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Role of AKAP5 in postsynaptic signaling complexes

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ROLE OF AKAP5 IN POSTSYNAPTIC SIGNALING COMPLEXES

by

Mingxu Zhang

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacology
in the Graduate College of
The University of Iowa

JULY 2010

Thesis Supervisor: Adjunct Professor Johannes W. Hell

ABSTRACT

Noradrenergic signaling has important functions in the central nervous system (CNS) with respect to emotion, learning and memory. Activation of β- adrenergic receptors (β ARs) stimulates protein kinase A via Gs-protein, adenylyl cyclase, and cAMP. Synaptic β_2 -adrenergic receptors, targets of the neurotransmitter norephinephrin, are associated with the GluA1 subunit of AMPA-type glutamate receptors, which mediate most excitatory synaptic transmission in mammalian CNS. PKA-mediated phosphorylation of GluA1 on Ser845 is important for GluA1 surface expression, activity induced postsynaptic accumulation, and synaptic plasticity. Postsynaptic localization of PKA is mediated by a major scaffolding protein 'A kinase anchor protein 5 (AKAP5)'. AKAP5 associates with AMPA receptors via SAP97 and PSD95.

We have two strains of AKAP5 mutant mice: AKAP5 knockout and AKAP5 D36. AKAP5 KO mice have a complete loss of AKAP5 gene expression. D36 mice miss the last 36 residues (PKA binding site) of AKAP5 but without affecting other interactions. These mutant mice provide us with appropriate *in vivo* models for studying the functional roles of AKAP5.

We compared the functional and physical association of β_2AR and AMPA receptors among wild type, AKAP5 KO, and AKAP5 D36 mice. Although AKAP5 was not necessary for the assembly of the β_2AR / GluA1 complex, we found that AKAP5 anchored PKA activity was required for full β_2AR stimulation-induced GluA1 Ser845 phosphorylation. Recording and analysis of field EPSPs (fEPSPs) of CA1 pyramidal neurons with brief bath perfusion of the β_2AR agonist isoproterenol indicated a role of

AKAP5 anchored PKA in the regulation of postsynaptic AMPAR responses by norephinephrin.

Moreover, we observed a delayed extinction of contextual fear memory in AKAP5 D36 mice, which suggests the involvement of AKAP5 anchored PKA in memory formation and modification.

Abstract Approved: _____

Thesis Supervisor

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Title and Department

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Date

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Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Mingxu Zhang

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Pharmacology at the July 2010 graduation.

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To
My parents Liqing Zhang, Fangying Jia
My husband Yan Li and daughters Emily, Yaya
For their love and support

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ABSTRACT

Noradrenergic signaling has important functions in the central nervous system (CNS) with respect to emotion, learning and memory. Activation of β- adrenergic receptors (β ARs) stimulates protein kinase A via Gs-protein, adenylyl cyclase, and cAMP. Synaptic β_2 -adrenergic receptors, targets of the neurotransmitter norephinephrin, are associated with the GluA1 subunit of AMPA-type glutamate receptors, which mediate most excitatory synaptic transmission in mammalian CNS. PKA-mediated phosphorylation of GluA1 on Ser845 is important for GluA1 surface expression, activity induced postsynaptic accumulation, and synaptic plasticity. Postsynaptic localization of PKA is mediated by a major scaffolding protein 'A kinase anchor protein 5 (AKAP5)'. AKAP5 associates with AMPA receptors via SAP97 and PSD95.

We have two strains of AKAP5 mutant mice: AKAP5 knockout and AKAP5 D36. AKAP5 KO mice have a complete loss of AKAP5 gene expression. D36 mice miss the last 36 residues (PKA binding site) of AKAP5 but without affecting other interactions. These mutant mice provide us with appropriate *in vivo* models for studying the functional roles of AKAP5.

We compared the functional and physical association of β_2AR and AMPA receptors among wild type, AKAP5 KO, and AKAP5 D36 mice. Although AKAP5 was not necessary for the assembly of the β_2AR / GluA1 complex, we found that AKAP5 anchored PKA activity was required for full β_2AR stimulation-induced GluA1 Ser845 phosphorylation. Recording and analysis of field EPSPs (fEPSPs) of CA1 pyramidal neurons with brief bath perfusion of the β_2AR agonist isoproterenol indicated a role of

AKAP5 anchored PKA in the regulation of postsynaptic AMPAR responses by norephinephrin.

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LIST OF ABBREVIATIONS

ABR	auditory brainstem response
ACSF	artificial cerebrospinal fluid
AKAP	A kinase anchor protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
AR	adrenergic receptors
BCA	bicinchoninic acid
CA1	Cornu Ammonis area 1
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	central nervous system
CPP	conditioned place preference
CREB	cAMP response element-binding protein
CS	conditional stimulus
Dbh	dopamine β-hydroxylase
DG	dentate gyrus
DOC	sodium deoxycholate
ECG	electrocardiogram
FC	fear conditioning
fEPSPs	field excitatory postsynaptic potentials
GK	guanylate kinase-like
HRP	horse radish peroxidase
ICV	intracerebroventricular
ISO	isoproteronol
KO	knockout
LTP	long-term potentiation
LTD	long-term depression

MAP2B	microtuble associated protein 2B
mEPSC	miniature excitatory post-synaptic current
NE	norepinephrine
NMDA	*N*-methyl-D-aspartate
PDZ	PSD-95/Discs Large/ zona occludens 1
PFC	prefrontal cortex
PKA	cyclic AMP (cAMP)-dependent protein kinase
PKC	protein kinase C
PP1	protein phosphatase-1
PP2B	protein phosphatase 2B
PPF	pair-pulse facilitation
PSD	postsynaptic density
PSD-95	postsynaptic density protein of 95 kDa
PTSD	posttraumatic stress disorder
PVDF	polyvinyldifluoride
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SH3	the Src homology 3
SAP97	synapse-associated protein 97
TBS	Tris buffered saline
TTX	tetrodotoxin
US	unconditional stimulus
WT	wild type

CHAPTER 1
INTRODUCTION

Overview

Norepinephrine (NE) signaling via β adrenergic receptors has been studied as a central factor for emotional arousal and learning, especially under novel and emotionally charged situations (Berman and Dudai, 2001; Cahill et al., 1994; Hu et al., 2007; Minzenberg et al., 2008; Nielson and Jensen, 1994; Stranger and Dolan, 2004; Stranger et al., 2003; Tully and Bolshakov, 2010). Manipulation of NE signaling may be a potential treatment for anxiety disorder, addiction, or posttraumatic stress disorder (PTSD), based on studies on animals and human. The molecular mechanisms downstream of NE signaling are still under investigation.

NE acts via the β_1 and β_2 adrenergic receptors (AR) to activate the trimeric G_s protein, adenylyl cyclase, and ultimately PKA. Activation of PKA will increase neuronal excitability through ion channels and receptors, or stimulate downstream targets for gene expression (Figure 1-1). PKA regulated ion channels and receptors include L-type calcium channel $Ca_v1.2$, NMDA and AMPA glutamate receptors and so on. The synaptic localization of PKA in neuronal cells is mainly mediated by a postsynaptic A kinase anchoring protein AKAP5 (Carr et al., 1992). Our previous study has identified that β_2 adrenergic receptors form a complex with AMPAR GluA1 subunit via AKAP5, PSD95 and SAP97 (Joiner et al., 2010).

The goal of this thesis work is to further clarify the role of AKAP5 in the β adrenergic receptor /glutamate receptors signaling complex. As a broad introduction, I

will summarize the following subjects: 1) fear memory and extinction; 2) noradrenergic signaling system; 3) glutamate receptors, especially AMPA receptors; 4) PKA and AKAP5.

Fear memory and extinction

Learning and memory are higher order cognitive processes involving the acquisition, encoding, and consolidation of new information in the brain. The molecular mechanism underlying this process is still not clear yet. Synaptic plasticity, which allows neurons to change the strength of synapses based on activity, is widely assumed to be the neurochemical mechanism for memory formation (Bliss and Collingridge, 1993; Dunwiddie and Lynch, 1978; Malenka and Nicoll, 1999; Neves et al., 2008; Whitlock et al., 2006).

Hippocampus is involved in learning and memory, eg. declarative, relational and spatial memory. Recently contextual fear conditioning has become popular for testing hippocampal-dependent learning. Fear conditioning is a Pavlovian response which associates the unconditional stimulus (US), usually an aversive electric foot shock, with the conditional stimulus (CS), for example, the contextual cues or the tone (Fanselow, 2000). In contextual fear conditioning, the hippocampus is the place for forming and storing the integrated representation of context and temporally keeping the memory stable (Anagnostaras et al., 2001; Fanselow, 2000; Sanders et al., 2003).

Fear responses can be indicated as ‘freezing’ (lack of all bodily movements expect those for respiration), ‘potentiated startle’ (an increased acoustic startle response),

an increased blood pressure, respiration changes, and so on (Myers and Davis, 2007). Freezing is a most common indicator for fear (Myers and Davis, 2007).

Fear memories are formed initially in a short-term format and converted later into a long-term form (consolidation). This long-term memory is dependent on several molecular events such as protein synthesis. Hippocampus and amygdala are the places for memory storage and consolidation (Sanders et al., 2003). Conditioned fear memory is modulated by reexposure to the stimulus. This retrieval of the memory induces two independent processes: reconsolidation and fear extinction (Myers and Davis, 2007; Ouyang and Thomas, 2005).

The retrieval of the memory renders the memory labile and susceptible to amnestic treatment. This process is named as memory reconsolidation (Nader et al., 2000a; Tronson et al., 2006). Noradrenergic blockade by propranolol disrupts the reconsolidation of auditory fear conditioning in rats (Debiec and LeDoux, 2004, 2006). This finding is especially important for the clinical use of propranolol for posttraumatic stress disorder (PTSD) (Schiller et al., 2010).

Fear extinction is a process that causes reduction in the conditioned fear responses. Different from forgetting, extinction needs the repeated exposure to the CS (tone or context) in the absence of the US (foot shock). The extinguished fear responses (extinction) can be recovered after several tests, such as reinstatement, renewal, and spontaneous recovery (Myers and Davis, 2007). The mechanism of fear extinction is not clear yet although studies suggest the multiple level of regulation. Hippocampus, norepinephrine, and PKA are all involved in the regulation of fear extinction (Myers and Davis, 2007).

Fear conditioning is not only a good example for studying the mechanism of learning and memory but also a practical model for understanding fear- and anxiety-related disorders such as phobias and PTSD (Debiec and LeDoux, 2006). The study on the mechanisms of fear inhibition would be beneficial for the clinical interventions to these disorders in patients.

NE and noradrenergic signaling system

NE is synthesized from dopamine by dopamine β-hydroxylase in catacholaminergic neurons. As a neurotransmitter it is released from the locus ceruleus and lateral brain stem tegmentum. These noradrenergic neurons project bilaterally to many brain regions, including hippocampus and amygdala, the places for learning and memory. Noradrenergic signaling facilitates several forms of LTP in the hippocampal dentate gyrus and CA1 region (Gelinas and Nguyen, 2005; Hu et al., 2007; Katsuki et al., 1997; Lin et al., 2003; Thomas et al., 1996; Walling and Harley, 2004) and the gating and polarity of cortical plasticity (He et al., 2009).

NE acts on the target cells by binding to adrenergic receptors (α_1, α_2, β_1, β_2 and β_3). α_1 and α_2 receptors are mainly localized on smooth muscles while β_1 receptor is expressed in heart muscles. These groups of adrenergic receptors largely regulate vasoconstriction and vasodilation. In the brain, β_1, β_2, and α_2 adrenergic receptors are widely distributed in multiple regions, including cortex and cerebellum. We are mainly interested in the β-adrenergic signaling.

Both β_1AR and β_2AR are expressed in the dentate gyrus (DG) of the hippocampus (Milner et al., 2000), and the cornu ammonis (CA)1 and CA3 regions as well (Guo and Li, 2007). The β_2AR is enriched at excitatory postsynaptic sites of pyramidal neurons where it forms a signaling complex with the AMPAR GluA1 subunit, G_s, and adenylyl cyclase for highly localized GluA1 phosphorylation and regulation (Colledge et al., 2000; Davare et al., 2001; Joiner et al., 2010; Tavalin et al., 2002).

Noradrenergic β-receptor signaling is involved in the memory consolidation by emotional arousal (Tully and Bolshakov, 2010). Recent studies emphasize the involvement of noradrenergic signaling system in memory modulation, especially in extinction (Mueller and Cahill, 2010; Tully and Bolshakov, 2010). Adrenergic activation may promote and adrenergic blockade impair memory consolidation. The memory modulatory effects of GABA are involved in the downstream modulation of noradrenergic signaling (Myers and Davis, 2007).

Studies on the dopamine β-hydroxylase (Dbh) knockout mice (Ouyang and Thomas, 2005) found that pharmacological or genetic block of adrenergic signaling impaired fear memory extinction. Similarly, studies on cocaine-induced anxiety (Schank et al., 2008) found the resistance of Dbh knockout (Dbh -/-) mice to cocaine-induced anxiety in the elevated plus maze. Pretreatment of adrenergic antagonist propranolol blocked cocaine-induced anxiety-like behavior in Dbh +/- and wild type mice.

Propranolol is the most studied β-blocker on fear memory, anxiety disorders and PTSD although the findings are controversial. Systemically-administered propranolol in rats (Rodriguez-Romaguera et al., 2009) or mice (Cain et al., 2004) reduced conditioned fear during extinction learning but did not change retention of extinction. Some clinical

studies on human found that propranolol had a preventing effect against PTSD (Brunet et al., 2008; Pitman et al., 2002; Vaiva et al., 2003) while other studies found no effect of propranolol on PTSD (Stein et al., 2007). It seems that the timing of propranolol administration may play a role in the effects of the drug.

