To evaluate possible variables that could contribute to the learning results, we measured anxiety, locomotor activity and pain between the genotypes. The first basic measurement, which looks at baseline freezing in the CF paradigm, shows no difference in freezing between genotypes (Figure 2A). Next, we measured anxiety on a 3 d EPM paradigm between WT and DKO mice (Figure 5) as previous results show that AC8KO mice have a decrease in anxiety over time (7). The present results follow a similar pattern as seen in the aforementioned study with DKO mice showing decreased anxiety. However, DKO mice show decreased anxiety on Day 1 with no differences on Day 2 or 3, while the converse is true with AC8KO mice. We further evaluated anxiety on an open-field paradigm (Figure 6A). Indices of anxiety-like behavior, such as time spent in the center vs periphery of the open field, were similar between all three groups, WT (65% periphery vs 35% center ± 9.0), DKO (75% periphery vs 25% center ± 2.8) and AC8 Rescue (75% periphery vs 25% center ± 4.5) mice. We used the open-field paradigm to test locomotion as well. Locomotion measures were increased (Figure 6B; $p = 0.02$), as assessed by the total number of grid crossings in 5 min, in both the DKO (165 grid crossings) and AC8 Rescue mice (166 grid crossings) relative to WT mice (83 grid crossings). However, since AC8 Rescue and DKO mice show different learning phenotypes, it is unlikely that the increased locomotion alters interpretation of the memory findings. Basal pain sensation has also been previously studied with no major differences seen between WT and DKO mice (24). We further support this finding by showing no differences in qualitative responses to increasing shock intensities (Figure 6C).

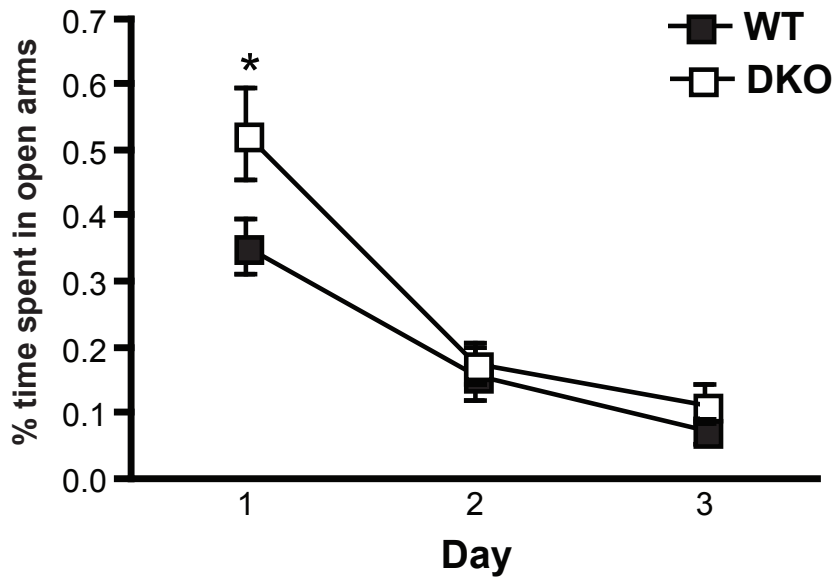

Figure 5. Absence of Ca^{2+}-stimulated AC activity decreases anxiety on the EPM. DKO mice display increased time spent in the open arm on Day 1 relative to WT, which indicates a decrease in anxiety. This difference in anxiety is absent by day 2 and 3. n = 7-8/genotype, *$p \leq 0.05$

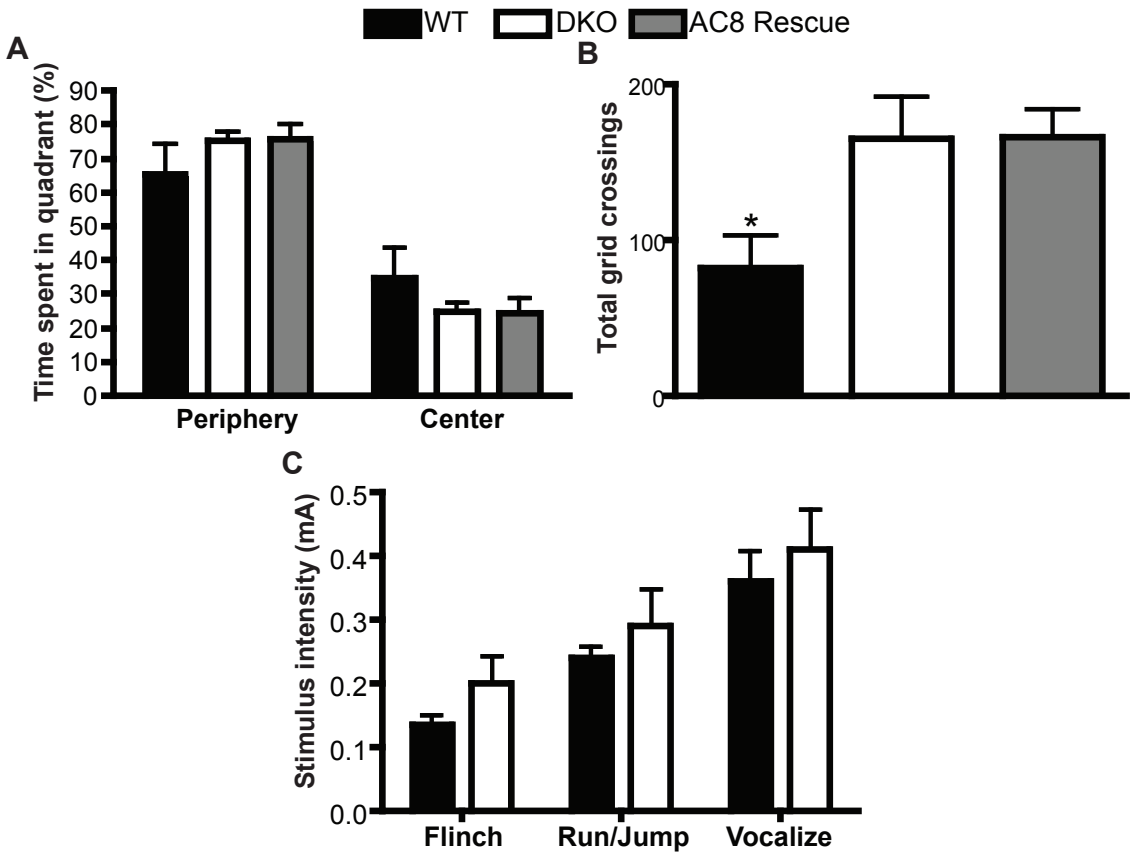

Figure 6. Assessment of anxiety, locomotion and pain threshold in DKO and AC8 Rescue mice. (A) Indices of anxiety-like behavior, such as time spent in the center vs periphery of the open field, were similar between all three groups,. **(B)** Locomotion measures were increased (*p = 0.02) as assessed by the total number of grid crossings in 5 min, in both the DKO and AC8 Rescue mice relative to WT mice. **(C)** DKO mice reacted in a qualitatively similar manner as WT mice to increasing shock intensities.

DISCUSSION

We were able to successfully generate a mouse line that allows temporal restoration of forebrain AC8 in mice on a DKO background. This provides a useful tool for beginning to delineate the temporal and regional importance of Ca^{2+}-stimulated AC activity on learning and memory. Past literature supports the theory that Ca^{2+}-stimulated AC activity plays a pertinent role in learning and memory. Although disrupting just one of the two Ca^{2+}-stimulated ACs can cause memory impairments (25), data show that AC single knockout mice show intact CF behavior while DKO mice show alterations in CF behavior (10). Testing in the CF paradigm reveals that DKO mice have intact memory at 24 h, but impaired memory at 1 wk. Moreover, when tested on a novel object recognition paradigm, DKO mice have impaired object recognition at 1 h, but not 5 min (8). Overall, these data suggest Ca^{2+}-stimulated AC activity is not necessary for acquisition of a task, but necessary for long-term consolidation of a memory and that a single AC (AC1 or AC8) can compensate for loss of the other AC. Our data support these findings as AC8 Rescue mice that have AC8 turned off during the consolidation CF training period show memory impairment, while AC8 transgene expression throughout training and testing restores long-term conditioned responses. Additionally, we recapitulate the novel object recognition data with DKO mice showing impairments, but also provide evidence that memory can be restored if AC8 expression is turned on in the forebrain before training. Overall, the above data suggests that Ca^{2+}-stimulated AC activity is necessary for the consolidation of memory elicited by both an aversive and non-aversive stimulus.

The forced swim learning paradigm used in this study demonstrates that acute restoration of forebrain AC8 is not sufficient to rescue memory deficits in DKO mice on

all stress-facilitated learning paradigms. AC8 Rescue mice showed the same deficits as DKO mice. The literature supports the concept that differential circuitry is involved in the processing of different stressors (26), and therefore, the forced swim and CF learning paradigms may be processed differentially based on the stress response elicited. This suggests that Ca^{2+}-stimulated AC activity during development may be necessary for intact forced swim memory, or the results may highlight the differential role or regional localization of AC1 and AC8.

Not only is memory consolidation impaired in DKO mice, but memory retention is as well. This is evident when Ca^{2+}-stimulated AC activity is turned off in AC8 Rescue mice after CF training. These mice fail to show a normal conditioned response when tested 1 wk after training. These data are in apparent conflict with analysis of passive avoidance behavior in DKO mice. DKO mice exhibit impaired passive avoidance behavior that can be restored with a single administration of forskolin into the hippocampus of DKO mice 15 min prior to training, even when memory testing occurs as long as 8 d after training (10). This would imply that Ca^{2+}-stimulated AC activity is only necessary for consolidation of a memory as the memory was retained 8 d after initial cAMP activation. However, passive avoidance is a less complex task than CF learning; therefore, a one time injection of forskolin may be sufficient to rescue memory deficits in passive avoidance, but for CF learning, a more sustained activation of biological pathways might be necessary to maintain the memory. This theory is supported by transcriptional data discussed in Chapter 3.

Finally, we assessed general anxiety, locomotor activity and pain to see if these factors are differentially regulated and may confound the learning and memory

interpretations. Open field results reveal no differences in anxiety, but the EPM suggests a decrease in anxiety. Previous studies found no baseline anxiety differences in AC8KO, but a decrease in anxiety after exposure to a stressor (7, 9). Although no thorough examination has been conducted to assess whether the anxiety-like behavior measured in the open field test is similar to the EPM, our data suggest that there may be subtle differences, and possibly, the EPM could elicit a greater stress response, which brings to light the anxiety differences in WT versus DKO mice. Moreover, locomotor was similarly increased in DKO and AC8 Rescue mice. Previous literature shows that AC8KO mice display an increase in locomotor activity (9), which would suggest that DKO mice might show similar results. Our findings suggest again that Ca^{2+}-stimulated AC activity is necessary during development to elicit WT levels of locomotor activity, or perhaps, the difference in genetic background is altering locomotor activity. Regardless, both genotypes show increased locomotor activity, but different memory results, suggesting that this measure is not significantly altering the memory results. Finally, a qualitative assessment of pain shows no differences in DKO mice as previously shown (24).

These results highlight a pertinent role of Ca^{2+}-stimulated AC activity in modulating multiple forms of memory. Moreover, it displays the importance of this activity during memory consolidation and retention. Although some subtle differences are seen with anxiety and locomotor activity, the results fully support the conclusion that deficits in memory processing result from a lack of Ca^{2+}-stimulated AC activity and that acute restoration of forebrain AC8 is able to rescue many of these memory deficits. The

subsequent chapters will analyze and discuss in detail the possible mechanisms by which this is occurring.

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CHAPTER 3

Ca^{2+}-stimulated adenylyl cyclase activity is critical for transcriptional regulation during fear learning

(as partially published in Wieczorek et al. *PLoS One*. 2010 Oct 14; 5(10):e13385)

INTRODUCTION

The cAMP signal transduction pathway has been repeatedly implicated in learning and memory using both invertebrate and vertebrate models. More specifically, the Ca^{2+}-stimulated adenylyl cyclase (AC) pathway, which couples neuronal activity and intracellular Ca^{2+} increases to the production of cAMP, is crucial for normal memory processes (1). This essential role is evident by memory impairments seen in the rutabaga *Drosophila* mutant, which shows a lack of Ca^{2+}-stimulated AC activity (2). Of the ten AC isoforms in mammals, AC1 and AC8 are the only two that are primarily stimulated by Ca^{2+}/calmodulin (3-5). Murine models have demonstrated the importance of these isoforms in memory processing. For example, both AC1 knockout (AC1KO) and AC8 knockout (AC8KO) mice display learning impairments in the Morris water maze (6, 7). Moreover, there appears to be functional redundancy in these two isoforms as passive avoidance and conditioned fear (CF) memory are intact in AC1KO or AC8KO mice but are impaired in AC1 and AC8 double knockout (DKO) mice (5). Interestingly, DKO mice show normal CF memory at 24 hr, but not 1 wk, suggesting that Ca^{2+}-stimulated activity is necessary for long-term memory changes.