Glutamate receptors

Glutamate, a major excitatory neurotransmitter in brain, is released from the pre-synaptic neurons and plays the role in synaptic plasticity by binding to the post-synaptic glutamate receptors. Two groups of glutamate receptors exist (Dingledine et al., 1999): ionotropic glutamate receptors (iGluRs, ligand-gated ion channels) and metabotropic glutamate receptors (mGluRs, G-protein coupled receptors). AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole) receptor (AMPARs) and NMDA (N-methyl-D-aspartate) receptors (NMDARs) are two major subtypes of the ionotropic glutamate receptor family and have important functions in synaptic plasticity. I will focus on AMPA receptors in the following section.

AMPA receptors mediate most of the postsynaptic response at glutamatergic synapses during basal synaptic transmission. AMPA receptors are encoded by four GluA1-4 subunits. Each subunit has two isoforms, 'flip' or 'flop', from alternative transcription. Flop isoforms desensitize faster than flip form (Dingledine et al., 1999; Mosbacher J et al., 1994). Different from the others, postnatal GluA2 subunit is posttranslational edited from Glu to Arg on a single residue site (Q/R site) within the M2 region (Egebjerg et al., 1994; Higuchi et al., 1993; Puchalski et al., 1994). This edited

GluA2 subunit changes AMPAR channel properties from Ca^{2+} permeable, more highly conducting, inwardly rectifying, to a linear current-voltage relationship, Ca^{2+} impermeable, and low conductance (Bowie and Mayer, 1995; Hume et al., 1991; Sommer et al., 1991).

Within the cortex and hippocampus, GluA1/2 heteromers are the predominantly expressed AMPARs while GluA2/3 contributing a significantly lower portion under basal conditions (Dingledine et al., 1999; Hollmann and Heinemann, 1994; Lu et al., 2009; Wenthold et al., 1996). GluA1 homomers comprise less than 10% of the receptor population. Normal function of AMPARs requires a family of transmembrane AMPAR associated proteins (TARPs). These auxiliary AMPAR subunits are important for AMPAR trafficking, surface expression, and channel kinetics (Bats et al., 2007; Kessels et al., 2009; Milstein et al., 2007).

PKA phosphorylates GluA1 on serine 845 (Ser845) (Roche et al., 1996), which is important for GluA1 surface expression (Ehlers, 2000; Man et al., 2007; Oh et al., 2006; Sun et al., 2005; Swayze et al., 2004), activity-induced postsynaptic accumulation (Esteban et al., 2003) and various forms of synaptic plasticity (He et al., 2009; Hu et al., 2007; Lee et al., 2003; Lee et al., 2010a). Phosphorylation of Ser831 by CaMKII and PKC (Barria et al., 1997; Boehm et al., 2006; Mammen et al., 1997), and Ser818 by PKC(Boehm et al., 2006; Roche et al., 1996) , also facilitates synaptic AMPAR delivery and monitors synaptic transmission (Kessels and Malinow, 2009; Song and Huganir, 2002). A number of studies have suggested that synaptic plasticity through AMPAR trafficking underlies the molecular mechanisms for experience-dependent behavioral

modifications, such as hippocampus- or amygdala-dependent learning and memory, emotional arousal and addiction (reviewed in (Kessels and Malinow, 2009)).

PKA and AKAP5

PKA holoenzyme consists of two regulatory subunits (R) and two catalytic subuints (C). Under low level of cAMP, catalytic subunits bind with regulatory subunits and PKA is inactive. With an increase in cAMP concentration, cAMP binds to regulatory subunits while catalytic subunits will be released and activated to phosphorylate its substrate. There are two major forms of PKA, type I and type II. Two catalytic subunit isoforms are denoted as Cα and Cβ. For each type of regulatory subunit, there are two subtypes (RIα, RIβ, and RIIα, RIIβ). RIα and RIIα are widely distributed in different tissues; RIβ is mainly expressed in brain and developing sperm cells and RIIβ is found majorly in mammalian central nervous system (CNS). For subcellular distribution, above 90% of RIIα is existed in the soluble cytoplasm, while ~70% of RIIβ fractionates are tightly associated with particulate components of cerebral cortex (Ventra et al., 1996).

Specificity and efficiency of PKA in cell signaling are achieved by a group of PKA anchoring proteins (AKAPs), which anchor PKA holoenzyme through binding to the N-terminus of the R subunits and specifically localize PKA to its functional sites through their unique targeting domains (Dell'Acqua et al., 2006; Wong and Scott, 2004). In addition to anchoring PKA, many AKAPs bind other signaling or scaffolding

molecules, or membrane receptors and therefore play important roles as the signaling complexes (Michel and Scott, 2002).

The AKAPs family consists of at least 50 members with diverse structure but similar function. Most AKAPs have a PKA-anchoring domain, unique localization signals, and binding sites for other signaling molecules (Wong and Scott, 2004). The AKAP5 family contains three well-studied orthologues: human AKAP79, bovine AKAP75, and murine AKAP150 (Bergman et al., 1991; Carr et al., 1992). These orthologues have conserved functional domains but different numbers of inserted segments with unknown function (Wong and Scott, 2004).

The structural organization of AKAP5 is shown in Figure 1-2. The C-terminal 36 residues of AKAP5 bind the N-termini of the regulatory RII subunits of PKA to mediate PKA anchoring under basal conditions (Carr et al., 1992). The N-terminus of AKAP5 binds PKC, F-actin, cadherin, and PIP_2 and targets AKAP5 to dendritic spines (Dell'Acqua et al., 1998; Gomez et al., 2002; Gorski et al., 2005; Klauck et al., 1996; Tavalin, 2008). This region also has a binding site for adenylyl cyclase 5 and 6 (Efendiev et al., 2010) in the polybasic region B (Figure 1-2). The central region of AKAP5 binds the Ca^{2+} and calmodulin-activated phosphatase calcineurin (PP2B) (Coghlan et al., 1995; Oliveria et al., 2007; Oliveria et al., 2003).

As a major PKA-binding protein in postsynaptic density, AKAP5 associates with the Src homology 3 (SH3) domain and the guanylate kinase-like (GK) region of synapse-associated protein 97 (SAP97) and postsynaptic density protein of 95 kDa (PSD-95) (Bhattacharyya et al., 2009; Colledge et al., 2000; Leonard et al., 1998). PSD-95 interacts through its first two PDZ domains with the C-terminus of stargazin ($\gamma 2$) and its

homologues γ3, γ4, and γ8 ("TARPs"), which in turn associate with AMPARs for their postsynaptic targeting (Chen et al., 2000; El-Husseini et al., 2000; Schnell et al., 2002). SAP97 directly binds with its first two PDZ domains to the C-terminus of GluA1 (Leonard et al., 1998). Therefore through association between SAP97, PSD95 and the GluA1 subunit of AMPA receptors, AKAP5 anchors PKA, PKC, and PP2B close to GluA1 and provides a platform for the phosphorylation and dephosphorylation events of postsynaptic GluA1 subunit (Bhattacharyya et al., 2009; Colledge et al., 2000; Leonard et al., 1998; Roche et al., 1996; Tavalin, 2008; Tavalin et al., 2002; Wong and Scott, 2004).

In addition to AKAP5, there are other AKAPs localized at the post-synaptic sites, e.g. Yotiao and MAP2B. Yotiao, a splice variant of AKAP350, directly binds to the cytoplasmic tail of NR1 and anchors PKA and protein phosphatase-1 (PP1) to post-synaptic NMDA receptors (Westphal et al., 1999). MAP2B associates PKA with neuronal class C L-type calcium channels (Davare et al., 1999).

AKAP5 is concentrated in the mouse brain areas involved in learning and memory (Ostroveanu et al., 2007). The studies on AKAPs by disrupting PKA anchoring have suggested the role in the coordination of higher order neuronal events such as the regulation of synaptic strength, inhibition of neuronal excitability and auditory fear conditioning (Moita et al., 2002; Nijholt et al., 2008b; Smith et al., 2006; Snyder et al., 2005). In addition to facilitate cAMP-PKA pathway, AKAP5 also coordinates Epac-mediated signaling pathway (Nijholt et al., 2008a; Pidoux and Tasken, 2010), which is important for NE mediated memory retrieval (Ouyang et al., 2008).

Central hypothesis

AKAP5 is the major AKAP at postsynaptic site in mouse and rat to anchor PKA to GluA1 in post-synaptic density. β_2AR forms a complex with GluA1 via PSD95 and the third intracellular (i3) loop and C terminus of β_2AR is necessary for the β_2AR/AMPA receptor complex (Joiner et al., 2010; Wang et al., 2010). This signaling complex includes adenylyl cyclase, AKAP150, PKA and PP2A (Wang et al., 2010). AKAP5 directly associates with β_2AR (Dai et al., 2009). The specific function of AKAP5 in the synaptic complex of β_2AR-GluA1 is still unknown. We hypothesize that AKAP5 is important for the synaptic function of β_2AR-GluA1 signaling complex.

To investigate the specific function of AKAP5 *in vivo*, we used two strains of AKAP5 mutant mice made by Dr. Stanley McKnight's lab at the University of Washington: AKAP5 knockout, and AKAP5 D36 (Lu et al., 2007; Weisenhaus et al., 2010) (Figure 1-3.). D36 mice were generated by insertion of a stop codon to delete the last 36 residues of AKAP5 and were used to test the functional roles of AKAP5 with respect to PKA targeting but without affecting the other interactions (Lu et al., 2007; Lu et al., 2008; Weisenhaus et al., 2010). AKAP5 KO completely lost AKAP5 gene. These mutant mice provide us with appropriate models for studying the role of AKAP5 in the complex formation, function and regulation of the postsynaptic β_2AR-GluA1 signaling complex.

D36 and KO mice have very limited and specific phenotypes with normal basal synaptic transmission and normal behavior. However, LTP is nearly absent during early development (P12-14) and in adult D36 mice and LTD is impaired at P12-14 in D36

mice (Lu et al., 2007; Lu et al., 2008; Weisenhaus et al., 2010). AKAP5 KO mice had normal LTP and LTD likely because the deletion of AKAP5 eliminated not only PKA but also PP2B anchoring, causing a milder phenotype than the D36 mutation (Weisenhaus et al., 2010) (but see (Tunquist et al., 2008) for deficits in LTD (but not LTP) in another AKAP5 KO mouse). General behavior (e.g., open field) and learning (e.g., Morris water maze, novel object recognition, operant conditioning) is normal in D36 mice but reversal learning of the operant task is delayed although it still occurs (Weisenhaus et al., 2010).

In the following parts of the thesis, we investigated the association of β_2AR and GluA1 in D36 and KO mice. Since PKA mediated phosphorylation on GluA1 Ser845, which is important for GluA1 surface expression and synaptic plasticity, we tested the basal and induced Ser845 phosphorylation in WT, D36 and KO mice. In addition, we analyzed the changes in synaptic transmission and LTP in D36 mice. The general behavior and fear responses were observed in littermated WT, D36 and KO mice.

Figure 1-1. Noradrenergic signaling enhances membrane excitability and synaptic plasticity.

NE release evoked by CS presentation activates β-receptors and PKA. PKA increases excitability through phosphorylation of ion channels and receptors (here AMPA receptors as shown), and stimulates downstream targets, including CREB, leading to gene transcription and translation. The subsequent synaptic modifications result in the stabilization of memory information. (Adapted from Mueller and Cahill, 2010.)

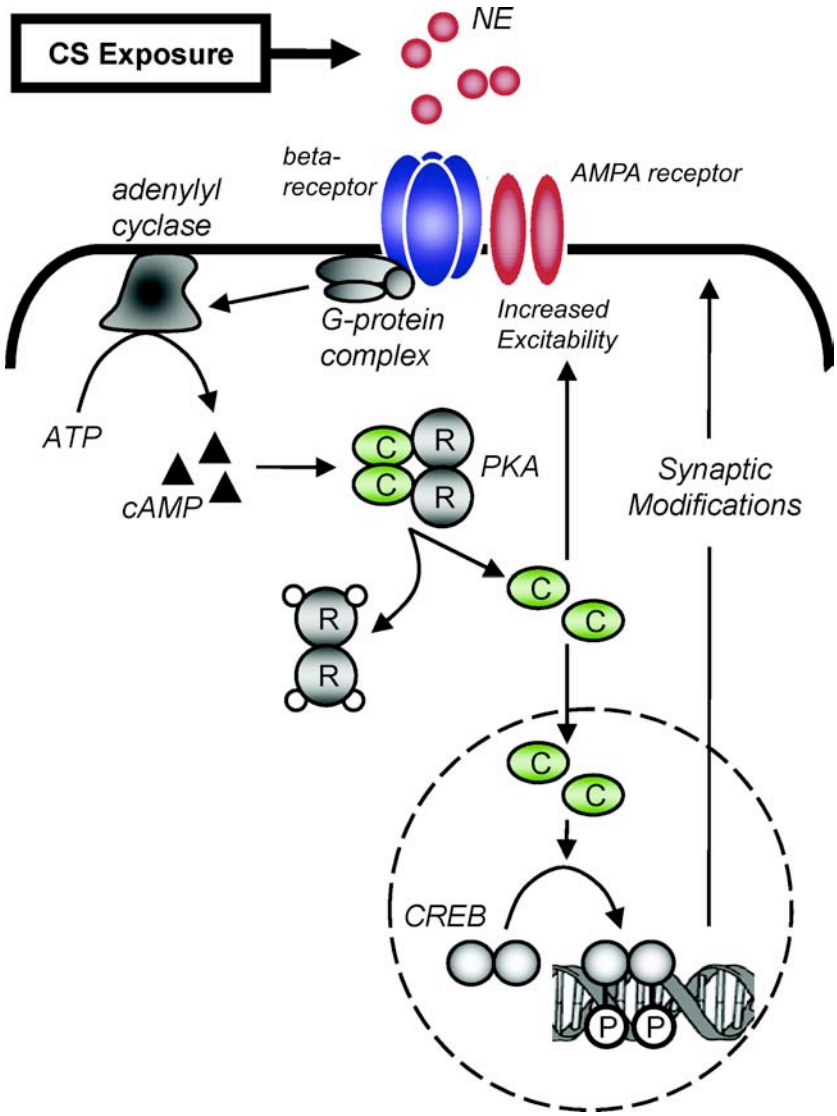

CS Exposure
NE
beta-
receptor
AMPA receptor
adenylyl
cyclase
G-protein
complex
Increased
Excitability
ATP
cAMP
C
R
C
R
PKA
Synaptic
Modifications
R
R
C
C
C
C
CREB
P
P

Figure 1-2. Modular organization of AKAP5.

The N-terminus of AKAP5 binds PKC, F-actin, cadherin, and PIP_2 and targets AKAP5 to dendritic spines. The central region of AKAP5 binds the Ca^{2+} and calmodulin-activated phosphatase calcineurin (PP2B). The C-terminal 36 residues of AKAP5 bind the N-termini of the regulatory RII subunits of PKA. AKAP5 associates with the SH3 - GK region of the postsynaptic scaffolding proteins PSD-95 and SAP97 and links to GluA1. (Adapted from Dell'Acqua M.L., et al, 2006.)

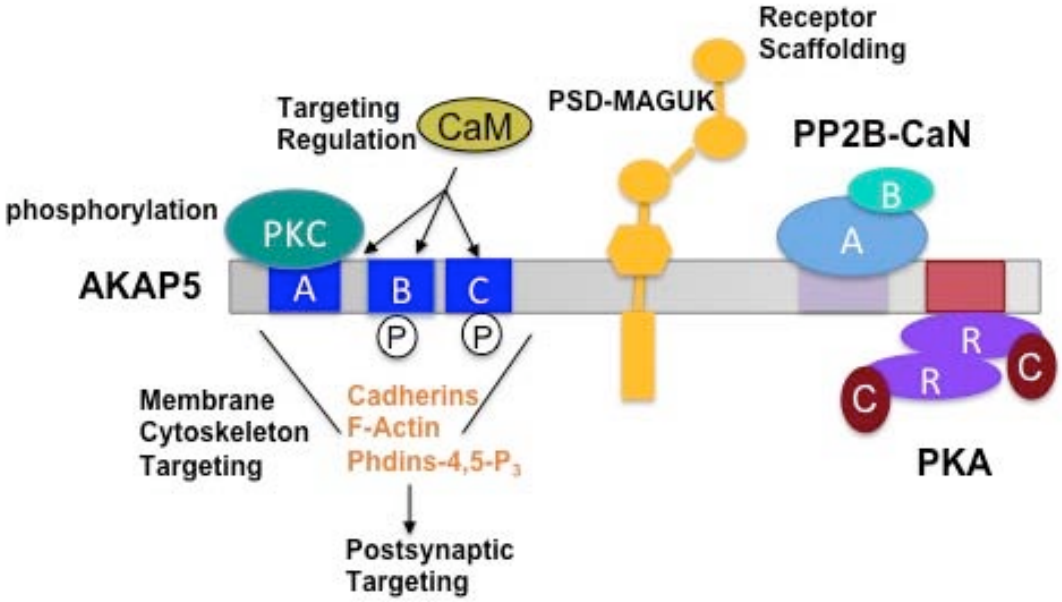

Receptor
Scaffolding
Targeting
Regulation
CaM
PSD-MAGUK
PP2B-CaN
phosphorylation
PKC
B
A
AKAP5
A
B
C
P
P
R
C
R
C
Membrane
Cytoskeleton
Targeting
Cadherins
F-Actin
Phdins-4,5-P3
PKA
Postsynaptic
Targeting

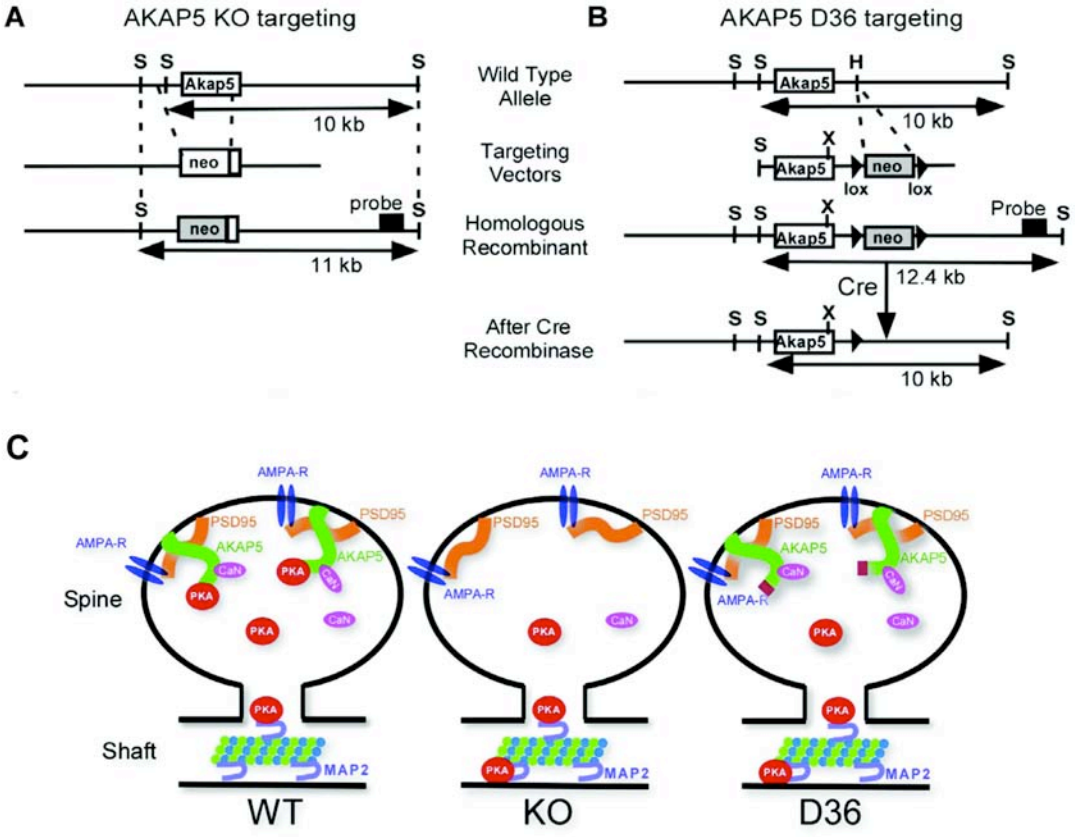
Figure 1-3. Generation of AKAP5 KO and D36 mutant mice.

A. Targeting strategy for generating AKAP5 KO mice. The AKAP5 coding region was replaced by a neomycin selectable marker (neo) cassetttee insertion.

B. Targeting strategy for generating AKAP5 D36 mice. A stop codon was inserted into Akap5 coding sequence Leu 710 (denoted as an "X") with a downstream insertion of the neo gene flanked by loxP sites.

C. Model of the AKAP5 scaffold domain at the PSD in WT, KO and D36 mice. In AKAP5 KO PSDs, the relative amounts of PKA and PP2B are reduced and PKA redistributes to the dendritic shafts and the cell body. In D36 PSDs, PP2B still binds to AKAP5 D36 and only PKA redistributed.

(Adapted from Weisenhaus, M. et al, 2010.)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AKAP5 KO targeting
S S
Akap5
S
10 kb
neo
probe
S
neo
S
11 kb
B
AKAP5 D36 targeting
Wild Type Allele
S S
Akap5
H
S
10 kb
Targeting Vectors
S
X
Akap5
neo
lox
lox
Homologous Recombinant
S S
X
Akap5
neo
Probe
S
12.4 kb
Cre
After Cre Recombinase
S S
X
Akap5
S
10 kb
C
AMPA-R
PSD95
AKAP5
CaN
PKA
Spine
Shaft
MAP2
WT
KO
D36

CHAPTER 2
ROLE OF AKAP5 IN THE COMPOSITION OF β_2AR – GLUA1 SIGNALING COMPLEX

Rationale

Central noradrenergic signaling mediates multiple neuronal activities, including arousal and learning and memory. The β_2AR is one of the major receptors for the neurotransmitter norepinephrine; and is concentrated at the postsynaptic sites in pyramidal neurons (Davare et al., 2001). It is well known that stimulation of β_2AR activates cAMP dependent protein kinase PKA via G_s protein, adeneylyl cyclase, and cAMP. This signaling pathway is highly localized in the plasma membrane and regulates neuronal activity through PKA mediated phosphorylation.

AMPARs are responsible for the majority of synaptic activity. Surface expression and trafficking of AMPARs to the postsynaptic membrane are critical for the regulation of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD). Among the four AMPARs subunits (GluA1-4), GluA1 subunit is the major subunit for trafficking of AMPARs. Phosphorylation of GluA1 by PKA on Serine 845 is important for surface expression of GluA1 and synaptic plasticity.

Our recent studies (Joiner et al., 2010) have identified the signaling complex including β_2AR, G_s, adenylyl cyclase, PKA, and GluA1 subunit, which foster the highly localized cAMP signaling in neurons. AKAP5 is the major neuronal AKAP for the targeting of PKA to the postsynaptic sites. Therefore we want to assess the specific function of AKAP5 in the association of β_2AR- GluA1 complex in this section.

AKAP5 targets to the plasma membrane (Dell'Acqua et al., 1998) and has the binding sites for PKA, PKC and PP2B. It has direct interaction with β_2AR (Dai et al., 2009; Davare et al., 2001) and also binds to GluA1 through SAP97 and PSD95 (Colledge et al., 2000). It is unknown whether AKAP5 is necessary for the β_2AR-GluA1 complex formation. To test the role of AKAP5 in the association of β_2AR and GluA1, we used coimmuprecipitation to *in vivo* analyze the complex association in AKAP5 D36 and KO mice. In D36 mice, AKAP5 does not have the last 36 residues that are the PKA binding sites but other interaction sites still exist. In AKAP5 KO mice, complete loss of AKAP5 will influence the anchoring of all the associated proteins.

Both β_2AR and AMPARs are concentrated in the postsynaptic density. We further compared the protein components in the PSD fractions from WT, D36 and KO mice. This would suggest whether AKAP5 is involved in the recruitment of β_2AR to PSD.

Results

AKAP5 is not necessary for β_2 AR - GluA1 interaction

Previously we have found that β_2AR associated with GluA1 subunit (Joiner et al., 2010). To assess the role of AKAP5 in the complex formation, we used coimmunoprecipitation (coIP) to assess the association of β_2AR with GluA1 in AKAP5 mutant mice.

Forebrains were isolated from WT, D36 and KO mice and homogenized in 1% Triton X-100 to extract the membrane associated proteins. 1% Triton X-100 is a nonionic

surfactant and used to solubilize membrane proteins in their native state. Sodium dodecyl sulfate (SDS) and sodium deoxycholate (DOC) are also commonly used detergents for membrane proteins extraction. However our prior studies found that Triton X-100 extracted relatively more AMPA receptors, $Ca_v1.2$ and β_2AR in the same time compared to SDS and DOC. Moreover we want to optimize the possibility of detecting even weaker interactions between β_2AR and other proteins in the synapse. Therefore we primarily used 1% Triton X-100 for coimmunoprecipitations.

As showed in Figure 2-1A, coimmunoprecipitation of GluA1 with the β_2AR from brain extracts was identical for Wt, D36 and KO mice. Because coimmunoprecipitation signals for GluA1 were weak and control IgG precipitations typically also had some detectable signal, the latter was quantified and subtracted from the former values for each experiment. The resulting values, which reflect specific coimmunoprecipitation, did not significantly vary between the three genotypes (Figure 2-1B) suggesting that neither the D36 mutation nor the full elimination of AKAP5 affected the formation of the β_2AR-GluA1 complex.

AKAP5 is not necessary for postsynaptic targeting of the β_2 AR

AKAP5 is present in the postsynaptic density (Colledge et al., 2000). Analogous to the preceding considerations, AKAP5 could be involved in linking the β_2AR in general to the PSD. In order to prove that the synaptic localization of β2AR and AMPAR was not affected in AKAP5 D36 or KO mice, we analyzed the relative composition of AMPAR

subunit GluA1, β_2AR, and L-type calcium channel $Ca_v1.2$ in purified PSD fractions from WT, D36 and KO mice.

We isolated PSD fractions from brains of WT, D36 and KO mice. About 10 μg of each fraction (crude lysate, P2, synaptosomal-enriched, and PSD) during the purification process were separated via SDS-PAGE and immunoblotted to show the relative enrichment of the various proteins (Figure 2-2A and 2-3A). The excellent purity of the final PSD fraction is reflected by the loss of any synaptophysin (a presynaptic marker) signal and the strong enrichment of the PSD marker, PSD-95. The presence of equal amount of PSD-95 in PSDs from D36 / WT mice and KO / WT mice served as a second loading control in addition to our protein assay. It indicated that the amount of PSD material obtained from WT or AKAP5 mutant mice was equal.

Immunoblotting for the β_2AR showed no difference between WT and D36 (Figure 2-2) and between WT and KO brains (Figure 2-3), indicating that neither the D36 deletion nor the complete KO affected postsynaptic targeting of the β_2AR. The enrichment of GluA1 in the PSD fraction was modest but was unchanged in D36 mice as reported earlier (Lu et al., 2007) (Figure 2-2). We also detected slightly enrichment of $Ca_v1.2$ in the PSD fractions of WT, D36 and KO mice. The quantification analysis showed that the relative content of β_2AR in the PSD was not statistically different in the D36 mice, compared to WT ($p > 0.05$, n=5, Student's t test) (Figure 2-2B). We also analyzed the levels of $Ca_v1.2$ and GluA1. There was no statistically significant difference between WT and D36 mice for either of these PSD components as well (Figure 2-2B). Similarly we did not test the changes of protein components in the PSD from KO mice compared to WT (Figure 2-3B).