AC1 and AC8 are both localized to brain regions known to play essential roles in memory processing, such as the cortex, cerebellum, and hippocampus (8-10). At the cellular level, AC1 and AC8 are localized to the synapse, specifically the postsynaptic region for AC1 and presynaptic region for AC8 (11). The regional and subcellular location of these two isoforms clearly has physiological implications as AC1KO and AC8KO mice show impairments in mossy fiber long-term potentiation (LTP) (12).

Although Ca^{2+}-stimulated AC activity has been implicated in modulating

behavior, the mechanism by which this occurs has still not been thoroughly defined. There is evidence highlighting deficits in acute, short-term activation of the MAPK/ERK pathway 30 min after CF training (13). However, since long-term memory and LTP are both dependent on transcription and are disrupted in AC knockout models, we hypothesize that the primary effect of Ca^{2+}-stimulated AC activity during CF is to modulate gene expression (14-16). We assessed the effect of Ca^{2+}-stimulated AC activity on global gene expression via microarray analysis. Moreover, we assessed gene expression changes at several time points across learning. The contextual CF paradigm, which relies on the structural integrity of the hippocampus and amygdala (17), was used as our paradigm to define the network changes that result during memory processing in the context of disruption of AC expression with a knockout mouse model. We demonstrate that there is an overall attenuation of transcriptional changes in mice lacking both Ca^{2+}-stimulated AC isoforms, which may contribute to the memory impairments seen in DKO mice after CF learning.

MATERIALS AND METHODS

Animals. All mouse protocols were in accordance with 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 12 hr/12 hr light/dark cycle with *ad libitum* access to rodent chow and water.

DKO (5, 6), AC1KO (6) and AC8KO (18) mice were generated as previously described. AC8 rescue mice were generated as described in Chapter 2. C57Bl/6 were used as non-littermate control mice (WT).

Tissue collection and RNA extraction. We collected hippocampal and amygdala samples from WT and DKO mice at baseline and four different time points after a 5 min CF training trial. The four time points (and the respective memory stages each time point corresponded to) were as follows: 0 hr (acquisition), 1 hr (consolidation), 48 hr (retention), and 1 wk after CF training (retrieval). For the 1 wk after CF training time point, mice were tested in a 5 min CF testing trial prior to killing. Micropunches using a 1 mm diameter capillary tube were used to extract bilateral hippocampal and amygdala punches approximately 2 mm thick. Tissue was quickly preserved using RNA later (Qiagen) at 4°C and moved to -80°C 24 hr later until RNA preparation. The Qiagen miRNAeasy Mini Kit was used to extract RNA, which uses a two-step process, both Trizol and column extraction, to isolate purified, intact RNA.

Microarray analysis. RNA samples from 5-10 mice were pooled per array with one array per genotype/brain region/time point for a total of 20 arrays. Prior to processing the tissue for microarray analysis, RNA quality was verified for each sample by an Agilent

2100 Bioanalyzer and only samples with a RIN value greater than 8.0 were used. mRNA was then reverse transcribed, labeled, and hybridized to Affymetrix Mouse Gene ST 1.0 Arrays (~29,000 transcripts) by the Vanderbilt Functional Genomics shared resource core using standard procedures. The Affymetrix Gene Chip Command Console was used for all instrument control and data acquisition, while the Expression Console (Affymetrix) was used for normalization (RMA) and primary data analysis. All microarray data is MIAME compliant and the raw data has been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (GEO accession number: GSE23008), while fold change (fc) values relative to baseline can be found in Tables S1-4 of previously published work (19). Genes that were further characterized and thought to be of interest were categorized into two lists. One list represents genes that did not change over time despite exposure to CF testing, but remained consistently different between genotypes (Table 1). These genes were classified as developmental gene changes that resulted from knocking out AC1 and AC8 from birth. Statistical analysis here pooled across the time points and considered each time point to be an n of 1 per genotype (total of 5 arrays/genotype/brain region). We used Qvalue (20) to assess statistically significant gene changes across the arrays comparing DKO and WT mice, which takes a list of p-values resulting from the simultaneous testing of many hypotheses and estimates their q-values in order to determine the false discovery rate. A q-value of ≤ 0.1 was considered significant. The other list represents genes that changed acutely as a result of learning changes and these genes displayed at least a ± 1.5 fc relative to baseline at one or more time points (Table S5 and S6 (19)). These genes could not be assessed statistically as only one array was run per condition.

Real time RT-PCR. The same individual RNA samples used on the microarrays were also used for the microarray RT-PCR validation experiments. To prepare the cDNA, 500ng of total RNA was reversed transcribed in a 20uL reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time RT-PCR was performed with SYBR Green Master Mix (Sigma Aldrich) in an Applied Biosystems 7900HT Fast Real-time PCR System at Vanderbilt University's DNA Resource Core. Each sample was run in duplicate and dissociation curves were used to assess the specificity of each primer pair. GAPDH was used as the standard control. Primers used for amplification are listed in Table S7 of previously published work (19). For RT-PCR validation of microarray results, a random selection of genes was evaluated in WT and DKO mice across all time points in the amygdala and hippocampus to confirm that microarray results correlated with RT-PCR results across all arrays (Fig S1).

Functional analysis. To assess the most represented functional annotations among the genes that showed a ±1.5 fc relative to baseline, the GO Slimmer program from Gene Ontology (www.geneontology.org) was used. The number of genes falling into each functional annotation was determined to assess the most represented functional annotations per condition. A minimum parameter of 3 was used. Any GO category whose level in the GO hierarchy is below this parameter was not included in the GO analysis in order to eliminate functional categories that are too broad. The category, Biological Process, is defined to be at level 1 in the hierarchy. The level of any other term is the length of the longest path to level 1 in terms of the number of categories on the path.

Gene cluster analysis. The software program Short Time-Series Expression Miner (STEM) (21) was used to cluster and analyze the gene expression data from the microarray experiments. Genes were filtered so that only genes with a ±1.5 fc at one or more time points were assigned to a cluster. The STEM program calculates the significance of a cluster based on the ratio of the number of assigned genes versus the number of expected genes to a profile. The program has the ability to implement a novel method for clustering short time series expression data that can differentiate between real and random patterns as previously described (22).

Transcription factor analysis. We used Whole Genome rVISTA (http://genome.lbl.gov/vista) to identify transcription factor binding sites that are conserved between mice and humans and overrepresented in the 2.5Kbp upstream region of genes that were regulated similarly during the CF paradigm.

Behavioral analysis. Behavioral experiments were conducted 2-5 hr after lights on. Male mice ages 2-4 mo were used for all behavioral experiments. All mice were on a C57Bl/6 inbred background.

Cognitive skills were evaluated using a test of Pavlovian fear conditioning. The CF chamber was a standard grid box (20.3X15.9 X10.0cm; Med Associates) with a small vial of coconut oil, which served as an olfactory cue. During CF training, mice were put in the chamber for 2 min to assess basal (pretraining) freezing levels, followed by a 2 sec 0.7mA foot shock. Subsequently, postshock (posttraining) exploration was monitored for an additional 2 min. Mice were left undisturbed until tissue was extracted for RNA analysis.

Data analysis. Results are expressed as the mean ± SEM. Student's t-test was used to compare pairs of means. In cases with multiple conditions, a two-way ANOVA was used followed by Bonferroni *post hoc* tests when appropriate. A one-way ANOVA was used for single condition analysis followed by Tukey's Multiple Comparison *post hoc* tests when appropriate. Chi-square analysis was used to determine the significance of gene expression changes between WT and DKO mice across time. A p-value of ≤ 0.05 was considered statistically significant. All statistical comparisons were done with Prism 4 software (GraphPad) unless otherwise stated.

RESULTS

DKO mice show consistent long-term, but not short-term, CF memory deficits (5). Impaired gene expression changes within the hippocampus have been correlated with impairments in long-term memory consolidation (23). Therefore, to assess whether learning deficits resulting from a lack of Ca^{2+}-stimulated AC activity are imparted by impaired gene expression, we obtained amygdala and hippocampal tissue samples from WT and DKO mice at different time points after CF learning. Samples were extracted at baseline (-5 min before training), 0 hr, 1 hr, or 48 hr after a 5 min CF training trial, or at 1 wk after a 5 min CF testing trial, and subjected to microarray analysis and validated with RT-PCR (Fig S1). The different time points reflect when the different stages of memory processing are thought to occur (24).

To provide context for gene expression changes found during CF in the DKO mice, we measured basal gene expression differences that were consistent before testing and across all time points after training. As expected, AC8 mRNA was significantly decreased in both the amygdala (-2.76 fc) and hippocampus (-4.06 fc) of DKO mice compared to those of WT mice; however, AC1 only met the FDR threshold in the hippocampus (-2.97 fc), reflecting the low abundance of AC1 in WT amygdala (-1.48 fc, p = 3.6E-04) (Table 1). Decreased expression of a few other genes was observed using microarray in both the amygdala and hippocampus of DKO mice compared to those of WT mice (Table 1; Fig 1A,C). Confirming our microarray data, we found using RT-PCR that Slc11a2 and Lynx1 are significantly decreased in DKO mice compared to expression levels in WT mice (Fig 1B, D). Additional RT-PCR analysis of these genes with single

Table 1. Genes that are modulated during development

Amygdala			
Accession ID	**Gene symbol**	**Mean fold change**	**P-value**
NM_008732	Slc11a2	-1.32	2.8E-08
NM_009623	Adcy8	-2.76	3.8E-07
NM_025931	Rabl4	-1.38	9.9E-07
NM_146014	Ccm2	-1.37	1.2E-05
NM_146776	Olfr821	1.17	2.9E-05
NM_029787	Cyb5r3	-1.64	3.9E-05
Hippocampus			
Accession ID	**Gene symbol**	**Mean fold change**	**P-value**
NM_009622	Adcy1	-2.97	6.2E-08
NM_009623	Adcy8	-4.06	4.8E-07
NM_011838	Lynx1	-1.40	2.1E-06
NM_011124	Ccl21a	-1.64	3.1E-06
NM_023052	Ccl21c	-1.64	3.1E-06
NM_011335	Ccl21b	-1.64	3.1E-06
NM_175408	Tmem139	-1.22	1.9E-05
NM_009342	Dynlt1	1.26	6.0E-05
NM_029787	Cyb5r3	-1.47	7.3E-05
NM_026681	Ccdc88c	-1.18	7.7E-05

The genes, with their respective fold change (from WT level) and p-value, are listed in order of significance. Only genes that met a q-value of ≤ 0.1 were listed.