Discussion

The β_2AR is linked to GluA1 via PSD-95 and stargazin and its homologues (Joiner et al., 2010). More precisely the very C-terminus of the β_2AR constitutes a PDZ domain-binding site (DSPL in rodents, DSLL in humans) that interacts with the third PDZ domain of PSD-95 (Joiner et al., 2010). The β_2AR also binds directly to AKAP5 (Dai et al., 2009; Fraser et al., 2000). More precisely, the ~80 residue long C-terminus of the β_2AR interacts with the N-terminal ~200 residues of AKAP5 (Dai et al., 2009). Moreover AKAP5 also binds to the SH3-GK region of PSD-95 and SAP97. PSD-95 links AKAP5 to AMPARs via TARPs and SAP97 via a direct interaction specifically to GluA1. It is thus conceivable that AKAP5 is necessary for stabilization of the β_2AR-GluA1 complex. This possibility would be of universal interest as it would support a general model of the formation of signaling complexes by multiple rather than single interactions.

However, our coimmunoprecipitation results (Figure 2-1) suggested that neither the D36 mutation nor the full elimination of AKAP5 affected the formation of the β_2AR-GluA1 complex. Coimmunoprecipitation is one of the well-established biochemical techniques for detecting *in vivo* protein-protein interactions. We immunoprecipitated β_2AR and analyzed the coIP of GluA1 subunit and β_2AR in WT, D36 and KO mice. Here we did not immunoprecipitate GluA1 and determined the coIP of β_2AR and AKAP5 because: 1) previously we showed the association of β_2AR and GluA1 by immunoprecipitation of β_2AR (Joiner et al., 2010); 2) we have found a substantial

fraction of GluA1 (80-90% of total GluA1) that is not β_2AR linked (Joiner et al., 2010) therefore β_2AR may be undetectable from immunoprecipitation of GluA1 in our conditions (data not shown). We detected only the association of β_2AR and GluA1 due to previous studies that have shown that β_2AR complex was not composed of NMDAR subunits and other metabotropic glutamate receptors (Joiner et al., 2010).

Although in our studies we did not detect the requirement for AKAP5 in the complex formation, we cannot completely rule out the possibility that AKAP5 plays a role in the association of the β_2AR / GluA1 signaling complex. There are several limitations in our current coimmunoprecipitation conditions. First, we did not successfully coimmunoprecipitate β_2AR by using antibodies against GluA1. Second, we detected the nonspecific binding by control IgG and the relatively stronger specific binding by β_2AR antibody (Figure 2-1A). Even though we subtracted the signals of nonspecific binds from the specific signals during quantification, the reduction of the total value may exclude the possibility of detecting significant changes among WT, D36 and KO samples. Third, our β_2AR antibody detected two bands in the coimmunoprecipitation samples but one band in the lysates. Whether or not both bands are specific would need further investigation. To draw the conclusion more carefully, we will need more supportive results in the future, such as colocalization analysis of β_2AR and GluA1 by immunostaining in D36 and KO mice.

Both AMPA receptors and β_2AR are localized in postsynaptic sites. Previously we have detected the accumulation of GluA1 subunit in PSD (Lu et al., 2007). However it is unclear whether β_2AR is concentrated in PSD. It is the first time that we looked at the localization of β_2AR in PSDs. In wild type mice, immunoblotting of β_2AR (H-20

antibody from Santa Cruz) detected a major 72 kDa band in the non-PSD fractions (Lys, P2 and Synaptosome). However, this 72 kDa band was disappeared with a ~100 kDa band enriched in PSD fractions (data not shown). There are majorly 3 specific bands observed by using commercial β_2AR antibodies: 40-50 kDa of unglycosylated receptor; 60-70 kDa of glycosylated receptor and 90-100 kDa of dimerized receptor. This ~100 kDa band was also the only band detected by the mouse monoclonal antibody from Dr. Kobika (Stanford Univ.). We believed that β_2AR was accumulated in PSD in the dimer form.

In addition to AMPARs, β_2AR also forms a complex with L-type calcium channel $Ca_v1.2$ in neurons (Davare et al., 2001). AKAP5 has direct interaction with both β_2AR and $Ca_v1.2$ (Dai et al., 2009; Hall et al., 2007). Therefore we detected in parallel whether or not neuronal L-type calcium channel $Ca_v1.2$ was accumulated in postsynaptic density. It is believed that neuronal $Ca_v1.2$ is localized in the postsynaptic sites but there is no direct evidence for its localization in PSD. Electron microscopy studies did not find $Ca_v1.2$ in PSD (Tippens et al., 2008). In our study, we isolated the different fractions of brain tissues by ultracentrifugation and sucrose gradient. The pre- and post-synaptic enriched fraction (synaptosome) was further extracted by 1% Triton-X100. The insoluble PSD fractions were concentrated although some proteins including $Ca_v1.2$ may be dissociated from PSD by Triton-X100. The results showed a slightly accumulation of $Ca_v1.2$ in PSD. This highly suggests the enrichment of $Ca_v1.2$ in postsynaptic density. The quantified comparison of $Ca_v1.2$ level in PSDs from WT, D36 and KO mice indicated no changes in postsynaptic $Ca_v1.2$ localization in AKAP5 mutant mice. This

findings is consistent with previous studies that postsynaptic $Ca_v1.2$ distribution is independent of interactions with AKAP5 (Biase et al., 2008).

When we looked at the protein compositions in PSDs from WT, D36 and KO mice, there were no changes in GluA1 and β_2AR components in PSD. It is consistent with our previous results that GluA1 was unchanged in D36 mice although majority of PKA subunits were dislocated from PSD (Lu et al., 2007).

In closing, neither truncated AKAP5 nor full length AKAP5 is necessary for the formation of β_2AR-GluA1 complex. We observed that β_2AR was accumulated in PSDs independent of AKAP5.

Figure 2-1. AKAP5 is dispensable for the complex formation between β_2AR and GluA1.

A. Forebrains from WT, AKAP5 D36, and AKAP5 KO mice were extracted with Triton X-100 and immunoprecipitated with 8 mg of antibodies against β_2AR (H-20) before immunoblotting for GluA1, β_2AR, and AKAP5. A mix of 1/3 WT, 1/3 D36 and 1/3 KO extract was used for control immunoprecipitation with 8 mg of non-immune rabbit IgG. **B**. Immunosignals were quantified by densitometry. The non-specific IgG signal was subtracted from the GluA1 signal. The ratio of GluA1 to β_2AR from 4 independent experiments was quantified and normalized to the WT signal from the same experiment. There is no statistically significant difference between genotypes.

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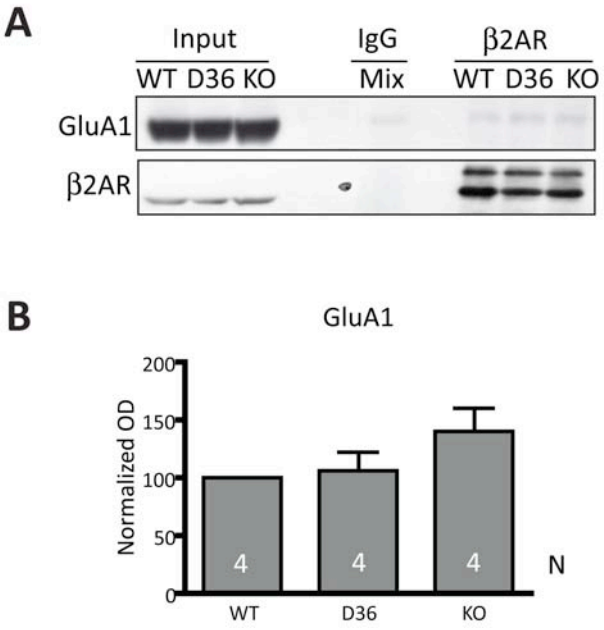
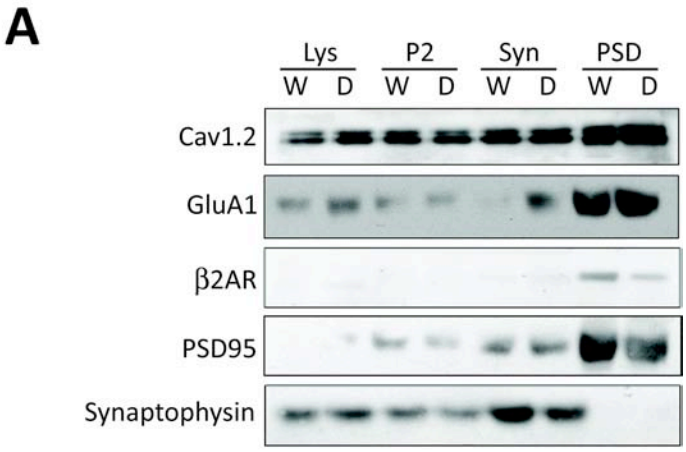

A
Input
IgG
β2AR
WT D36 KO
Mix
WT D36 KO
GluA1
β2AR
B
GluA1
Normalized OD
200
150
100
50
0
4
4
4
N
WT
D36
KO

Figure 2-2. **Effect of AKAP5 mutation on PSD components in WT vs D36 mice.**

A. Equal amount of protein fractions of crude lysate (Lys), P2, synaptosomal-enriched (Syn), and PSD from WT and D36 mice were separated by SDS-PAGE and immunoblotted for the indicated proteins.

B. PSD signals from D36 mice were quantified and normalized to the WT signal for each protein. The data represents the average ± SEM for independent experiments. (n is shown as indicated)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P2
Syn
PSD
W D W D W D W D
Cav1.2
GluA1
β2AR
PSD95
Synaptophysin

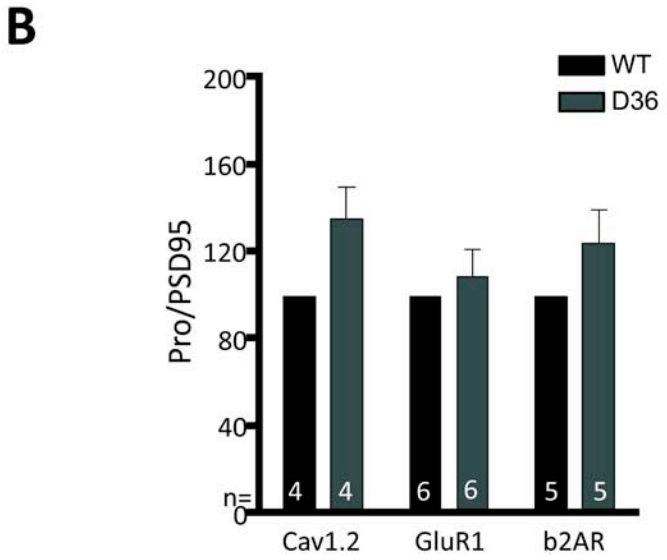
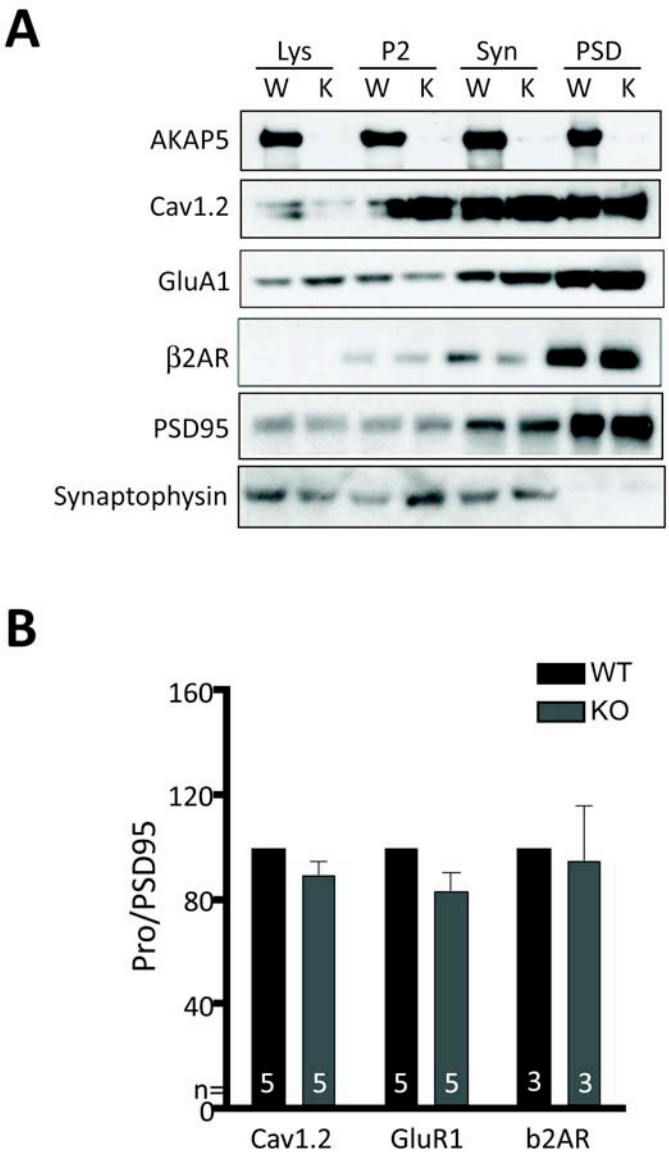

B
WT
D36
Pro/PSD95
200
160
120
80
40
0
n=
4 4 6 6 5 5
Cav1.2
GluR1
b2AR

Figure 2-3. Effect of AKAP5 knockout on PSD components.

A. Equal amount of protein fractions of Lys, P2, Syn, and PSD from WT and KO mice were separated by SDS-PAGE and immunoblotted for the indicated proteins.

B. PSD signals from KO were quantified and normalized to the WT signal for each protein. The data represents the average ± SEM for independent experiments (n as indicated).