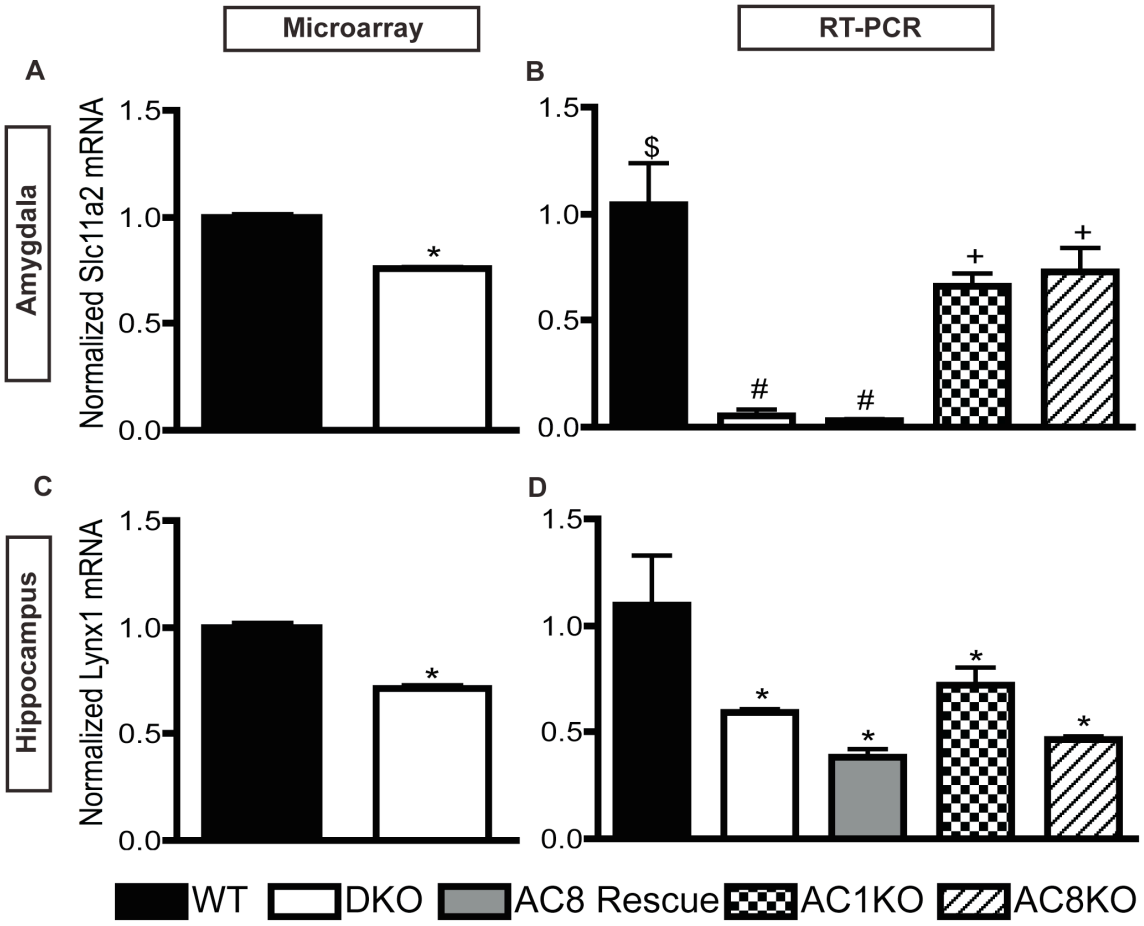

Figure 1. Developmental changes in DKO mice. Two genes identified with microarray analysis and showing the most significant change between WT and DKO mice, **(A)** Slc11a2 ($n = 5$, $*p \leq 0.0001$), with a -1.3 fold change, and **(B)** Lynx1 ($n = 5$, $*p \leq 0.0001$), with -1.4 fold change, were confirmed via RT-PCR. **(C-D)** Both RT-PCR results confirmed a significant decrease in Slc11a2 ($n = 4\text{-}5$, \$ vs # $p \leq 0.001$; \$ vs + $p \leq 0.05$; # vs +, $p \leq 0.001$) and Lynx1 ($n = 4\text{-}5$,$*p \leq 0.05$). RT-PCR results also reveal that these gene changes are not rescued in AC8 rescue mice, and therefore, do not modulate the CF learning changes, but rather, probably contribute to baseline changes. No differences in gene expression between AC1KO and AC8KO mice suggest that these genes are not targeted by one specific Ca^{2+}-stimulated AC.

knockout animals (AC1KO, AC8KO) suggests that these genes are not targeted by one specific Ca^{2+}-stimulated AC as both single knockouts show similar reduction in expression levels.

Microarray analysis of gene expression changes after CF learning

Amygdala: We find that the aggregate pattern of gene expression changes in the amygdala occurring after CF learning is similar between WT and DKO mice, but the relative number of genes differs between the two genotypes. Chi-square analysis reveals that WT mice have significantly more upregulated genes than DKO mice at 48 hr (94 vs 6; $p \leq 0.05$) and 1 wk (145 vs 66; $p \leq 0.05$) (Fig 2B), while DKO mice have more upregulated genes at 1 hr than WT mice (28 vs 6; $p \leq 0.05$). Additionally, WT mice have significantly more downregulated genes than DKO mice at every time point in the amygdala (Fig 2D).

Using the STEM software, we clustered individual genes into groups based on their changes across time and according to whether they showed up or downregulated gene expression (Fig 3; Tables S8 and S9 (19)). Within the amygdala, DKO mice show a predominance of clusters with downregulated expression over time, but 4 out of 9 clusters are similar between WT and DKO mice. Further analysis of these clusters (Table S8 (19)) reveals that there is minimal overlap in the specific genes that fall within the same cluster between WT and DKO mice. Therefore, we conducted a heat map analysis on the most significant WT cluster in the amygdala (Fig 4) to determine how the same set of genes changed between the WT and DKO mice. The WT heat map represents the expression changes of the genes falling into this cluster. The DKO heat map represents

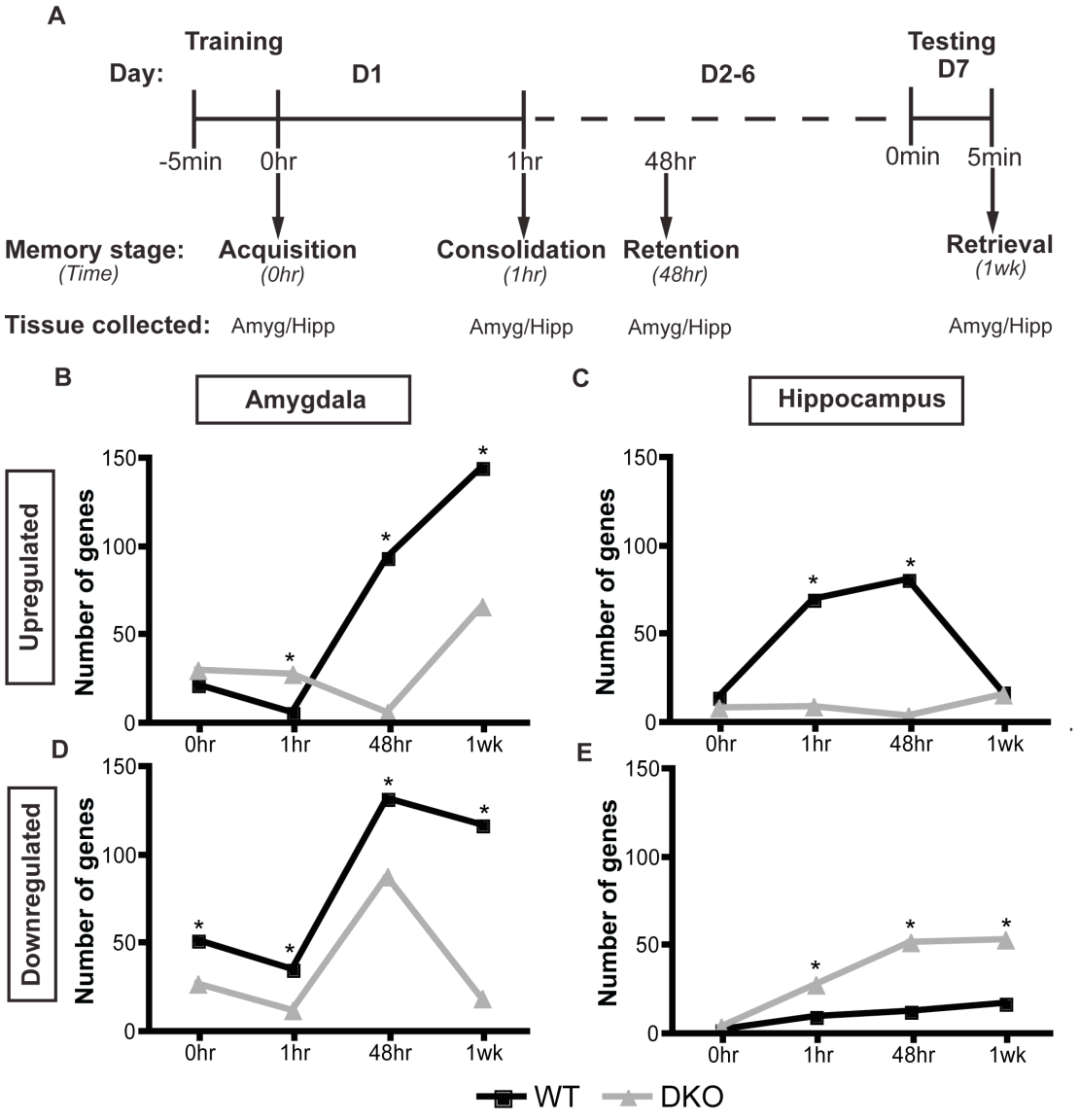

Figure 2. Microarray analysis setup and gene expression changes. (A) Amygdala and hippocampal micropunches were taken at baseline and four time points across CF learning (0 hr, 1 hr, 48 hr, 1 wk) in WT and DKO mice (1 array per genotype/brain region/time point). The number of genes showing a ±1.5 fold change from baseline is graphed. **(B, D)** WT mice show the largest change in gene expression at the 48 hr and 1 wk time points in the amygdala and **(C, E)** at the 1 hr and 48 hr time points in the hippocampus. Overall, DKO mice show a suppression in gene expression changes, except at 1 hr in the amygdala when DKO mice show more gene changes that WT mice (* Chi-square, WT vs DKO $p \leq 0.05$).

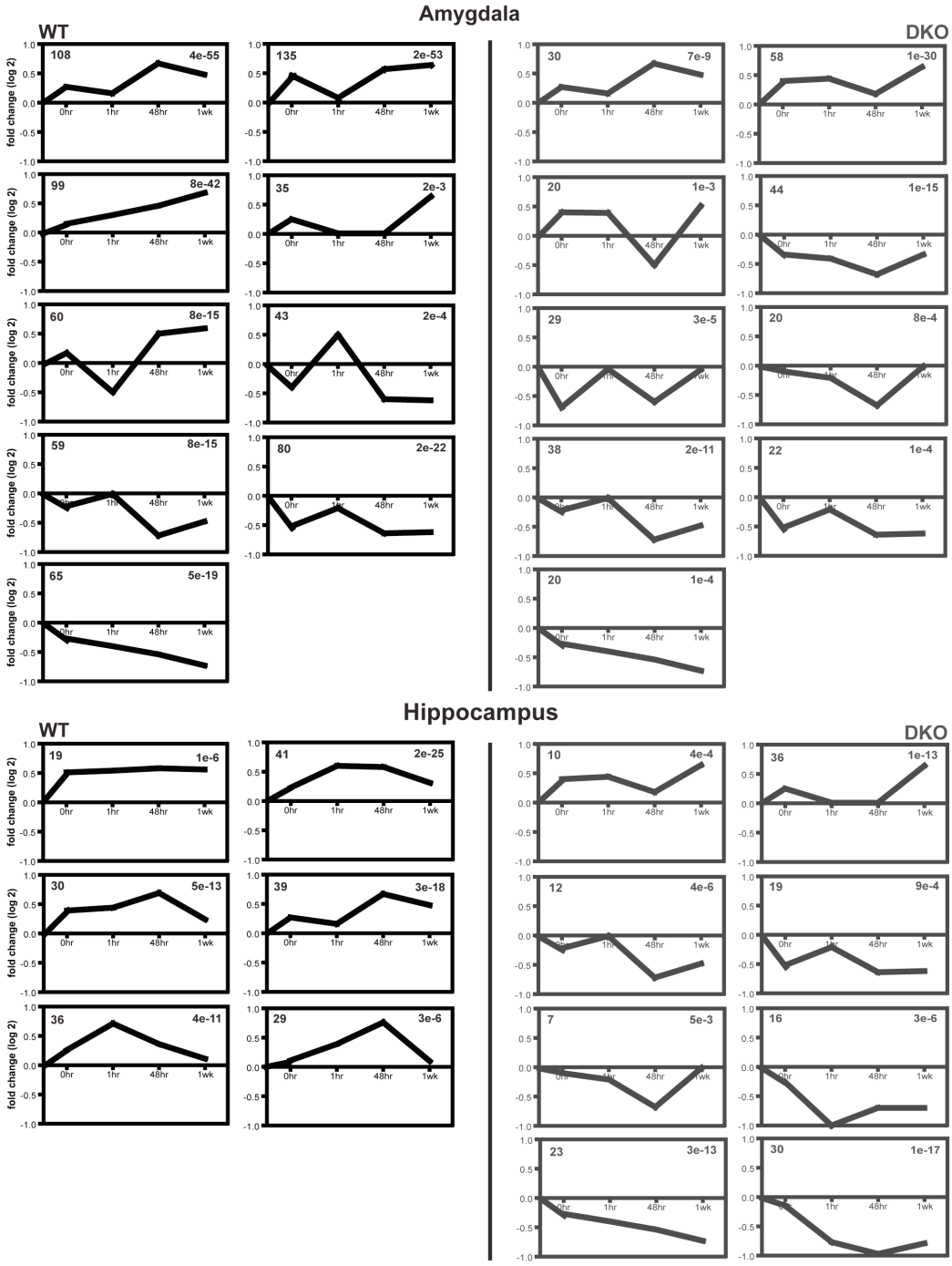

Figure 3. Cluster analysis reveals the most represented expression pattern changes occuring over time after CF learning. Amygdala results reveal a balance between up and downregulated gene changes in WT mice. DKO mice reveal an overall decrease in gene expression within the amygdala over time. Four similar clusters are found between both the WT and DKO mice in the amygdala. WT mice show a consistent upregulation in gene expression over time within the hippocampus; whereas, DKO mice genes tend to be downregulated over time except for two clusters. No two clusters are alike between WT and DKO mice. The number in the upper left corner represents the number of genes in the cluster and the number in the upper right reveals the p-value.

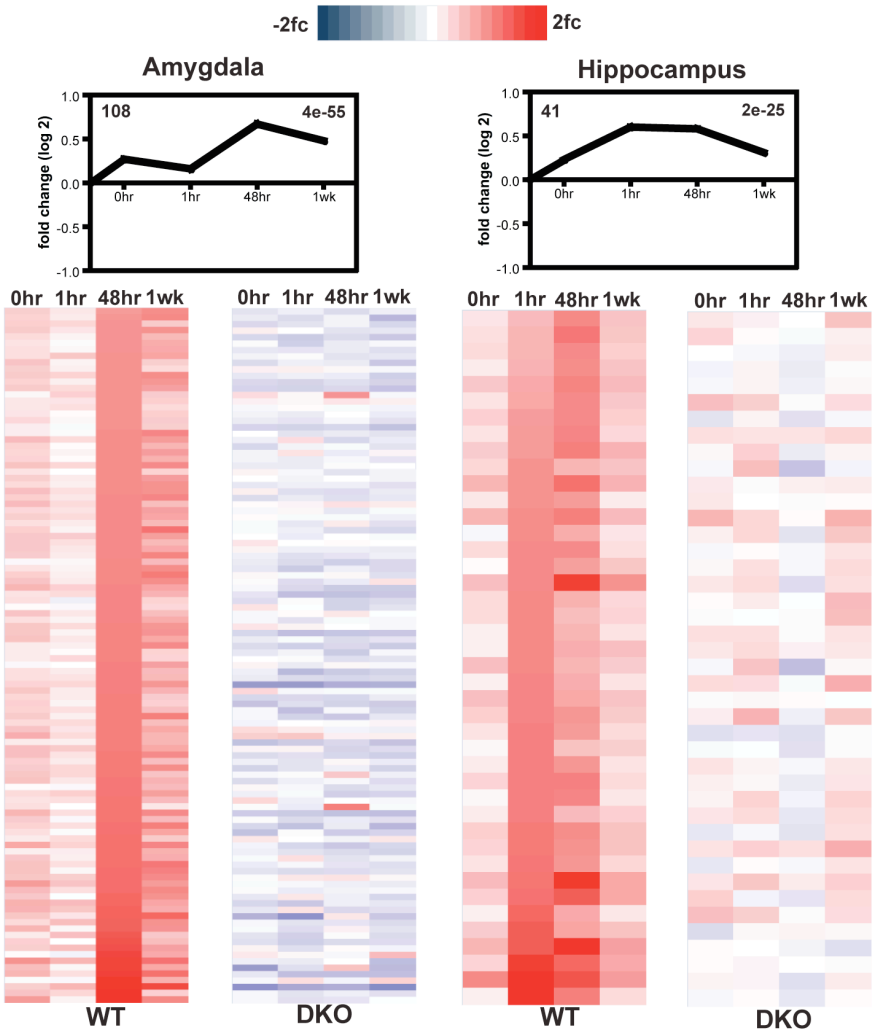

Figure 4. Heat map analysis reveals divergent gene expression changes. A heat map was generated for the most significant cluster in each brain region of WT mice. The gene changes are mapped in the WT mice, and the respective changes in expression of the same genes are mapped in the DKO mice. **(A)** The amygdala results reveal not only a suppression, but opposing regulation in gene expression in DKO mice. **(B)** The hippocampal results reveal an overall suppression in gene changes within DKO mice.

expression levels of those same genes and depicts how they are differentially modulated relative to WT mice. Virtually all of these genes show the opposite regulation within the amygdala between WT and DKO mice. This observation is further supported when comparing the ±1.5 fc gene lists between WT and DKO mice within the amygdala (Table S5 (19)).

We also determined whether the genes that changed after CF learning in DKO mice were functionally related to the genes that change in WT mice (Table 2). We defined the top 5 functional categories represented by genes changed after CF learning in WT mice and compared the number of genes that changed in DKO mice within the same 5 functional categories. Within the top 5 functional categories, DKO mice show considerably fewer genes relative to WT mice across all time points, except for genes upregulated at 1 hr where DKO mice show a greater number of gene changes, which is consistent with the gene expression pattern changes seen in Figure 2. Functional analysis reveals that the decrease in transcriptional changes may be largely due to the lack of changes in genes that modulate cellular transcription (GO: 0006350). Moreover, DKO mice have deficits in cell signaling as they show a decrease in number of genes that fall into the cell communication (GO: 0007154) and signal transduction (GO: 0007165) functional categories.

Hippocampus: WT mice have significantly more upregulated genes than DKO mice in the hippocampus at 1 hr (70 vs 9; $p \leq 0.05$) and 48 hr (81 vs 4; $p \leq 0.05$)(Fig 2C). However, the converse is true for downregulated genes, of which DKO mice have significantly more than WT mice at 1 hr (28 vs 10; $p \leq 0.05$), 48 hr (52 vs 13; $p \leq 0.05$), and 1 wk (53 vs 17; $p \leq 0.05$) (Fig 2E).

Table 2. Top 5 functions represented by each respective gene list

	Number of genes (compared to baseline)							
BIOLOGICAL PROCESS	**0 hr**		**1 hr**		**48 hr**		**1 wk**	
Amygdala, Upregulated	**WT**	**DKO**	**WT**	**DKO**	**WT**	**DKO**	**WT**	**DKO**
Cellular nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (GO:0006139)	5	3	1	11	21	0	34	13
Cell communication (GO:0007154)	2	7	2	6	17	0	34	14
Cell differentiation (GO:0030154)	2	3	2	8	21	0	30	7
Cellular transcription (GO:0006350)	4	2	1	8	19	0	26	7
Signal transduction (GO:0007165)	1	7	1	4	15	0	29	14
Amygdala, Downregulated								
Cell communication (GO:0007154)	11	4	5	2	30	18	25	7
Cellular nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (GO:0006139)	4	1	2	1	31	16	28	4
Signal transduction (GO:0007165)	9	4	5	2	27	13	23	7
Protein metabolic process (GO:0019538)	6	4	1	3	26	16	22	4
Transport (GO:0006810)	10	6	7	2	21	16	17	1
Hippocampus, Upregulated								
Cellular nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (GO:0006139)	6	0	20	2	21	0	5	4
Cellular transcription (GO:0006350)	5	0	10	2	15	0	5	0
Cell communication (GO:0007154)	3	1	12	1	15	1	4	0
Protein metabolic process (GO:0019538)	2	0	15	2	10	1	2	2
Signal transduction (GO:0007165)	2	1	8	1	13	1	2	0
Hippocampus, Downregulated								
Cell communication (GO:0007154)	0	1	3	5	4	12	5	8
Signal transduction (GO:0007165)	0	1	2	5	4	12	5	8
Transport (GO:0006810)	0	0	2	8	3	11	4	9
Cell differentiation (GO:0030154)	0	0	2	3	3	6	2	9

Cluster analysis of individual gene changes in the hippocampus reveals that there are no similar cluster patterns between the WT and DKO mice (Fig 3; Tables S8 and S9 (19)). Only clusters upregulated over time are statistically significant in WT mice with the 1 hr and 48 hr time points showing the largest increases in gene number. DKO mice, however, show a predominance of clusters with downregulated expression over time in the hippocampus. Heat map assessment of the most significant WT cluster in the hippocampus reveals that gene expression changes are attenuated in DKO mice, but the patterns appear in similar directions (Fig 4). The functional categories of the top clusters in WT hippocampus are similar to those of the amygdala with DKO mice showing a decrease in the number of genes falling within the cellular transcription (GO: 0006350), cell communication (GO: 0007154), and signal transduction (GO: 0007165) categories.

Normal CF learning displays over-representation of specific transcription factor binding sites in regulated genes

To further elucidate possible mechanisms for the network of gene expression changes associated with CF learning, we assessed the transcription-factor binding sites that were overrepresented in genes either upregulated or downregulated within the amygdala and hippocampus of WT and DKO mice at 48 hr (Top 10 listed in Table 3; full list in Table S10 (19)). The DKO mice show no overrepresented transcription factor binding sites in the amydala or hippocampus of upregulated genes at 48 hr; however, this may largely be due to only a small number of genes being upregulated at this time point in DKO mice. Downregulated genes, in contrast, appear to be regulated by a shared group of transcription factors. Nearly all the transcription factor binding sites (9 out of

10) that are overrepresented in the downregulated genes within the hippocampus of DKO mice are found in genes upregulated in the hippocampus of WT mice. This observation is consistent with the shift from predominantly upregulated to downregulated gene expression seen in the DKO mice.

Table 3. Top 10 TF binding sites overrepresented in genes regulated at 48 hr

WT				**DKO**			
Hippocampus		**Amygdala**		**Hippocampus**		**Amygdala**	
TF binding site	**P-value**	**TF binding site**	**P-value**	**TF binding site**	**P-value**	**TF binding site**	**P-value**
Upregulated							
Pou6f1	2.3E-11	Areb6	7.4E-04	N/A		N/A	
Pou1f1	2.5E-11	Lfa1	1.3E-03				
Tef	4.4E-11	E12	3.5E-03				
Crebatf	4.5E-09	Gata2	3.8E-03				
Gata3	1.9E-08	Hoxa7	5.1E-03				
Dr3	1.5E-07	Pbx1	5.4E-03				
Amef2	1.7E-07	Foxp3	5.8E-03				
Hp1sitefactor	6.0E-07						
Hfh3	7.3E-07						
Atf	1.0E-06						
Downregulated							
Pads	1.6E-04	Lhx3	8.9E-10	**TfIIa***	**1.3E-07**	E47	3.6E-05
Meis1	2.5E-04	Cart1	2.4E-08	**Foxo1***	**9.4E-07**	**Areb6**	**8.2E-04**
TfIIa	3.8E-04	Pou6f1	3.4E-08	**Gata3**	**1.9E-05**	Cmyb	2.3E-03
Hp1sitefactor	1.3E-03	Sox5	9.6E-08	**Foxo4***	**3.9E-05**	Crebp1cjun	5.0E-03
Gata1	2.7E-03	Sox9	5.2E-07	Lef1tcf1	4.8E-05	Nmyc	5.1E-03
Areb6	3.1E-03	Hif1	6.7E-07	**Dr3**	**9.3E-05**		
		Nkx61	1.0E-06	**Hp1sitefactor**	**1.6E-04**		
		Hp1sitefactor	2.2E-06	**Foxp3***	**2.8E-04**		
		Vbp	2.8E-06	**Hfh4***	**4.3E-04**		
		Gata3	2.9E-06	**Lhx3***	**7.2E-04**		

The 2.5kbp region upstream region of genes modulated during CF learning was assessed for common transcription factor (TF) binding sites. The significance value takes into consideration the total number of sites in the genes analyzed versus the total number of sites in the whole genome. **Bold** genes represent TF binding sites in DKO mice that are significantly upregulated within each respective WT brain region,while * indicates TF binding sites that are significantly upregulated in WT mice, but do not make the top ten list.

DISCUSSION

Here, we report that disruption of both Ca^{2+}-stimulated ACs (AC1 and AC8) causes an overall decrease in long-term transcriptional changes after CF learning. While changes between WT and DKO mice are evident at all time points, the most robust disparity occurs at the 1 hr and 48 hr time points, periods when memory consolidation and retention are thought to occur.