A
Lys
P2
Syn
PSD
W K W K W K W K
AKAP5
Cav1.2
GluA1
β2AR
PSD95
Synaptophysin
B
WT
KO
160
120
80
40
0
Pro/PSD95
n=
5 5 5 5 3 3
Cav1.2
GluR1
b2AR

CHAPTER 3
ROLE OF AKAP5 IN THE PHYSIOLOGICAL FUNCTION OF β_2AR-GLUA1 SIGNALING COMPLEX

Rationale

GluA1 is responsible for the regulation of AMPARs surface expression and AMPARs mediated synaptic transmission. Phosphorylation of GluA1 favors the trafficking of AMPARs and regulates the physiological function of AMPARs, including higher order brain functions. Phosphorylation sites on Ser845 by PKA and Ser831 by PKC and CaMKII are two important sites with most focused studies. The double mutant of Ser845 and Ser831 has been found to block LTP induced by epinephrine (Hu et al., 2007). Our studies also found that AKAP5-anchored PKA activity is important for LTP and LTD (Lu et al., 2007; Lu et al., 2008).

10-15% of GluA1 is phosphorylated by PKA on Ser845 under basal conditions. After stimulation of cAMP signaling, phosphorylation of Ser845 is induced to a higher level and fosters surface expression of AMPARs (Ehlers, 2000; Man et al., 2007; Oh et al., 2006; Sun et al., 2005; Swayze et al., 2004), therefore upregulates synaptic plasticity. The association of β_2AR and GluA1 forms a highly localized stimulation of cAMP-PKA pathway. There was only a small fraction of β_2AR-linked GluA1 phosphorylated in response to β_2AR stimulation although a substantial fraction of GluA1 is available (Joiner et al., 2010).

Therefore it is interesting to test the specific role of AKAP5 during this selective GluA1 phosphorylation by stimulation of β_2AR and whether it is important for the AMPARs mediated synaptic transmission.

To address this question, we compared the changes of Ser845 phosphorylation in response to β_2AR stimulation among WT, D36, and KO mice. Then we tested the changes in θ-LTP between WT and D36 mice.

Results

Basal phosphorylation of Ser845 GluA1 in D36 and KO mice

AKAP5 anchors PKA to GluA1 for Ser845 phosphorylation. To observe the basal level of Ser845 phosphorylation in AKAP5 mutant mice, we homogenized the cortex fractions from WT, D36 and KO mice by 1% Trition X-100 buffer and cleared by ultracentrifugation. The pellet was resuspended by 1% SDS followed by ultracentrifugation. Both supernatant and pellet fractions were separated by SDS-PAGE and blotted by phospho-Ser845 (Millipore) and total GluA1 antibodies. A fraction of GluA1 is phosphorylated on Ser845 under basal conditions. This fraction was unchanged in adult D36 and KO mice as was the total amount of GluA1 (Fig 3-1 A, B), which is consistent with our previous observation (Lu et al., 2007).

Previous studies showed that only 10-15% of GluA1 are actually phosphorylated on Ser845 under basal conditions (Boehm et al., 2006; Oh et al., 2006), and it is unclear whether this phosphorylation occurs at postsynaptic sites. We further compared the basal

Ser845 phosphorylation in the PSD fractions from AKAP5 mutant mice (Figure 3-1 C). The signal quantification showed that GluA1 phosphorylation on Ser845 under basal conditions was unaltered in D36 mice (Figure 3-1 D), indicating that this phosphorylation does not depend on AKAP5 anchored PKA.

We also detected the basal phosphorylation of Ser845 and Ser831 in the acute cortical slices (data not shown). As observed earlier by our lab, basal phosphorylation levels for Ser845 were not statistically different in D36 and KO slices (mean ± SEM from 4 independent experiments are 100 ± 40.5% for WT (mean normalized to equal 100%), 87.0 ± 22.6% for D36, and 190.5 ± 40.5% for KO (the unexpectedly large values for KO slices were not significantly different from WT). Basal phosphorylation levels for Ser831 were also not statistically different in D36 and KO slices (100 ± 31.4% for WT, 109.9 ± 39.4% for D36, and 152.7 ± 53.7% for KO).

Increased phosphorylation of Ser845 GluA1

after β stimulation

Previously we have tested that the basal phosphorylation of Ser845 was unchanged in AKAP5 mutant mice. We then examined whether a β agonist isoproteronol (ISO) induced β2AR-cAMP/PKA signaling leads to phosphorylation of GluA1. The mouse acute prefrontal cortex (PFC) slices were incubated with 10 μM ISO for 2,5,15 mins or 15 min followed by 15 min washout. The phosphorylation of Ser845 was increased dramatically 2 min after ISO incubation (Figure 3-2). The elevated phosphorylation was maintained during 15 min washout of ISO after 15 min ISO

incubation. This is consistent with our electrophysiological findings that increased mEPSC responses were maintained after removal of ISO (Joiner et al., 2010).

Since adenylyl cyclase is part of the main β_2AR signaling pathway and is present in the β_2AR-GluA1 complex, we also tested whether ISO induced Ser845 phosphorylation was mediated through adenelyl cylase. Prefrontal cortex slices were treated with 10 μM ISO for 15 min in the absence or presence of 20 μM SQ22536, an adenylyl cyclase inhibitor. The increase of Ser845 phosphorylation induced by ISO was reduced by 42%, suggesting that it was largely mediated by adenylyl cyclase (Figure 3-2).

Reduced ISO-induced Ser845 phosphorylation

in D36 and KO mice

The GluA1 subunit of the AMPAR has two major phosphorylation sites, Ser845 (PKA site) and Ser831 (PKC/CaMKII site), which regulate GluA1 trafficking and function *in vitro* and *in vivo*. Acute forebrain slices from WT, D36, and KO mice were treated with vehicle or ISO for 15 min before Ser845 phosphorylation was determined by immunoblotting using a phosphospecific antibody. ISO treatment caused a dramatic increase in the phosphorylation of GluA1 at Ser845 (527 ± 108% of control, Figure 3-3. A and B). The stimulation of β_2AR by ISO caused an increase in Ser845 phosphorylation in D36 slices (Figure 3-3. A and B), but this increase was reduced by 46% compared to wildtype slices. Similarly complete knockout of AKAP5 reduced the increase in Ser845 phosphorylation by 63%.

We also monitored phosphorylation of Ser831, which occurs by PKC and CaMKII (Mammen et al., 1997; Roche et al., 1996) and could be indirectly triggered by β AR/G_s stimulation (e.g., augmented Ca^{2+} influx through NMDARs by PKA (Chalifoux and Carter, 2010; Skeberdis et al., 2006)). In fact, ISO increased Ser831 phosphorylation in wild type mice (221 ± 67.6% of control, Figure 3-3. C) but this increase was comparable in all three genotypes (Figure 3-3. A, C).

Reduced ISO-induced Ser845 phosphorylation

in β_1 KO and β_2 KO mice

Another study suggests that the β_1AR, but not β_2AR, regulates Ser845 phosphorylation (Vanhoose and Winder, 2003). We used β_1AR and β_2AR KO mice (from Dr. Brian Kobilka lab in Stanford University) to evaluate the role of both βARs in the PKA-mediated phosphorylation of GluA1 on Ser845. Treatment of acute forebrain slices with ISO for 10 min increased Ser845 phosphorylation by nearly 5-fold in WT slices but only 2 fold in β_2AR KO slices and not at all in β_1AR KO slices (Figure 3-4). This suggests both βARs are critical for effective stimulation of Ser845 phosphorylation by ISO with β_1AR apparently being indispensable.

Influence of fEPSPs after ISO stimulation

LTP induced by prolonged theta rhythm tetani (θ-LTP) at Schaffer collateral – CA1 synapses depends on β adrenergic stimulation (Gelinas and Nguyen, 2005; Hu et al., 2007; Katsuki et al., 1997; Thomas et al., 1996). This LTP is deficient in GluA1 S831A/S845A double KI mice (Hu et al., 2007). Therefore, we evaluated whether PKA

anchoring by AKAP5 is required for θ-LTP. As reported earlier by us (Joiner et al., 2010), in acute slices from WT mice ISO by itself increased fEPSP initial slopes in ~60% of the recordings (Figure 3-5 A) with little to no effect in the remaining ~40% (Figure 3-5 B). D36 slices never showed any significant increase in the postsynaptic response upon perfusion with ISO, suggesting that the ISO effect itself depends on AKAP5-anchored PKA under otherwise basal conditions.

In WT slices induction of θ-LTP increased fEPSPs by 57% if ISO by itself had initially no effect and by 35% otherwise (Figure 3-5). The reduced degree of potentiation in slices that showed an increase to the ISO perfusion suggests that this increase might occlude a portion of θ-LTP. Accordingly, the two regulatory mechanisms may share molecular mechanisms. Importantly, induction of θ-LTP increased the fEPSP response by only ~40% in D36 mice (Figure 3-5 C, D). Accordingly, θ-LTP is significantly higher in WT than D36 slices. This difference is independent of whether or not ISO itself lead to a potentiation of fEPSPs.

Discussion

Basal level of phosphorylation on GluA1 Ser845

AKAP5 is the prominent AKAP in rats and mice and enriched in post-synaptic density of neurons's dendritic spines (Carr et al., 1992). There is PKA binding site in the C-terminus of AKAP5 while PKC and PP2B bind with the N-terminus and middle part of AKAP5 respectively. Our previous studies (Lu et al., 2007) have shown that the majority of PKA was dislocated from PSD when PKA binding site was truncated in

AKAP5 D36 mice and the basal phosphorylation level of GluA1 Ser845 was unchanged in D36 whole brain lysate. Consistently we found here that the basal phosphorylation level of Ser845 in cortex and cortical slices were not affected in AKAP5 mutant mice (Figure 3-1. A B).

Moreover, it was the first time that we observed the Ser845 basal phosphorylation level in PSDs. In wild type mice, we clearly detected the enrichment of GluA1 and the relatively higher enrichment of phospho-Ser845 in PSDs (data not shown). This finding is supported by the electron microscopy observations that phospho-Ser845 GluA1 was mainly localized in PSD (unpublished data from Dr. Julius Zhu at Univ. of Verginia). However we did not detect the dramatic change of Ser845 basal phosphorylation in D36 PSDs compared to wild type (Figure 3-1. C D). Our results suggest that basal phosphorylation level of GluA1 Ser845 in PSD is not maintained by AKAP5-anchored PKA. The other AKAPs at PSD (such as MAP2B, yotiao) may play an important role in maintaining the basal Ser845 phosphorylation level.

We found no change in the basal Ser845 phosphorylation in KO mice. However, Tunquist reported a reduction in phosphorylation of Ser845 (51.2 ± 11%) under basal conditions and in LTD (Tunquist et al., 2008) in AKAP5 -/- mice, which is at variance to our own findings: no significant differences in basal Ser845 phosphorylation and normal LTD for our KO slices (Weisenhaus, 2010). It is possible that the details of the KO mice from both groups are different genetically. The other possibility is that the balance between phosphorylation and dephosphorylation by AKAP5 anchored PKA and PP2B is altered under various treatment conditions and were different in our hand vs Tunquist's (Tunquist et al., 2008) so that Tunquist found differences in KO when we did not.

Changes on Ser845 phosphorylation and θ-LTP

in D36 and KO mice after βAR stimulation

AKAP5 D36 and KO mice showed an ~ 50% reduction in ISO-stimulated Ser845 posphorylation. At the same time D36 mice exhibited a similar reduction in θ-LTP. Ser845 phosphorylation via PKA fosters GluA1 surface expression due to reduced endocytosis rate and enhanced re-insertion rate (Ehlers, 2000; Man et al., 2007). The augmentation of θ-LTP upon β_2AR stimulation is thus likely due to enhanced postsynaptic accumulation of GluA1-containing AMPAR, although Ser845 phosphorylation could also increase the activity of GluA1-containing AMPARs by upregulating their open probability (Banke et al., 2000)(but see (Joiner et al., 2010)).

The postsynaptic expression of θ-LTP is supported by the finding that PPF of fEPSPs was unaltered by θ-LTP induction for all interstimulus intervals tested (unpublished data from Hai Qian). Our current and previous (Joiner et al., 2010) results collectively argue that the main effect of β adrenergic stimulation on synaptic transmission of glutamatergic synapses on pyramidal neurons is via AMPAR trafficking. Although it is tempting to speculate that Ser845 plays thus an important role in θ-LTP, final proof will have to await additional experiments.

In addition to the increase of Ser845 phosphorylation induced by β_2AR stimulation, we also observed the increase of Ser831 phosphorylation in wild type cortical slices after ISO stimulation but no changes in the phosphorylation level in D36 and KO mice. NE treatment on hippocampal slices and fox urine exposure also caused increased Ser831 phosphorylation (Hu et al., 2007). GluA1 Ser831 is phosphorylated by

PKC and CaMKII (Mammen et al., 1997; Roche et al., 1996). Phosphorylation of Ser831 can be indirectly induced by βAR signaling through the regulation of Ca^{2+} permeability of NMDARs by PKA. NMDARs interact more directly with yotiao (AKAP9) (Feliciello et al., 1999; Westphal et al., 1999), although AKAP5 might be linked to NMDAR via PSD-95 (Colledge et al., 2000). Thus AKAP5 might be fully dispensable for PKA mediated regulation of Ca^{2+} permeability through NMDARs. These results indicate that the deficit in GluA1 Ser845 phosphorylation in AKAP5 D36 and KO mice is not a universal deficit in PKA-mediated phosphorylation in dendritic spines but limited to selected phosphorylation sites as regulation of NMDARs by PKA appears unaffected.

β_1AR or β_2AR dependent GluA1 phosphoryaltion

Although we initially detected ISO-induced β_2AR-dependent phosphorylation of GluA1 on Ser845 in primary hippocampal cultures (Joiner et al., 2010), another study suggest that β_1AR is also involved in the Ser845 phosphorylation (Vanhoose and Winder, 2003). Therefore we detected ISO-induced phosphorylation of Ser845 in acute cortical slices of β_1AR KO and β_2AR KO mice (Figure 3-4). The results showed both adrenergic receptors were critical for effective stimulation of Ser845 phosphorylation by ISO with β_1AR apparently being indispensable. Moreover we tested the effect of highly selective inhibitors CGP-20712 (β_1AR antagonist) and ICI-118551 (β_2AR antagonist) on ISO-induced Ser845 phosphorylation in acute cortical slices (unpublished data from Hai Qian). These results suggest that both βARs are actually required for effective stimulation of phosphorylation by ISO.

In summary our results demonstrate that AKAP5-anchored PKA activity is responsible for the increased Ser845 phosphorylation in response to βAR stimulation. ISO-induced θ-LTP was reduced in D36 mice. Complete loss of AKAP5 also reduced the ISO stimulated phosphorylation level of Ser845. However, whether this change would affect ISO induced LTP is currently unknown.

Figure 3-1. Basal GluA1 Ser845 phosphorylation unchanged in brain lysates and PSDs from AKAP5 D36 and KO mice.

A. Cortex fractions from WT, D36 and KO mice were solubilized with 1% Trition X-100 in the presence of phosphatase inhibitors and cleared by ultracentrifugation. The pellets were resuspended by 1% SDS and cleared by ultracentrifugation. Equal amount of both samples were separated by SDS-PAGE and immunoblotted for phospho-Ser845 GluA1 and total GluA1.

B. The pS845/GluA1 signal was quantified for each mouse. Data shown represents the average ± SEM for n=3 experiments.

C. Equal amount of PSD protein fractions purified from WT and D36 or KO mice forebrains were immunoblotted for phospho-Ser845 GluA1 and total GluA1.

D. The pS845/GluA1 signals in the PSD for the WT and D36 mice were quantified. The data represents the average ± SEM for n=3 experiments.

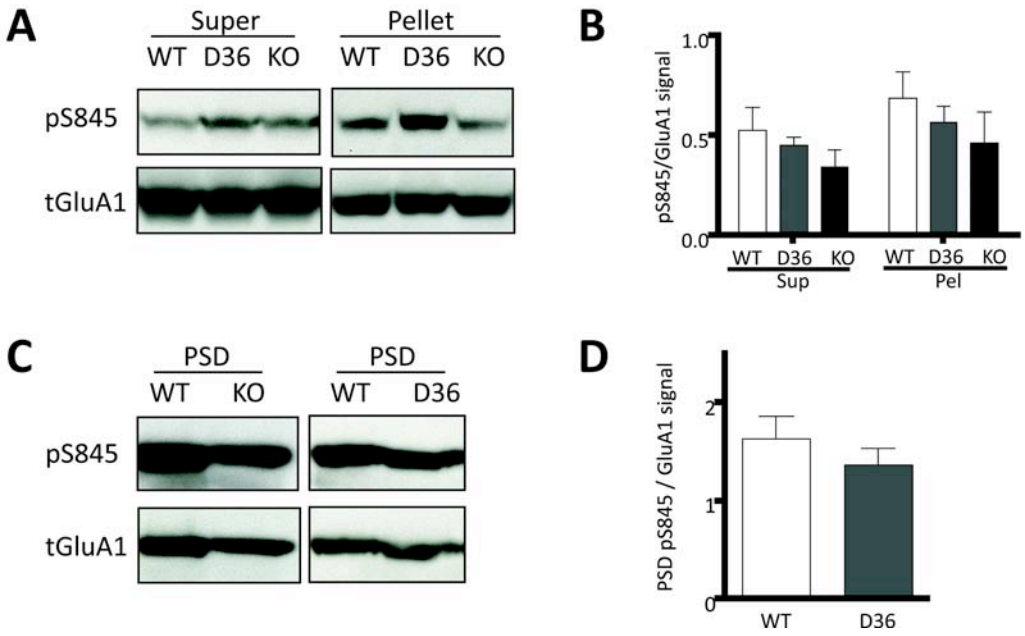

A
Super
Pellet
WT D36 KO WT D36 KO
pS845
tGluA1
B
pS845/GluA1 signal
1.0
0.5
0.0
WT D36 KO WT D36 KO
Sup
Pel
C
PSD
PSD
WT KO WT D36
pS845
tGluA1
D
PSD pS845 / GluA1 signal
2
1
0
WT
D36

Figure 3-2. Time course of ISO-stimulated GluA1 Ser845 phosphorylation in PFC slices.

A. Acute mouse PFC slices were treated with 10 μM ISO for 2-15 min, followed by a 15 min wash out period if indicated ("wash") or in the presence of an adenylyl cyclase inhibitor SQ22536 ("SQ"). Control group was left unstimulated ("control"). Slices were extracted with Triton X-100 and non-soluble material removed by ultracentrifugation. GluA1 was immunoprecipitated before mmunoblotting with antibodies against phosphorylated S845 (top) and total GluA1 (bottom).

B. Immunosignals were quantified by densitometry. Phospho-S845 (pS845) signals were corrected with respect to total GluA1 signals. Results are averages ± SEM from independent experiments (n as indicated within bars). ISO increased the phosphorylation within 2 min to near maximum. 15 min wash out did not reduce phosphorylation.

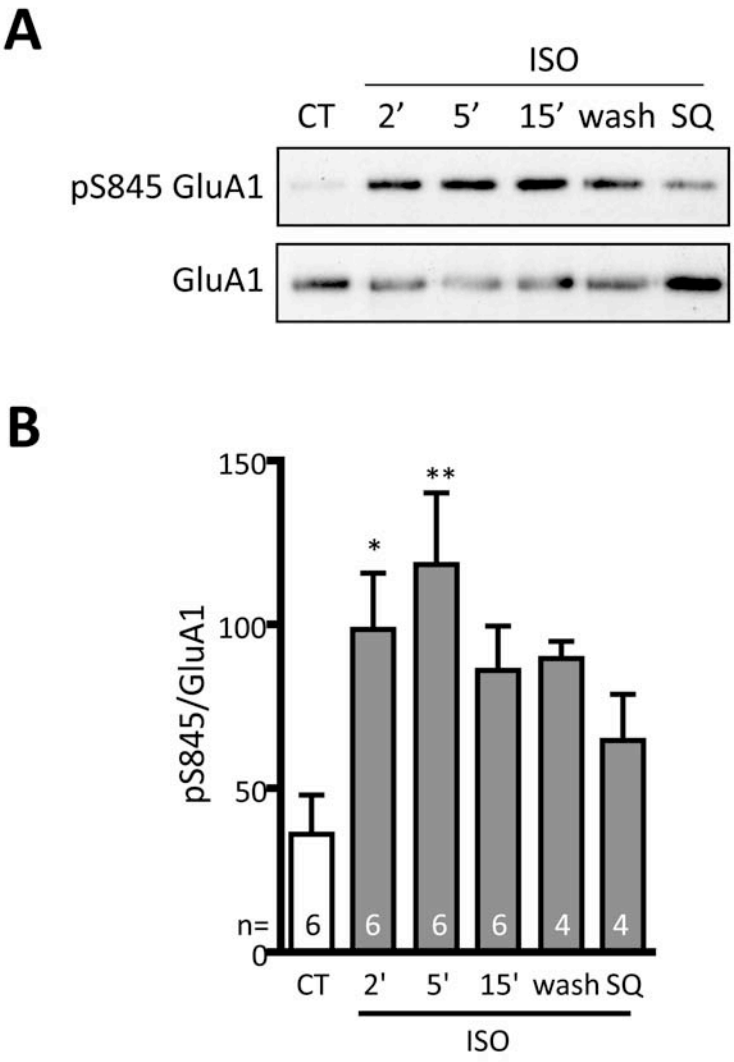

A
ISO
CT
2’
5’
15’
wash
SQ
pS845 GluA1
GluA1
B
150
100
50
0
pS845/GluA1
*
**
n=
6
6
6
6
4
4
CT
2'
5'
15'
wash
SQ
ISO

Figure 3-3. AKAP5-anchored PKA activity is required for phosphorylation of GluA1 on Ser845 upon ISO stimulation

A. Acute forebrain slices from 8 weeks old WT C57BL/6, AKAP5 D36, and AKAP5 KO mice were incubated with vehicle or ISO (10 μM) for 15 min before extraction, immunoprecipitation of GluA1, and immunoblotting with antibodies against phospho-S845 (top), phospho-S831 (middle) and total GluA1 (bottom).

B-C. Quantified immunosignals of phospho-S845 (**B**) and phospho-S831 (**C**) were corrected with respect to total GluA1 signals. Graphed are averages ± SEM of ISO-treated samples as % of control group (equaling 100%) for each genotype from independent experiments (N as indicated). * indicate $p<0.05$, one-way ANOVA.

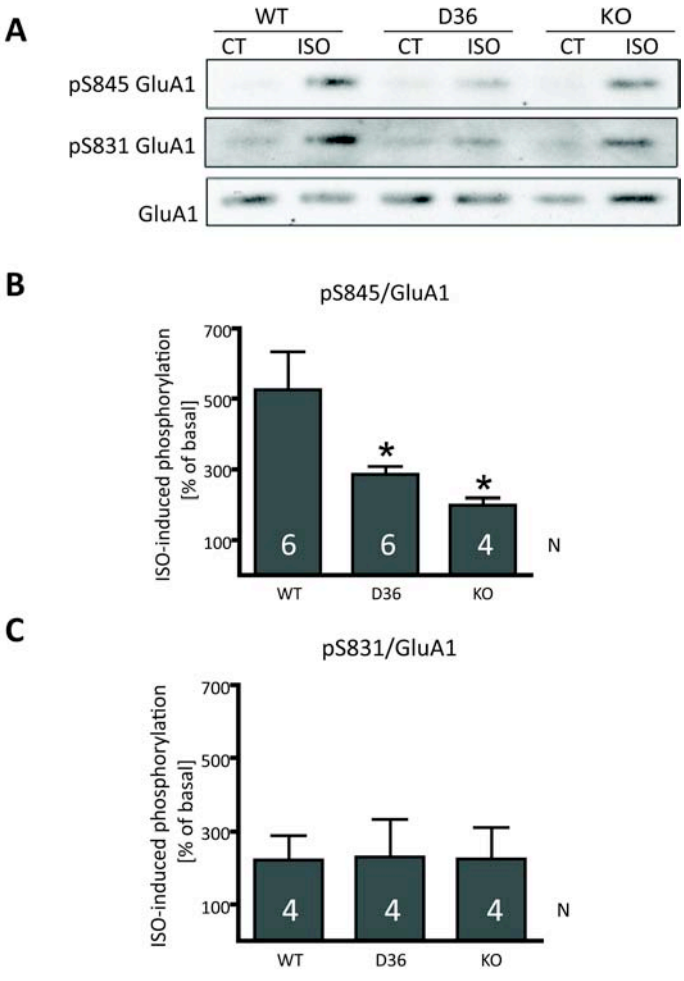

A
WT
D36
KO
CT
ISO
CT
ISO
CT
ISO
pS845 GluA1
pS831 GluA1
GluA1
B
pS845/GluA1
ISO-induced phosphorylation
[% of basal]
700
500
300
100
6
6
4
N
WT
D36
KO
C
pS831/GluA1
ISO-induced phosphorylation
[% of basal]
700
500
300
100
4
4
4
N
WT
D36
KO

Figure 3-4. Both β_1AR and β_2AR mediate the phosphorylation of GluA1 on Ser845 upon ISO stimulation

A. Acute forebrain slices from 8 weeks old WT C57BL/6, β_1AR KO, and β_2AR KO mice were incubated with vehicle or ISO (10 μM) for 15 min before extraction, immunoprecipitation of GluA1, and immunoblotting with antibodies against phospho-S845 (top) and total GluA1 (bottom).

B. Quantified immunosignals of phospho-S845 were corrected with respect to total GluA1 signals. Graphed are averages ± SEM of ISO-treated samples as % of control group (equaling 100%) for each genotype from 4 independent experiments. * indicates $p<0.05$, ** indicates $p < 0.01$, Tukey's test after one-way ANOVA.