Initially, we assessed changes in baseline gene expression to eliminate the possibility that baseline gene expression differences are contributing to the CF deficits in DKO mice. The most notable changes are a decrease in Slc11a2 in the amygdala and Lynx1 in the hippocampus of DKO mice. Both of these proteins may play a role in learning and memory. Slc11a2, a divalent metal ion transporter, causes memory impairments on the Morris water task when disrupted within the forebrain (25), and Lynx1, which enhances nicotinic acetylcholine receptor function, causes memory enhancements on CF when globally disrupted (26). However, our data show that expression of these genes in AC8 rescue mice is not recovered to WT levels, and since AC8 rescue mice have intact memory, this suggests that these proteins do not cause the CF learning deficits seen in DKO mice.

Past literature supports the theory that Ca^{2+}-stimulated AC activity plays a pertinent role in learning and memory. Although disrupting just one of the two Ca^{2+}-stimulated ACs can cause memory impairments (6), data show that AC single knockout mice show intact CF behavior while DKO mice show alterations in CF behavior (5). The observation that Ca^{2+}-stimulated AC activity is important for learning and memory is supported by the microarray data. Transcriptional changes within hours after exposure to

a learning paradigm is required for the consolidation of long-term memory (14-16). Moreover, recent evidence shows that an age-related decline in memory is correlated with decreased hippocampal transcriptional changes (23). Together, these data suggest that the lack of long-term transcriptional changes displayed by DKO mice throughout the CF learning process contribute to the learning deficits seen at 1 wk post CF training. Furthermore, the residual transcriptional changes that do occur in DKO mice are mainly suppression of expression. This is evident in Figure 2 as upregulated gene expression is overall dampened over time in both the amygdala and hippocampus of DKO mice.

DKO mice display an impairment in transcriptional changes at 1 hr, a period when memory consolidation is occurring; therefore, the overall suppression in transcriptional changes may be contributing to the inability of these mice to form a strong enough memory in order to retain it for longer than 24 hr. DKO mice show an increased number of genes changing relative to WT mice at 1 hr in the amygdala. This data suggests that the amygdala in DKO mice may be sufficiently activated to form a stress response to the aversive shock administered during CF training. Therefore, the activation of the amygdala may be sufficient to retain the CF memory for short periods, such as seen at 24 hr (5), but the overall lack of hippocampal activation may lead to impairments in the overall strength of the memory formed.

DKO mice also display an impairment at 48 hr, a period when retention of the memory is occurring. The present data suggest that maintenance of positive gene regulation is necessary as DKO mice show an overall lack of upregulated gene changes, but downregulated gene changes are maintained in the hippocampus. This is further seen by the overall lack of common transcription factor binding sites found in the genes

upregulated within the amygdala or hippocampus of DKO mice at 48 hr. Moreover, many of the transcription factors that modulate positive gene expression are found overrepresented in the genes that modulate negative gene expression within the hippocampus of DKO mice. Finally, the gene cluster analysis in DKO mice reveals that gene expression is decreased in most of the significant clusters at 48 hr, and functionally, there is at most only 1 gene found within the top 5 functions represented by genes modulated in WT mice. The lack of transcriptional changes at the period when memory is supposed to be maintained supports the behavioral observations that Ca^{2+}-stimulated AC activity is needed for memory retention.

Finally, the present functional analysis data suggests that the lack of transcriptional changes may be contributing to deficits in communication occurring at the level of the synapse. WT mice show a large number of transcriptional changes that functionally contribute to cell communication and signal transduction; whereas, DKO mice show a minimal number of transcriptional changes related to these functions. While this is merely correlative, the presence of both Ca^{2+}-stimulated ACs at the level of the synapse [11] supports this finding.

Overall, we demonstrate that Ca^{2+}-stimulated AC activity is necessary for the expression and pattern of long-term transcriptional changes that are associated with CF memory processing. The network of changes that we define should serve as a valuable resource for studies of learning and memory in other genetically altered systems allowing fundamental common mechanisms to emerge.

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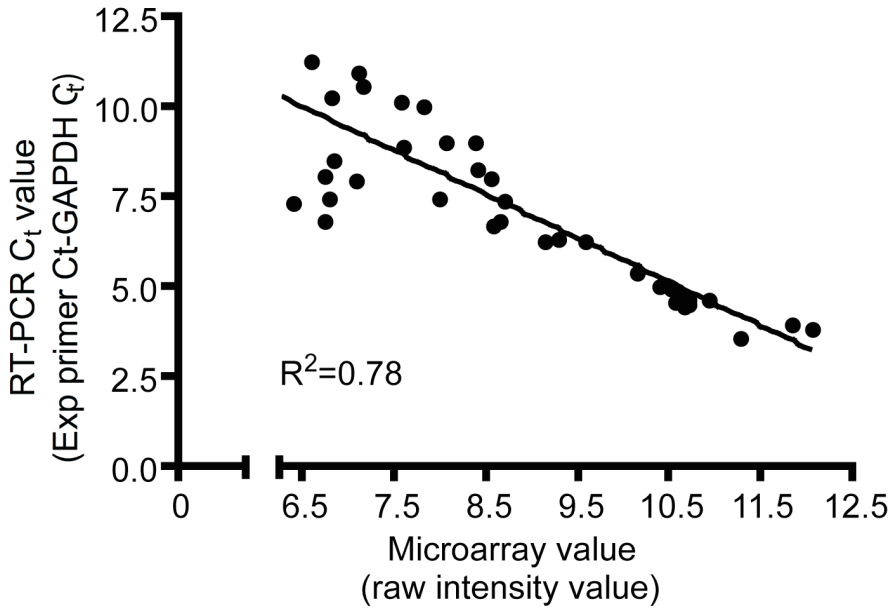
Figure S1.



Supplementary Figure 1. RT-PCR validation of microarray results. The graph shows a strong correlation of RT-PCR values with microarray values.

CHAPTER 4

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

INTRODUCTION

The Ca^{2+}-stimulated adenylyl cyclase (AC) pathway couples neuronal activity and intracellular Ca^{2+} increases to the production of cAMP. Through this activity-dependent increase in cAMP, the 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 (1), conditioned fear (CF) (1, 2), and novel object recognition (2, 3). Moreover, Schaeffer collateral-CA1 LTP is impaired in DKO mice (1, 4), while mossy fiber LTP is impaired in AC1 (5) or AC8 (6) single knockout mice. AC1 and AC8 are both localized to the synapse as evident by a synaptosome fractions study (7) and colocalization of AC8 with synapsin and synatophysin (6), 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-dependent manner (8). PKA is activated by Ca^{2+}-stimulated AC-mediated binding of cAMP (9), and therefore, initial activation of 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 (10). For example, environmental enrichment increases the expression of synaptic proteins, such as synaptophysin and postsynaptic density-95 (11, 12) as well as overall synaptic strength as evident by increases in LTP (8, 13-15).

Neurogenesis, the other major process shown to mediate experience-dependent changes in memory, is increased after environmental enrichment (16-18).

Consequently, we first evaluate Ca^{2+}-stimulated AC activity's roles 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 whether an absence of Ca^{2+}-stimulated AC activity affects experience-dependent changes in CF memory. Our data demonstrates that 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 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 12 h/12 h light/dark cycle with *ad libitum* access to rodent chow and water. DKO (19, 20) and AC8 Rescue mice (2) were generated as previously described and were backcrossed >10 generations to C57Bl/6 strain. Briefly, DKO mice have both AC1 and AC8 knocked out, while AC8 Rescue mice have AC8 replaced in the forebrain of mice on a DKO background. Through use of a tetracycline-inducible system, AC8 can be turned on or off by administration of doxycycline (200 mg doxycycline/1 kg; Research Diets). AC8 is not turned on until mice are weaned at 21 d. C57Bl/6 were used as control mice (WT).

Electrophysiology. LTP in the Schaffer collateral afferent fibers of the hippocampal CA1 region was induced as previously described (1) with minor modifications to the slice preparation as detailed below. Naïve mice were transported from the animal colony to the laboratory and placed in sound-attenuated cubicles for 1 h prior to slicing. They were then decapitated under isoflurane anesthesia. The brains were quickly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$ saturated with 95% O_2/5% CO_2. Transverse hippocampal slices (300 mm thick) were prepared using a Tissue Slicer (Leica). After dissection, slices were transferred to an interface recording chamber where they were perfused with heated

(~29°C), oxygenated (95% O_2/5% CO_2) ACSF at a rate of about 2 mL/min. Field potential recordings were measured as previously described (1).

Lentivirus production. The full length mouse cDNA AC8 sequence was cloned into a lentivirus by Applied Biological Materials, Inc. (ABM). The cloning of mAC8 was performed in three steps. First, the first 944 bp mAC8 was PCR amplified from the plasmid, pUHC-13-3- AC8 (pUHC-13-3) (21); AC8 cDNA, a gift from Dr. Richard Premont at Duke University), digested with XbaI and EcoR, and cloned into pLenti-III-EF1α (ABM) linearized by NheI and EcoRI. Next, the last 380 bp of mAC8 was PCR amplified from pUHC-13-3-AC8, digested with BamHI and XbaI, and cloned to the same pLenti-III-EF1α using BamHI and XbaI. Lastly, the middle portion of mAC8 was cut out of pUHC-13-3-AC8 with NheI and BamHI, and cloned to the pLenti-III-EF1α with the same enzymes. The newly generated lentivirus, LV-Adcy8, was sequenced to ensure proper insertion. Viral packaging and *in vitro* titering were completed at The Hope Center Viral Vector Core (Washington University in St. Louis).

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 (22, 23). 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% EDTA-free trypsin. Cells were plated at 500 000 cells/60mm 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). Heat inactivated FBS

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 uL of LV-Adcy8 (titer 3.1 x 10^8 IFU/ml) per 3 ml media/60mm dish and fixed at DIV 9 or 10 as described above.

Immunohistochemistry. Neurons were fixed with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS) for 20 min. Cells were permeabilized with 0.2% Triton X-100 for 5 min followed by a 1 h block with 20% goat serum in PBS. Antibodies were diluted in 5% normal goat serum (NGS) in PBS. Primary antibodies used were SV2 (1:500 for 1 h, Developmental Studies Hybridoma Bank) and β3-tubulin (1:500 overnight at 4°C, Abcam). Secondary antibodies used were Alexa Fluor 555 (1:1000 for 1 h, Invitrogen) and Alexa Fluor 488 (1:500 for 1 h, Invitrogen). All washes were done with PBS for at least 5 min X3. Coverslips were mounted with Vectashield mounting medium with DAPI (Vector Labs).

SV2 clusters were quantified from four different WT and DKO cultures. From each culture, at least 45 dendrites from approximately 15-20 neurons were analyzed. For the DKO +LV-Adcy8 cultures, the same analysis occurred, except plates from two different cultures were analyzed and results verified with another round of plates from the same cultures. Images were taken on a Zeiss Axio Imager M2 microscope with a 63X oil objective. Image J was used to quantify SV2 clusters. Quantification was done by an experimenter blinded to genotype.

Brains used for KI-67 staining were perfused with 4% paraformaldehyde, cryoprotected with 20% sucrose, frozen, and cut at 35 uM. Slices were quenched of endogenous peroxidases with 0.3% H_2O_2/0.55% Triton X-100 for 1 h, washed with 1X PBS, and blocked in 3% NGS in PBS for 1 h. Sections were then incubated with a rabbit monoclonal KI-67 antibody (1:300, Vector Labs, VP-RM04) in 1% NGS overnight at 4°C. Signal was detected with a biotinylated goat anti-rabbit antibody (1:400, Vector Labs, BA-1000) and visualized by incubation with diaminobenzidine tetrahydrochlorid (DAB) for 5 min. Slices were counterstained with methyl green and positive cells were counted per area within the dentate gyrus. Quantification was done by an experimenter blinded to genotype. AC8 staining was conducted as previously described (7).

Western blot analysis. Tissue extraction and protein analysis was conducted as previously described (2). For phospho-synapsin, synapsin, and SV2 detection, 20 μg of whole cell extract from the hippocampus was isolated and probed with an anti-rabbit phospho-synapsin I/II (1:1000, Cell Signaling, 2311), synapsin (1:1000, Cell Signaling, 2312), or SV2A (1:1000, Abcam, ab32942) antibody overnight at 4°C. Signal was detected using an anti-rabbit HRP-conjugated secondary antibody (GE Healthcare, NA934) and visualized using chemiluminescence (SuperSignal West Pico or Dura, Pierce Biotechnology). Image J was used to quantify protein concentrations. Equal protein loading conditions were verified by immunodetection for mouse anti-actin protein.

Environmental enrichment. Male mice ages 2-5 mo 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 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, 2) *carefresh*-CareFRESH® Natural bedding. Mice were born and kept in their reared environment until 2 wks prior to CF testing at which point mice were subjected to one of 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 4 groups for a total of 8 groups all together: Wt-minimal, WT-enriched, DKO-minimal, DKO-enriched. . Refer to Figure 6A for a schematic.

Conditioned Fear. Contextual CF training began 2 wks after exposure to the experimental environment. CF training and analysis occurred as previously described (2). Mice were tested 1 wk after training.

Data analysis. Results are expressed as the mean ± SEM. Student's t-test was used to compare pairs of means. In cases with multiple conditions, a two-way ANOVA was used followed by Bonferroni *post hoc* tests when appropriate. A one-way ANOVA was used for single condition analysis followed by Tukey's Multiple Comparison *post hoc* tests when appropriate. A p-value of ≤ 0.05 was considered statistically significant. All statistical comparisons were done with Prism 4 software (GraphPad).

RESULTS

AC8 activity modulates the synaptic vesicle protein SV2 but not neurogenesis

The expression of synaptic vesicle protein 2, SV2 is modulated by changes in synaptic activity (24). To examine if Ca^{2+}-stimulated AC activity modulates the expression of SV2, 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 number of SV2 clusters in DKO, relative to WT, neurons (Figure 1A and 1B, t-test, $p = 0.01$). Additionally, the average size of the SV2 clusters was significantly decreased in DKO, relative to WT, neurons (Figure 1A and 1C, t-test, $p = 0.01$).

To assess whether 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 mice and looked at AC8 distribution between 1 and 2 wk after injection (Figure 2B and 2C). 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 protein levels are similar in WT and DKO with LV-Adcy8 as shown in Figure 1A and quantified in Figure 1B (DKO vs DKO +LV-Adcy8, $p < 0.05$) and Figure 1C (DKO vs DKO +LV-Adcy8, $p < 0.001$).

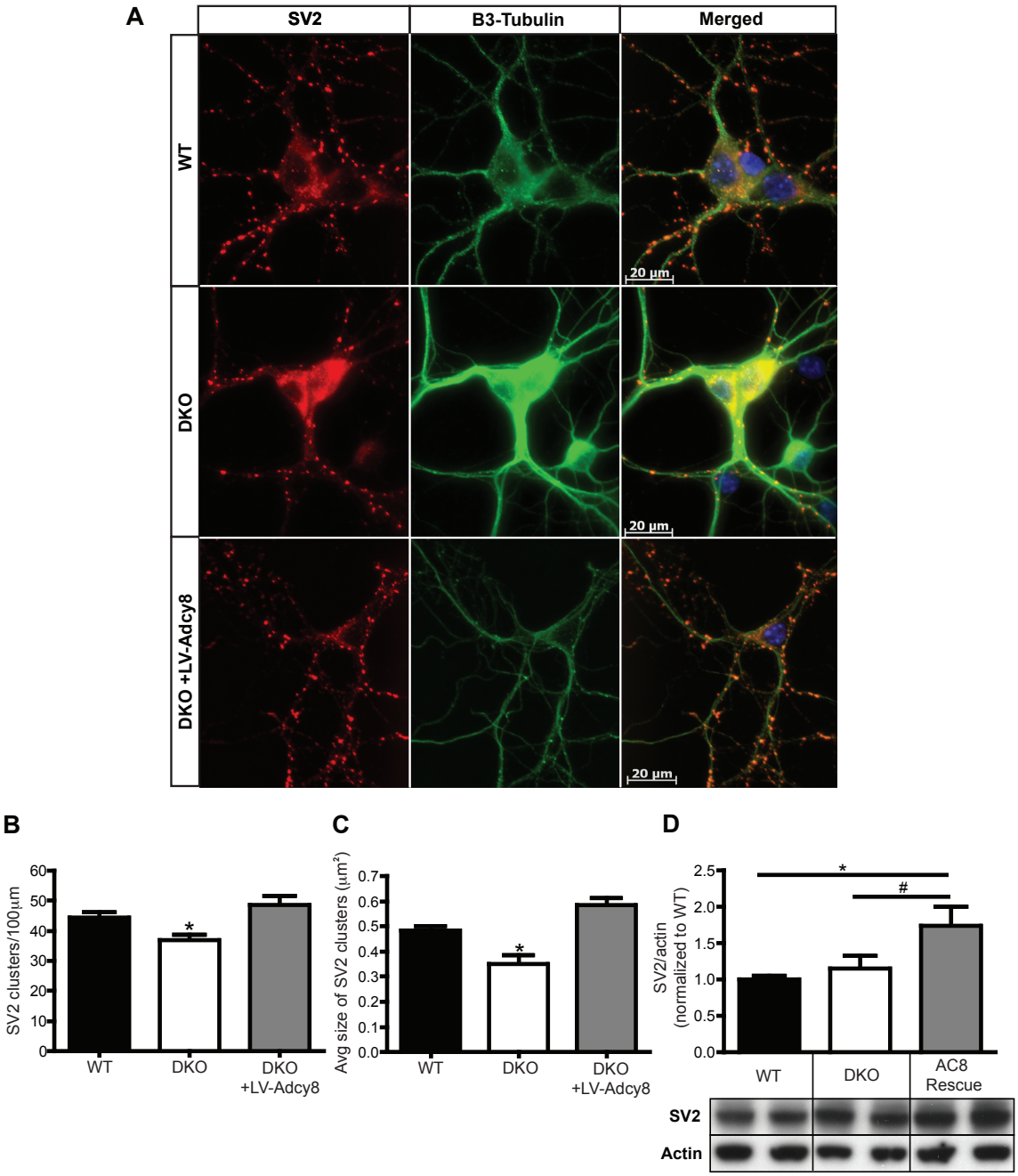


Figure 1. SV2 levels are reduced in hippocampal cultures, but not adult, hippocampal whole cell preps. (A) Representative images of SV2 distribution in DIV9-10 hippocampal neurons. SV2 is labeled with Alexa Fluor 555. β3-tubulin, a neuronal marker, is labeled with Alexa Fluor 488. Dapi is used to visualize nuclei. SV2 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. **(D)** SV2 levels in DKO mice are restored to WT levels in adult, hippocampal micropunches and AC8 Rescue mice show a trend towards an increase (n = 4/group). * WT vs AC8 Rescue $p < 0.05$, # DKO vs AC8 Rescue $p = 0.1$

SV2 protein levels were then measured using hippocampal micropunches (Figure 1D). A one-way ANOVA showed a main effect of genotype ($p = 0.05$). AC8 Rescue mice showed a significant increase in SV2 protein levels compared to WT mice (t-test, $p < 0.05$), while a trend persists for an increase compared to 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 Ca^{2+}-stimulated AC activity.

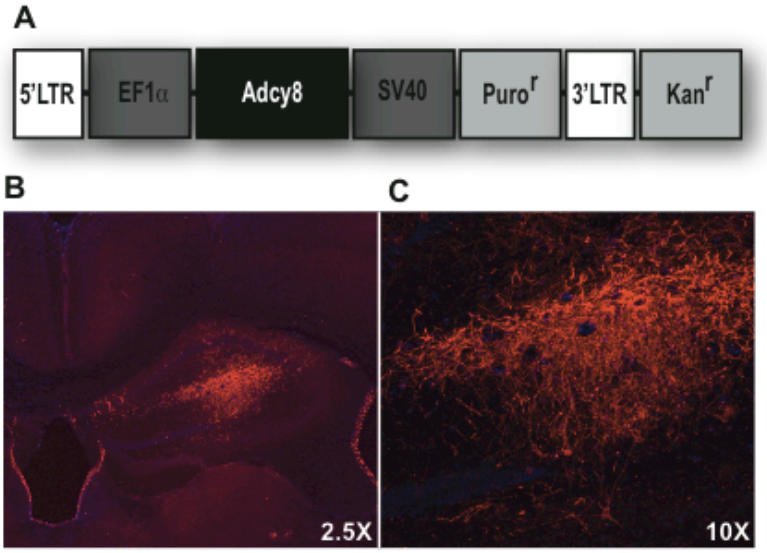


Figure 2. AC8 lentivirus expression. **(A)** A schematic of the major components of the LV-Adcy8 plasmid. A hippocampal image of LV-Adcy8 infection at **(B)** 2.5X and **(C)** 10X. LTR = long terminal repeat, EF1α = elongation factor, AC8= adenylyl cyclase 8, SV40 = Simian virus 40, $Puro^r$ = puromycin resistant, Kan^r = kanamycin resistant

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 (25-28). Because of the localization of Ca^{2+}-stimulated AC activity to the dentate gyrus (29) and the CF memory deficits in DKO mice (1, 2), we hypothesized that neurogenesis would be impaired in these mice. DKO mice displayed a small, but significant, decrease in neurogenesis compared to WT mice as measured by KI-67 staining ($p < 0.01$; Figure 3A and 3B). This decrease, however, was not rescued in AC8 Rescue mice (AC8 Rescue vs DKO $p > 0.05$), which have intact CF memory (2), suggesting that reductions in neurogenesis do not underlie the memory impairments seen in DKO mice.

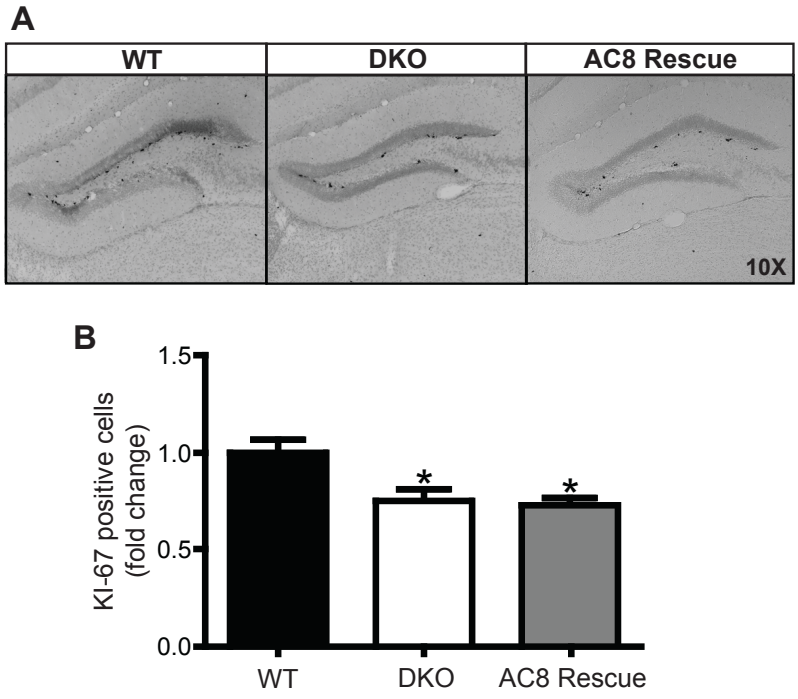


Figure 3. Neurogenesis is impaired in DKO mice, but not restored in AC8 Rescue mice. (A) Representative 10X images of KI-67 staining in the dentate gyrus. **(B)** KI-67 staining is decreased in DKO mice and not rescued by replacing forebrain AC8 in AC8 Rescue mice. * $p < 0.05$; WT n =5, 10 bilateral sections; DKO n = 6, 12 bilateral sections; AC8 Rescue n = 4, 8 bilateral sections

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 levels, 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 (1). These results imply that acutely increasing intracellular levels of cAMP through non 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 (1). We found that activation of a single Ca^{2+}-stimulated AC in the adult forebrain is sufficient to rescue CA1 LTP as measured in the AC8 Rescue mice (Figure 4A and 4B). Our results showed that DKO mice fail to show strong potentiation after HFS as measured by the % field EPSP 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 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%, $p > 0.05$).