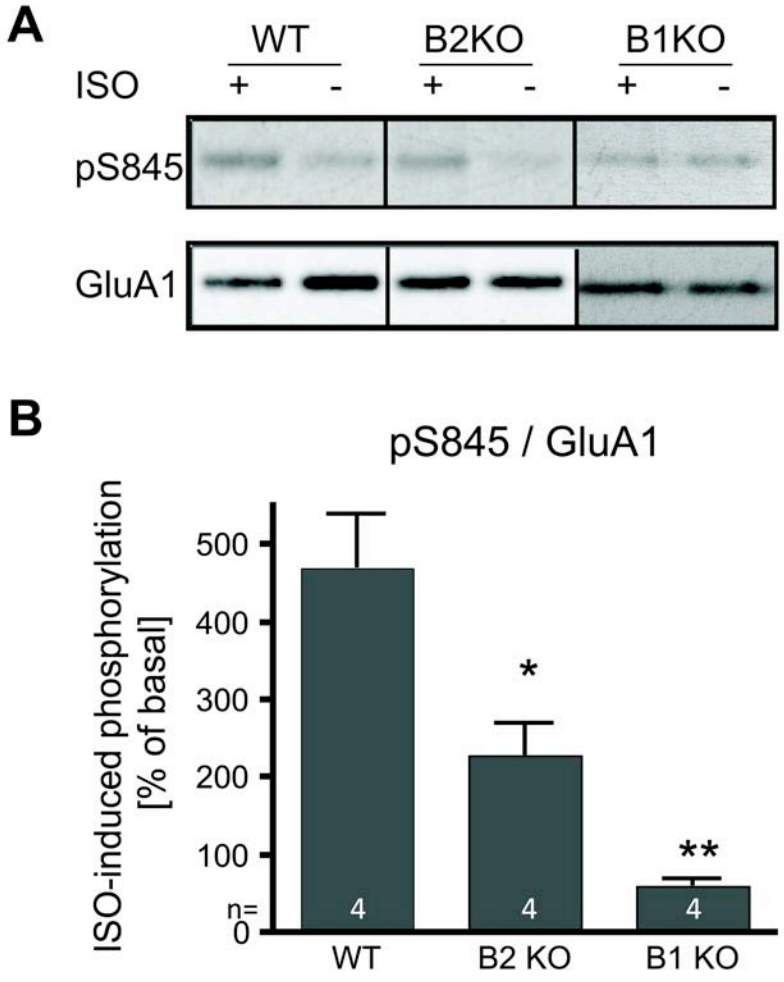
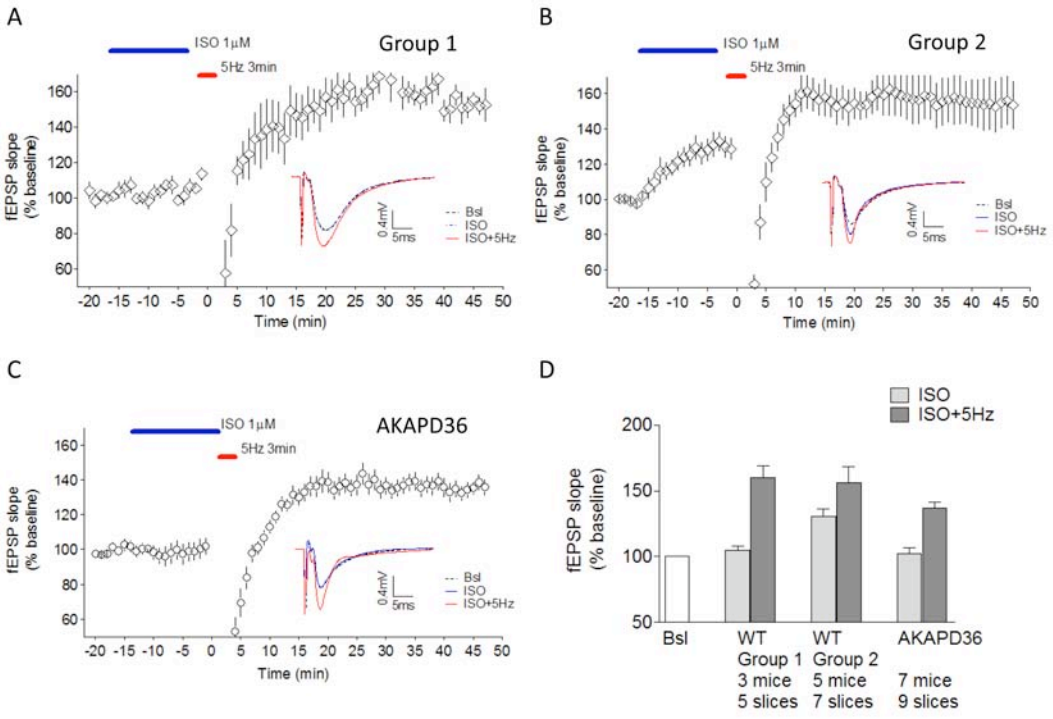

A
WT
B2KO
B1KO
ISO
+
-
+
-
+
-
pS845
GluA1
B
pS845 / GluA1
ISO-induced phosphorylation
[% of basal]
500
400
300
200
100
0
n=
4
4
4
*
**
WT
B2 KO
B1 KO

Figure 3-5. ISO-induced increases in basal synaptic transmission and θ-LTP by a 5 Hz / 3 min tetanus in the presence of ISO are impaired in AKAP5 D36 mice.

A-C. Time courses of fEPSPs before and after perfusion with ISO ((1 μM; upper bar) and delivery of the tetanus (lower bar) from recordings without (**A**) and with (**B**) an ISO baseline response in WT slice and from D36 slices (**C**). Shown are averages of initial slopes of fEPSP starting after baseline had stabilized. Inserts: examples of fEPSPs before (dashed lines) and after (solid lines) of θ-LTP induction. Graphed are averages of 10 consecutive fEPSPs recorded at the indicated times.

D. Summary data of θ-LTP. The baseline (Bsl) is the average of the fEPSP initial slopes from each individual experiment during the 5 min immediately preceding start of the ISO application and equaled 100% for each experiment. Compared to the interleaved WT recordings, the θ-LTP levels in D36 mice were significantly lower ($p < 0.05$).

(The experiments were performed by Hai Qian.)


A
ISO 1μM
5Hz 3min
Group 1
fEPSP slope (% baseline)
Time (min)
Bsl
ISO
ISO+5Hz
0.4mV
5ms
B
ISO 1μM
5Hz 3min
Group 2
fEPSP slope (% baseline)
Time (min)
Bsl
ISO
ISO+5Hz
0.4mV
5ms
C
ISO 1μM
5Hz 3min
AKAPD36
fEPSP slope (% baseline)
Time (min)
Bsl
ISO
ISO+5Hz
0.4mV
5ms
D
ISO
ISO+5Hz
fEPSP slope (% baseline)
Bsl
WT Group 1 3 mice 5 slices
WT Group 2 5 mice 7 slices
AKAPD36 7 mice 9 slices

CHAPTER 4
BEHAVIORAL ANALYSES ON AKAP5 MUTANT MICE

Rationale

Adrenergic receptors mediates many important neuronal activities, including learning and memory, sleep cycle, etc. Many studies have found the involvement of β-adrenergic signaling in fear memory. The mechanism of fear extinction is not clear yet although lots of studies suggest the multiple level of regulation involving hippocampus, norepinephrine, and PKA. The effect of adrenergic signaling on fear memory is still controversial (Cain et al., 2004; Ouyang and Thomas, 2005). It appears that in fear extinction, as in fear acquisition, adrenergic activation promotes and adrenergic blockade impairs memory consolidation.

Stimulation of β adrenergic receptor activates PKA, which phosphorylates a series of downstream targets. PKA is localized to postsynaptic sites by AKAP5. We have demonstrated that the β_2 adrenergic receptor forms a complex with the GluA1 subunit of AMPARs (Joiner et al., 2010) while AKAP5 binds to GluA1 via PSD95 and SAP97.

Experience induced neuronal plasticity underlies the molecular mechanism for higher order cognitive processes, such as learning and memory. Trafficking of AMPA receptors into postsynaptic sites contributes to neuronal activity through the regulation of phosphorylation and dephosporylation. PKA mediated phosphorylation of Ser845 on GluA1 mediates surface expression of AMPARs, synaptic transmission, and memory formation.

In the previous chapters we have found that AKAP5-anchored PKA is important for the Ser845 phosphorylation and LTP in response to noradrenergic stimulation. The phosphorylation of Ser845 together with Ser831 play important functions in emotion-involved learning and memory (Hu et al., 2007). However it is unknown that whether AKAP5 anchored PKA or complete loss of AKAP5 has an effect on the higher order brain functions, especially emotional arousal associated memory. The purpose of this section is to test the role of AKAP5 in fear memory.

Previously we have detected the neuronal plasticity and some behavioral responses in our AKAP5 mutant mice. D36 and KO mice have very limited and specific phenotypes with normal basal synaptic transmission and normal behaviors. LTP is nearly absent during early development (P12-14) and in adult D36 mice while LTD is impaired at P12-14 in D36 mice (Lu et al., 2007; Lu et al., 2008; Weisenhaus et al., 2010). Our AKAP5 KO mice had normal LTP and LTD likely because the deletion of AKAP5 eliminated not only PKA but also PP2B anchoring, causing a milder phenotype than the D36 mutation (Weisenhaus et al., 2010). However another group found deficits in LTD, not LTP in their AKAP5 KO mice (Tunquist et al., 2008). General behavior (e.g., open field) and learning (e.g., Morris water maze, novel object recognition, operant conditioning) is normal in D36 mice but reversal learning of the operant task is delayed although it still occurs (Weisenhaus et al., 2010).

In the following studies we will mainly detect the contextual and cued fear memory and extinction in D36 and KO mice. Both genotypes have litter mated C57BL6 mice as the wild type control.

Results

Normal locomotor activities in D36 and KO mice

In order to test whether AKAP5 plays a role in the motor activity, both AKAP5 KO and D36 with their litter mated controls received an open field test for 30 minutes. Mice were observed and analyzed by counting the numbers of different types of activity (jump, vertical but no jump, or ambulatory movements) using an automated system (Med Associates). Both KO and D36 groups compared to wild type (Figure 4-1. A and B) expressed comparable levels of general exploratory locomotion ($p > 0.05$, T test).

In addition, Figure 4-1.C and D showed total distance traveled during a 10 min period in mutant AKAP5 mice and the wild type controls. Analysis revealed no difference between the groups ($p > 0.05$, ANOVA).

Normal anxiety-related behavioral responses in D36 and KO mice

Anxiety and fear are very similar phenotypes in response to the startle stress. Different from fear, anxiety is a common reaction to stress without a noticeable triggering stimulus and helps one cope with future negative events. To detect whether AKAP5 is important for the anxiety-related behavior, we performed open-field exploration test and dark-light test.

Mice were allowed to freely explore an open field area. Two factors are suggested to influence anxiety-like behavior. One is the physical isolation from cage mates during the test. The other is the stress from facing the unprotected novel

environment. Typically mice tend to spend much more time exploring the periphery of the arena than the center area. Mice spending significantly more time in the center area would demonstrate anxiolytic-like behavior. In this experiment, we defined the center area as 80% of the whole area while the periphery as 20% of the whole area. Mice spent relatively less time (60%) in the center in wild type group (n=8, Figure 4-2. A). No significant change was observed in the percentage of time spent in the center among AKAP5 KO, D36 and wild type groups (n=8 for each group, $p > 0.05$, ANOVA test) (Figure 4-2. A). The results suggest normal anxiety response in both AKAP5 KO and D36 mice.

Dark-light exploration test is another type of examination for anxiety-like behavior in mice. When mice are exposed to a novel environment including a dark (protected) compartment and a light (unprotected) compartment, their exploratory activities may be inhibited by the conflict between risk avoidance and exploratory drive. Most mice show a preference for the dark compartment. We measured the time spent in each compartment during a 30 min session to assess anxiety-related behavior. Figure 4-2. B and C showed the percentage of time spent in the light compartment for D36 / wild type group (n= 18, Figure 4-2. B) or KO / wild type group (n=7, Figure 4-2. C). In both groups wild type mice spent less time (30-35%) in the light. There is no significant increase in time spending for D36 and KO mice ($p > 0.05$, T test). Consistent with the results in open field test, anxiety-related behaviors were normal in AKAP5 D36 and KO mice compared to litter mated wild type mice.

Delayed contextual fear extinction in D36 mice

Adrenoreceptors are believed to be involved in the formation and storage of fear memory. In order to examine the role of AKAP5 on learning, memory consolidation, and extinction, we performed several fear conditioning tests. The first one is to analyze the hippocampus-related contextual fear conditioning in D36 and wild-type littermates. In this paradigm, mice were trained by giving 2 foot-shocks (0.7 mA, 1sec) with 1 min interval after 3 min pre-exposure for a novel chamber. The long-term memory consolidation was tested 24 hr later in the same chamber. Fear extinction was examined for 7 continuous days.

A strong training paradigm may cause the difference in memory between the two groups undetectable because freezing is effectively saturated (a 'ceiling effect') (Shan et al., 2008). Here we used a relatively weak paradigm to train the mice and got around 30% freezing immediately after training (Figure 4-3. A). Both D36 and wild-type littermates exhibited similar hippocampus-dependent contextual memory after 1d (wildtype 59.40 ± 4.02% vs. D36 67.29 ± 4.79%, t test $p = 0.43$) (Figure 4-3. B).

Then we wondered whether AKAP5 anchored PKA would affect extinction of contextual memory, an active form of forgetting in which the animal learns that the context is no longer associated with a shock. The D36 and wild-type littermate mice, trained for contextual memory, were returned to the training apparatus and observed the freezing of 5 min for 7 continuous days. Freezing differed between two groups during the extinction test ($F_{(1, 147)}=5.739$, $p = 0.0178$; Figure 4-3. C).

Cued fear conditioning in D36 mice

Previous studies on PKA-AKAP interaction inhibitors showed that AKAP-anchored PKA is necessary for consolidation of auditory FC within the first few hours (Moita et al., 2002; Schafe and LeDoux, 2000). Thus we tested whether auditory FC was altered in AKAP D36 vs. control WT litter mates.

The auditory fear memory (assessed by freezing behavior in response to the CS alone) was evaluated in D36 and wild-type littermate mice as shown in Figure 4-5. Mice were conditioned to the CS by three pairs of tone-foot shock (tone: 5 kHz, 90 dB, 30 sec, 1 min apart; shock: 0.7 mA 1 sec at the end of each tone) on day 1 and received a memory reactivation trial by the same tone without shock 24 hrs later in a chamber with shape, floor, and smell that differed from the conditioning chamber. Similar to contextual FC, the shocks caused the same level of freezing in WT and D36 mice, indicating normal sensation of the shock and normal spontaneous fear reaction (Figure 4-5 B). After 24 hrs, freezing was minimal in the altered chamber before tone, but the tone induced nearly 80% freezing in D36 and WT mice (Figure 4-5 C). The outcome of these auditory FC tests is different from earlier findings that auditory FC requires AKAP-anchored PKA for expression several hours after the conditioning (Moita et al., 2002; Schafe and LeDoux, 2000).

Because extinction was delayed in D36 mice in the contextual paradigm we tested whether this was perhaps also true for extinction of cued FC. Following memory retrieval mice were tested auditory fear extinction on day 4, 8 and 16 by giving four CSs with variable intervals and analyzing the freezing during each tone (Figure 4-5. D) or average of the four freezing times during each tone (Figure 4-5. E). On the day 17, mice

received a single US (0.7mA, 1s). Exposure to the US following memory extinction has been shown to reinstate the expression of the original fear memory (Bouton and Bolles, 1979). The fear memory was then tested again 24 hr later (day 18). Analysis revealed that mice from both groups showed no significant different freezing response ($F_{(1,109)} = 3.464$, $p = 0.065$; Figure 4-5. E). However when we analyzed the individual freezing response during each CS on the different test days, D36 mice showed a tendency for higher freezing during the consecutive tests: day 4 $F_{(1, 104)} = 6.659$, $p = 0.011$; day 8 $F_{(1, 104)} = 3.407$, $p = 0.0678$; day 16 $F_{(1, 64)} < 1$, $p = 0.4081$; day18 $F_{(1, 68)} = 6.00$, $p = 0.0168$; total $F_{(1, 366)} = 13.58$, $p = 0.0003$ (Figure 4-5. D). Reinstatement on day 17 by an unsignaled tone in the test chamber resulted in a slightly larger freezing level in D36 vs. WT mice, which was not statistically significant but could indicate that extinction was less complete for D36 than WT mice allowing for a somewhat higher freezing level after reinstatement.