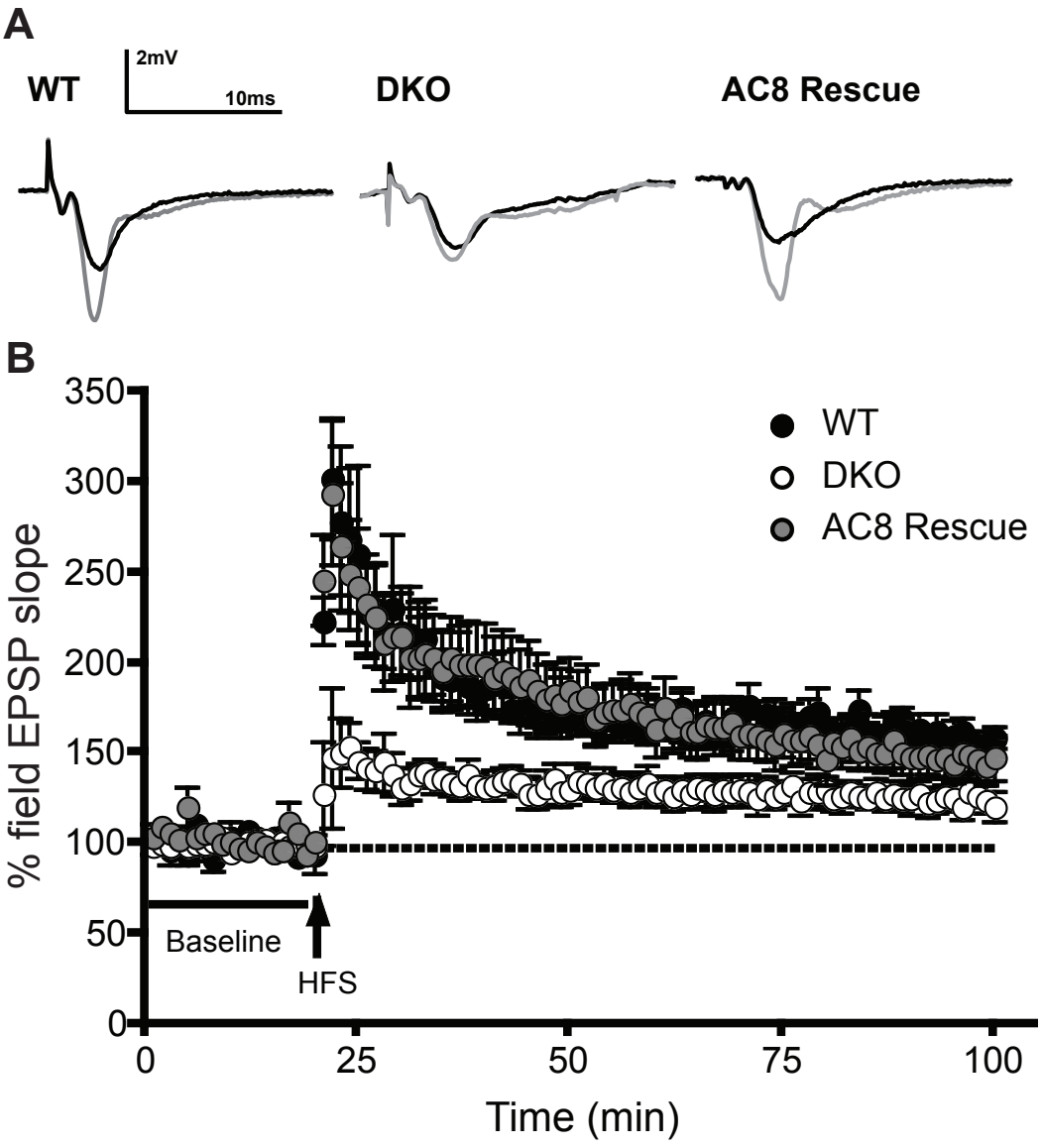


Figure 4. Forebrain AC8 is sufficient for intact CA1 LTP. (A) Averaged representative traces of pre-tetanus (black) and 80 min post-tetanus (grey). **(B)** LTP is impaired in DKO mice (open circles, n = 7) relative to WT mice (closed circles, n = 7), but replacing AC8 within the forebrain, AC8 Rescue mice (grey circles, n= 7), is sufficient to restore initial LTP deficits. Scale bar 2 mV, 10 ms

AC8 activity modulates synapsin I phosphorylation after conditioned fear 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 (30, 31). Previous data have shown an increase in hippocampal phosphorylation of synapsin I after CF (32). Therefore, we investigated whether the impairments in fear memory (1, 2) and LTP (Figure 4) (1) 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 (32), 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 (Figure 5A and 5C), 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 after CF learning ($p < 0.05$; Figure 5E). However, CF-induced increases in p-synapsin I 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 are also regulated by mechanisms independent of AC8, probably AC1. p-Synapsin II was increased after CF training in all genotypes (WT $p < 0.01$, DKO $p = 0.01$, AC8 Rescue $p = 0.01$; Figure 5B, 5D, and 5F), and the magnitude of change was similar between genotypes, suggesting that AC1 and AC8 do not regulate CF-induced increases in p-synapsin II.

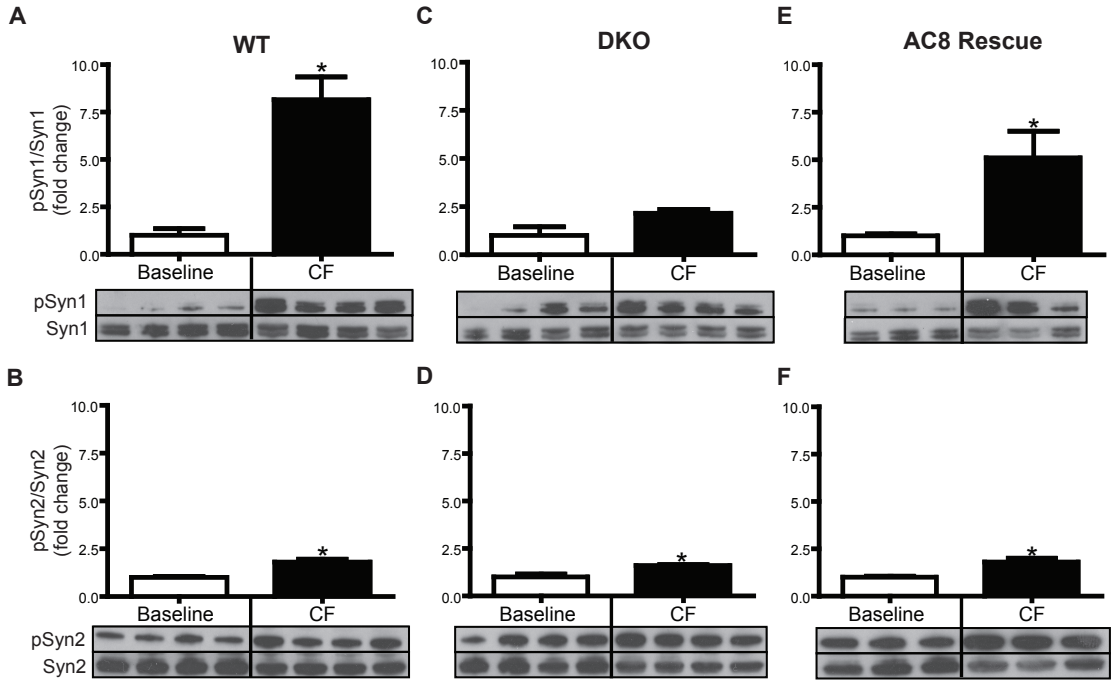


Figure 5. DKO mice show a reduction in p-synapsin 1 1 h after CF training. (A, C, E) p-Synapsin I levels measured at baseline and 1 h after CF training. WT and AC8 Rescue mice show an 8-fold and 5-fold increase after CF training, respectively. DKO mice fail to show an increase. **(B, D, F)** p-Synapsin II 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

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 (8, 33, 34) and synaptic plasticity (10). Moreover, gene-environment interactions have been widely implicated in influencing memory in a variety of paradigms (10, 35, 36). Therefore, we investigated the gene-environment influence on fear memory in DKO mice.

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.

For Figure 6D and 6E, 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, while DKO mice show a lack of this behavioral plasticity, regardless of environmental exposure.

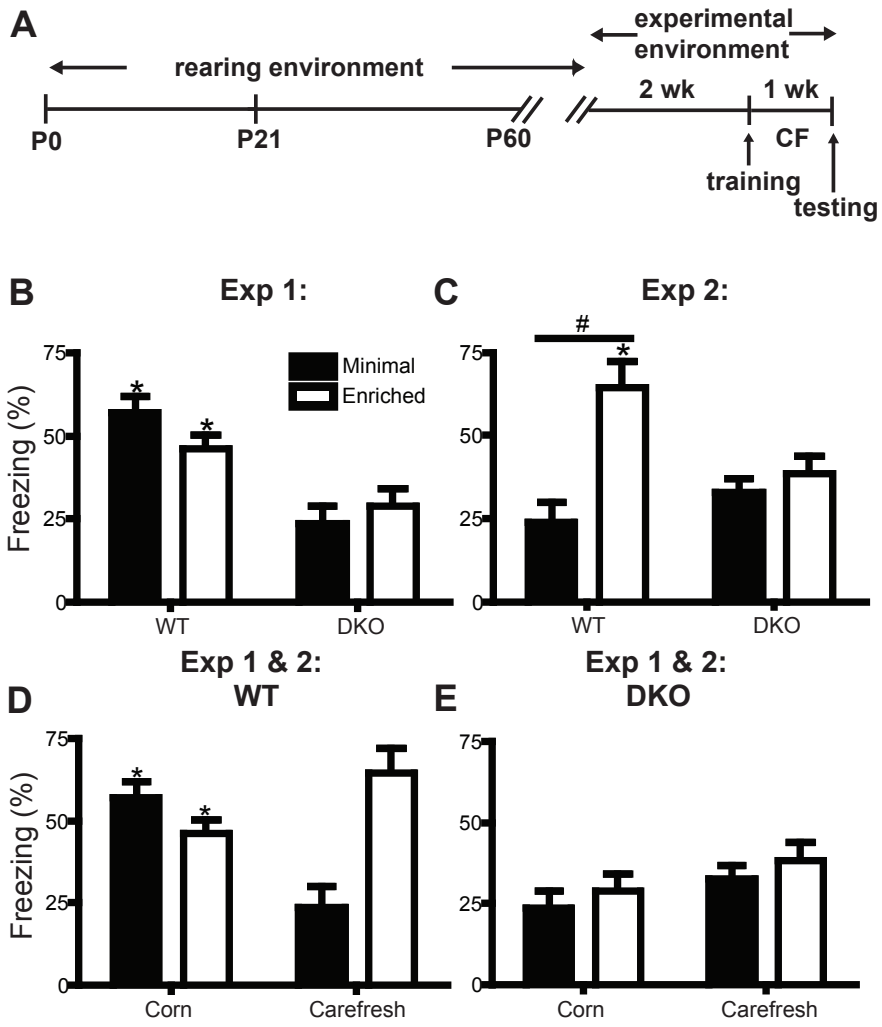


Figure 6. Experience-dependent fear memory is unaltered in DKO mice. **(A)** Mice reared in two different housing conditions were experimentally exposed to a minimal or enriched environment for 2 wks prior to CF training as shown in the schematic. **(B)** Experiment 1: WT mice (reared in corn bedding) show enhanced freezing compared to 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 to 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

DISCUSSION

Overall, the present study examines how Ca^{2+}-stimulated AC activity affects markers of synaptic activity as measured at the molecular, physiological, and behavioral levels. We not only demonstrate that Ca^{2+}-stimulated ACs modulate synaptic plasticity and neurogenesis, but we also highlight a novel gene-environment interaction as an absence of the Ca^{2+}-stimulated ACs leads to an impairment in experience-dependent fear memory. The first evidence we provide for Ca^{2+}-stimulated AC activity's influence on synaptic activity was measured by the abundance of the synaptic marker SV2. Due to the regional localization of the Ca^{2+}-stimulated ACs to the synapses (7), it is not surprising that Ca^{2+}-stimulated AC activity can directly affect SV2 levels. It should be noted that the DKO hippocampal cell culture SV2 levels are significantly reduced, while DKO adult hippocampal SV2 levels are not reduced. This may be a result of different sample preparations or techniques used to quantify protein levels, or it could suggest the ability of adult DKO mice to compensate for the loss of Ca^{2+}-stimulated AC activity through other mechanisms. Regardless, adult DKO mice continue to show deficits in learning, such as induction of LTP or contextual recall of an aversive stimulus, where a larger recruitment of synaptic activity is needed. DKO mice show deficits in potentiation after HFS in accord with previous studies (1, 4), and rescuing AC8 within the adult forebrain is sufficient to restore these deficits, which is consistent with AC single knockout studies (1). The results also suggest that no irreversible downstream deficits result from a loss of Ca^{2+}-stimulated AC activity during development. AC8 Rescue mice show a slightly faster rate of LTP decay as they are 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 ACKO mice (1). This finding may reflect that AC8 is over five times less sensitive to Ca^{2+} than AC1 (2) and hippocampal Ca^{2+}-stimulated AC activity in AC8 Rescue mice is 50% of WT mice (37).

Along with the induction of LTP comes an increase in a variety of synaptic markers, such as p-synapsin I (38). The regional localization of synapsin I, along with AC8, to the presynaptic terminal (39, 40) suggests that synapsin activity is downstream of AC8. Therefore, to further assess Ca^{2+}-stimulated AC activity's function 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 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 Ca^{2+}-stimulated AC activity in modulating learning-induced changes in synaptic activity.

Not only do our results show that Ca^{2+}-stimulated AC activity modulates synaptic activity, but we also show that 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 (25, 26), 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 (41). 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 (29), which would suggest Ca^{2+}-stimulated AC activity's modulation of neurogenesis 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 (1, 2). Moreover, 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 (42). 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 Ca^{2+}-stimulated AC activity prevents this behavioral plasticity.

Collectively, 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 Ca^{2+}-stimulated AC activity. Overall, the data suggest that experience-dependent fear memory may be driven by Ca^{2+}-stimulated AC-induced

changes in synaptic plasticity. 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 (43, 44), and bipolar disorder (45, 46), 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 (47, 48). 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.

ACKNOWLEDGEMENTS

The SV2 antibody developed by Dr. Kathleen M Buckley was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The pUHC-13-3 plasmid and AC8 cDNA were generous gifts from Dr. Hermann Bujard and Dr. Richard Premont, respectively. We would like to thank Drs. Benedict Kolber and Yarimar Carrasquillo for review of the manuscript. This work was supported by grants from the National Institute of Health to LW (F31MH843913), LJM (R01AG18876; R01MH079010), DJW (MH071674), DGW (R01AA019455) and TAW (F32AA019847).

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CHAPTER 5

Summary and future directions

SUMMARY AND CONCLUSIONS

The studies in this thesis examined the role of Ca^{2+}-stimulated AC activity in learning and memory, particularly fear memory. I analyzed the regional and temporal importance of Ca^{2+}-stimulated AC activity during learning. Using the CF paradigm as a model for fear learning, I also examined possible downstream targets being influenced by Ca^{2+}-stimulated AC activity during fear memory.

Throughout the studies described herein, I used a novel transgenic mouse model to investigate learning and memory. Chapter 2 describes the development and characterization of these mice, AC8 Rescue mice. Under the control of a tetracycline-inducible system, I am able to acutely turn on or off forebrain-specific AC8. These mice are on a Ca^{2+}-stimulated AC-deficient background. This background is important as the absence of both Ca^{2+}-stimulated ACs leads to a variety of memory impairments, while the absence of only a single Ca^{2+}-stimulated AC activity often does not. Therefore, I was able to examine whether forebrain AC8 is sufficient to rescue the memory impairments seen in DKO mice. Furthermore, this also allowed me to examine the necessity of Ca^{2+}-stimulated AC activity at different stages of memory by acutely turning on AC8 at different points throughout learning. We recapitulated previous results, showing that DKO mice have CF and novel object recognition memory deficits (1, 2); however, we also showed that they had memory deficits on the forced swim learning paradigm. When forebrain AC8 is turned on acutely after development, memory deficits were rescued on the CF and novel object recognition paradigms, but not the forced swim learning paradigm. Furthermore, if AC8 is turned off during periods coinciding with memory consolidation or retention, AC8 Rescue mice no longer show intact memory on the CF

paradigm. When examining differences in anxiety, locomotion, or pain, as these factors may contribute to learning results, DKO mice showed a significant decrease in anxiety on the EPM, but not the open field test. Moreover, both AC8 Rescue and DKO mice showed increased locomotion in the open field test. No differences in pain were seen.

Overall, these results suggest a broad role of Ca^{2+}-stimulated ACs in modulating learning on a variety of paradigms. Ca^{2+}-stimulated AC activity appears to be necessary particularly during memory consolidation and retention. Although acute restoration of forebrain AC8 is sufficient to rescue most memory deficits, the AC8 Rescue memory deficits on the forced swim paradigm suggest that AC1 and AC8 are not interchangeable and the presence of one may not make up for the absence of the other. This is evident in single AC knockout studies, which show memory impairments. For example, AC1KO mice display impairments on the Morris water maze (39), while AC8KO mice demonstrate memory deficits on a novel objection recognition task (40). However, additional studies need to be done to prove that AC1 and AC8 possess unique functions as the current findings may just be a result of those tasks requiring more Ca^{2+}-stimulated AC activity than a single AC can produce. Another possible explanation is that Ca^{2+}-stimulated AC activity is necessary during development for subsequent learning in adulthood. This could be further evaluated by maintaining the AC8 Rescue matings and newly born litters off doxycycline (ie normal rodent chow), which would turn AC8 on during development. Finally, the subtle differences in anxiety seen on the EPM further supports previous data that shows the Ca^{2+}-stimulated ACs may play a role in mediating the stress response.

To define the mechanism by which Ca^{2+}-stimulated ACs act, we conducted a genome-wide study of gene expression changes that occur after CF learning. We analyzed both WT and DKO mice after CF learning to identify Ca^{2+}-stimulated AC-dependent gene regulatory changes that occur across different stages of memory in the amygdala and hippocampus. We found that overall the number of genes upregulated after CF learning in the hippocampus or amygdala was significantly decreased in DKO mice. However, the largest influence occurs in the hippocampus, which coincides with previous data showing greater levels of Ca^{2+}-stimulated AC expression in the hippocampus (5, 6). Moreover, the largest increase in transcription changes occurred at 1 h and 48 h after CF learning, which coincides theoretically with when memory consolidation and retention, respectively, are occurring. This data supports the behavioral results above that reveal Ca^{2+}-stimulated AC activity is necessary during these two stages of memory. Although transcriptional changes do not appear to be as largely influenced in the amygdala, heat map analysis interestingly reveals divergent gene expression changes in the amygdala. A cluster of genes significantly upregulated in WT mice were analyzed and the results revealed that DKO mice show opposing regulation. Additionally, many of the transcription factors within the hippocampus that modulate enhanced gene expression in WT mice are found overrepresented in the genes that display reduced gene expression in DKO mice at 48 h. Both results support that an absence of Ca^{2+}-stimulated AC activity may reverse the pattern of gene expression changes or simply cause an overall decrease in transcriptional changes. Finally, the functional analysis data reveals the lack of transcriptional changes may be contributing to deficits in communication occurring at the level of the synapse as WT mice show a large

number of transcriptional changes that functionally contribute to cell communication and signal transduction, whereas, DKO mice do not. While this is merely correlative, the results highlighted below from Chapter 4 will further support this finding.

Chapter 4 provides data that the Ca^{2+}-stimulated ACs play a necessary role in mediating synaptic activity. Initial analysis of the synaptic protein, SV2, in hippocampal cultures revealed decreased levels in DKO mice, which could be rescued through infection with an AC8 lentivirus. Furthermore, I went on to show that Ca^{2+}-stimulated AC activity could directly affect synaptic activity as acutely turning on forebrain AC8 can rescue CA1 LTP deficits known to persist in DKO mice (1, 7). As LTP is often correlated with learning, we looked at whether there were any differences in activation of synaptic protein markers after CF learning. Results revealed that DKO mice failed to increase p-synapsin I levels after CF learning unlike WT and AC8 Rescue mice. Finally, we assessed whether the lack of molecular plasticity in DKO mice impacted function at the behavioral level. To this end, we exposed mice to an experience-dependent CF learning paradigm. Memory in WT mice was readily influenced by the environment as WT mice showed varying levels of freezing based on their initial housing conditions and experimental environment, enriched or minimal. However, DKO mice consistently showed the same freezing levels despite the different environmental exposures. Thus, the data demonstrates that DKO mice show a lack of experience-dependent plasticity. The results, overall, reveal the importance of forebrain Ca^{2+}-stimulated AC activity on modulating fear memory. Additionally, the data also provides evidence for a novel gene-environment interaction, which could help with future therapeutic interventions as

dysfunction of Ca^{2+}-stimulated ACs have been implicated in several psychiatric diseases, such as mood disorder (8), bipolar disorder (9), and Alzheimer's disease (10, 11).

FUTURE DIRECTIONS

This research has identified a necessary role of the Ca^{2+}-stimulated ACs on fear memory, specifically dissecting the temporal and regional importance of these two isoforms. Moreover, downstream analysis of Ca^{2+}-stimulated AC targets has demonstrated a role of these isoforms in modulating synaptic activity. Although the LTP results suggest that Ca^{2+}-stimulated AC activity modulates synaptic activity to cause changes in learning and memory, the two are not always directly correlated, and therefore, further research has to be conducted in order to understand the link between Ca^{2+}-stimulated AC activation and synaptic activity. For example, disruption of $G_{i\alpha1}$ and overexpression of AC1 both increase Ca^{2+}-stimulated AC activity. However, AC1 overexpression leads to both increased CA1 LTP and memory (2), while conversely, disruption of $G_{i\alpha1}$ leads to increased CA1 LTP but severe memory loss (12). These results, along with LTP results discussed previously, suggest that synaptic activity is tightly regulated, and an imbalance in this regulation can lead to impairments. Moreover, there may be synapse-specific plasticity that is required for new memory, and increasing sensitivity to stimulation beyond a threshold can reduce this synapse-specific plasticity. Therefore, through a combination of pharmacological and genetic techniques targeting the Ca^{2+}-stimulated ACs in conjunction with other targets along the pathway, like G protein-coupled receptors or cAMP, the Ca^{2+}-stimulated AC pathway's role in mediating changes in memory through modulation of synaptic activity can be further evaluated. Using AC8 Rescue mice to further this research is ideal as Ca^{2+}-stimulated AC activity is

only 50% of WT levels, and therefore, can be easily be up or downregulated to understand the balance between Ca^{2+}-stimulated AC activity and synaptic activity. Furthermore, using the tetracycline-inducible system, Ca^{2+}-stimulated AC activity can be acutely turned on or off, which allows a within subject control.

Synaptic plasticity was not only found to be altered by Ca^{2+}-stimulated AC activity at the molecular and physiological levels, but also at the behavioral level. The experience-dependent CF results suggest a novel gene-environment interaction. The inability of environmental changes to influence memory in DKO mice demonstrates the need for Ca^{2+}-stimulated AC activity to integrate information from the environment. Although the data suggest the lack of experience-dependent plasticity may be a result of changes in synaptic plasticity, no direct causal link has been revealed. Therefore, the next step in research would be to directly show that the Ca^{2+}-stimulated ACs' modulation of synaptic activity is responsible for the experience-dependent changes in fear memory. This effect could be analyzed by determining how different environmental contexts affect LTP in both WT and DKO mice. Furthermore, measuring the effects of environment on the behavior and physiology of AC8 Rescue mice would also be informative. The ability to turn Ca^{2+}-stimulated AC activity on or off would help begin to elucidate whether acute activation is sufficient for modulation of experience-dependent memory, or whether Ca^{2+}-stimulated AC activity is necessary developmentally, such that it is needed to cause more long-term changes like alterations in dendritic morphology. If this is the case, assessing dendritic morphology in DKO and AC8 Rescue mice would also be informative as current research supports the theory that dendritic spines provide structural support for the processing of new memories (13).

Finally, finding a more direct link between Ca^{2+}-stimulated AC activity and cognitive changes in the human population would be beneficial. Previous data has already linked AC8 with psychiatric disorders, specifically bipolar disorder (14, 15) and mood disorder (8). Moreover, AC1 has been linked to Alzheimer's disease (10, 11). However, whether the Ca^{2+}-stimulated ACs are responsible for cognitive changes in these psychiatric diseases is still unknown. As research in murine models further examines Ca^{2+}-stimulated AC activity's role during learning, this will provide more insight by which humans may be aversely affected by alterations in Ca^{2+}-stimulated AC activity.

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