Contextual and cued fear extinction in KO mice

We also detected contextual fear memory on AKAP5 KO mice and wild-type littermates. After the training of 2 trials of 0.7mA 1S foot-shock, both groups showed similar level of freezing 24 hr later (t test, $p=0.10$, Figure 4-6 A, B). The extinction of contextual fear was tested and analyzed as for D36 mice. There was no significant difference in freezing between KO and WT mice ($F_{(1,70)} < 1$, $p = 0.47$; Figure 4-6 C).

Next, we examined the effects of AKAP5 knockout on cued fear expression and subsequent extinction learning. Mice (KO n = 9; WT n = 7) were conditioned to the CS by three pairs of tone-foot shock (tone: 5 kHz, 90 dB, 30 sec, 1 min apart; shock: 0.5 mA

1 sec at the end of each tone) on day 1 and received a extinction training trial by 20x CS only (5 kHz, 90 dB, 30 sec with 5 sec interval) 24 hrs later in a chamber B with shape, floor, and smell that differed from the conditioning chamber A. The freezing time during each tone was consistently reduced from 60% to 40% in both KO and WT group (Figure 4-7). The fear response level was tested on day 3 by a single tone (5 kHz, 90 dB, 30 sec) in chamber B. The percentage of freezing time during the tone was similar in both groups and slightly increased from day 2 (Figure 4-7).

Discussion

Contextual and cued fear conditioning

Because it is well established in humans that β adrenergic signaling augments arousal and emotional learning (Berman and Dudai, 2001; Cahill et al., 1994; Hu et al., 2007; Minzenberg et al., 2008; Nielson and Jensen, 1994; Stranger and Dolan, 2004; Stranger et al., 2003) and PKA is the major effector downstream of β AR signaling, we were surprised to find that contextual and cued FC were not impaired in D36 mice.

Hu et al reported that intraperitoneal injection of epinephrine augments contextual FC in rodents (Hu et al., 2007). They used an uncommon 3-day protocol to test the contextual fear conditioning of mice. After injection of epinephrine, mice were given very short pre-exposure (2min) to the conditioning chamber on day 1 and immediately given the foot shock after putting into the same chamber on day 2. The changes of the mice motion on day 3 suggested that epinephrine reduced the threshold for fear memory therefore fostered contextual fear conditioning. It is unclear whether epinephrine causes

this effect via the CNS or indirectly through upregulation of cardiovascular systems. More interestingly, GluA1 S831/845A double mutant mice did not show epinephrine response in this pre-exposure FC paradigm. This suggests phosphorylation of one or both sites is important for the contextual FC regulation.

Our D36 mice showed reduced level of increasing on Ser845 phosphorylation after activation of βAR by ISO. They would mimic GluA1 S845A mutant with partial phosphorylation. We expected to observe the similar contextual FC results when we followed the same protocol as Hu did. However, we did not detect any changes in D36 mice after systemic and intracerebroventricular (ICV) infusion of ISO (data not shown). There are several possibilities for the explanation: ISO may not have exactly the same central effect as epinephrine; mice may have different genetic background. It may also suggest that both Ser845 and Ser831 phosphorylation are important for the regulation of contextual FC.

Although it is widely accepted that noradrenergic signaling manipulates fear conditioning, how and where the modulation happens are still not clear. Number of studies tested the changes of FC by using β blocker propranolol to block noradrenergic pathway yet got variable results (Cain et al., 2004; Do Monte et al., 2008; Grillon et al., 2004; Ji et al., 2003; Murchison et al., 2004; Qi et al., 2008). It seems that the effect of propranolol on FC is time-specific and site-specific. The different experimental protocols for FC training, retrieval and testing may also affect the outcomes.

Fear extinction and reconsolidation

Extinction and reconsolidation are two independent processes although both happen during the retrieval of the memory. Fear extinction appears as extinguished fear responses during repeated exposure to the CS (tone or context) in the absence of the US (foot shock). Mechanism of fear extinction is unknown. PKA, CaMKII and NMDARs are involved in fear extinction (Szapiro et al., 2003). Infralimbic prefrontal cortex is believed to be a site of extinction consolidation. Activation of noradrenergic signaling in this region strengthens the extinction memory (Mueller et al., 2008). However systematic injection of propranolol caused reduced fear expression but not extinction (Rodriguez-Romaguera et al., 2009).

Our D36 mice showed reduced rate of extinguished contextual fear during repeated exposure to the conditioned chamber. This may suggest AKAP5-anchored PKA activity facilitates fear extinction or impairs reconsolidation. Since we expect downstream of adrenergic signaling was blocked due to loss of PKA anchoring in D36 mice, this finding is against the clinical use of propranolol as a treatment for PTSD (Kindt et al., 2009; Pitman et al., 2002) although the studies on propranolol are controversial.

Memory reconsolidation is a process in which the retrieval of the memory renders the memory labile and susceptible to amnestic treatment (Nader et al., 2000a, b). Noradrenergic blockade by propranolol disrupts the reconsolidation of auditory fear conditioning in rats (Debiec and LeDoux, 2004, 2006). In their studies (Debiec and LeDoux, 2004), systemic and amygdala injected propranolol given right after a single

CS for memory retrieval on day 2 (1 day after FC) reduced freezing measured on day 3, 9, 16; this could be due to enhanced extinction or reduced reconsolidation.

When we tried to test the similar effect in D36 mice, we did not observe the significant changes in freezing between wild type and D36 but a tendency of higher freezing in D36 mice. Most of the studies on reconsolidation were done by pharmacological manipulation. However in our study, we used genetic manipulated mice that would have interfered PKA regulation during the process of conditioning, information storage and consolidation, extinction, and reconsolidation. It is possible that the changes in reconsolidation were hidden or overcome by other changes during the learning process. In the future we should find a better way to mimic this genetic manipulation or limit its influence on the results.

In our fear conditioning studies, we observed the delayed contextual fear extinction but normal fear consolidation in D36 mice. This would fit the model of regulation on fear conditioning and extinction by local inhibition (Ehrlich et al., 2009). The inhibitory interneurons in the lateral amygdala are activated by the acquisition of extinction and inhibit the fear responses mediated by pyramidal cells (Li et al., 2009; Li et al., 2007; Likhtik et al., 2008). GluA2-lacking AMPA receptors are involved in the synaptic plasticity of interneurons in the amygdala (Mahanty and Sah, 1998). Therefore it is possible that AKAP5-anchored PKA activity plays a major role in the potentiation of interneurons during fear extinction. Further investigations would be needed to test this possibility.

In conclusion, we have shown that genetic removal of AKAP5 anchored PKA changed slightly the contextual fear extinction. When complete loss of AKAP5, we did not detect strong phenotype in fear memory and extinction.

Figure 4-1. Spontaneous locomotor activities are normal in AKAP5 D36 and KO mice

A, B. Open field analysis of D36 and KO mice vs. their respective littermate WT mice. Jumping, general vertical (standing up), and ambulatory activities were indistinguishable between D36 (**A**) or KO (**B**) and WT littermate mice.

C, D. Ambulatory distances traveled over 10 min intervals after being placed into the novel chamber were virtually identical between D36 (**C**) or KO (**D**) and WT littermate mice.

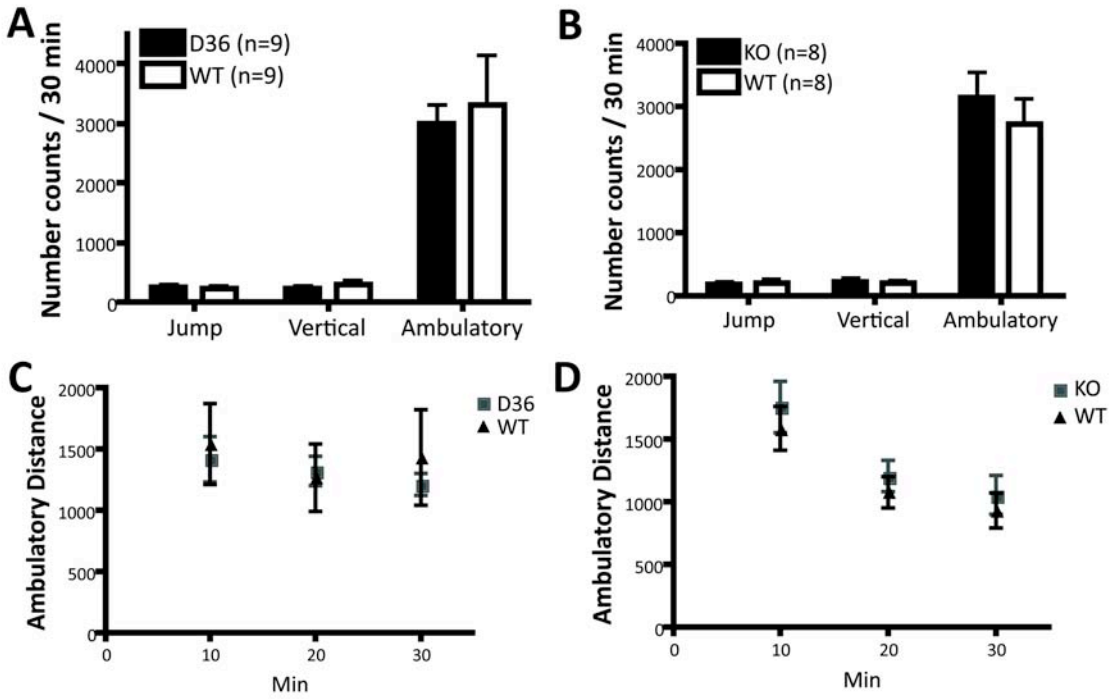

A
Number counts / 30 min
D36 (n=9)
WT (n=9)
4000
3000
2000
1000
0
Jump
Vertical
Ambulatory
B
Number counts / 30 min
KO (n=8)
WT (n=8)
4000
3000
2000
1000
0
Jump
Vertical
Ambulatory
C
Ambulatory Distance
D36
WT
2000
1500
1000
500
0
0
10
20
30
Min
D
Ambulatory Distance
KO
WT
2000
1500
1000
500
0
0
10
20
30
Min

Figure 4-2. Anxiety-related responses are normal in AKAP5 D36 and KO mice.

A. Open field analysis of WT, D36 and KO mice. Time spent in the center of the arena (80% of the whole area) over 30 min were the same for D36 and KO vs. WT mice (n=6 for each group).

B-C. Dark-light preference analysis. Time spent in the light chamber was indistinguishable between D36 (n=18, **B**) or KO (n=7, **C**) and WT littermate mice. All data are averages ± SEM.

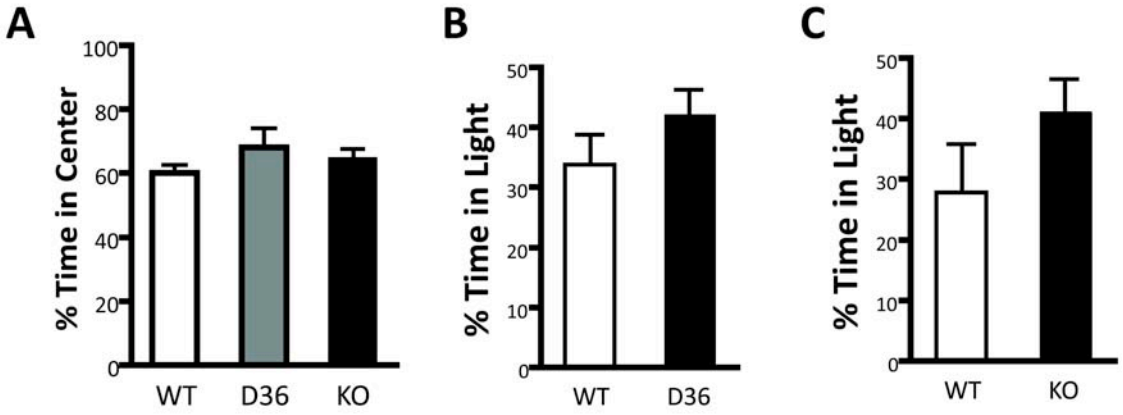

A
% Time in Center
100
80
60
40
20
0
WT
D36
KO
B
% Time in Light
50
40
30
20
10
0
WT
D36
C
% Time in Light
50
40
30
20
10
0
WT
KO

Figure 4-3. Extinction of contextual fear was delayed in AKAP5 D36 mice.

All data in this figure are the combined averages ± SEM from 2 independent experiments with two different cohorts of D36 and WT littermate mice (cohort 1: n=6 for D36 and 7 for WT; cohort 2: n=4 for D36 and 6 for WT).

A. Contextual FC on Day 1. Animals received two footshocks (0.7 mA, 1s) with one min interval after 3 min in the conditioning/test chamber, before being transferred into their home cages. Shown are averages ± SEM of times spent frozen for each min.

B. Contextual fear recall 24 hr after training during exposure to the conditioning chamber for 5 min. Shown are averages ± SEM of times spent frozen (in % of total chamber time).

C. Time course of contextual fear extinction. Mice were placed on 7 consecutive days each day for 5 min into the conditioning/test chamber without any footshock. Shown are averages ± SEM of times spent frozen (in % of total chamber time) for each day. The first time point is the freezing 24 hr after training shown in B. The reductions in freezing times on Days 3 and 4 are significantly smaller in D36 vs. WT (* $p < 0.05$; T-test).

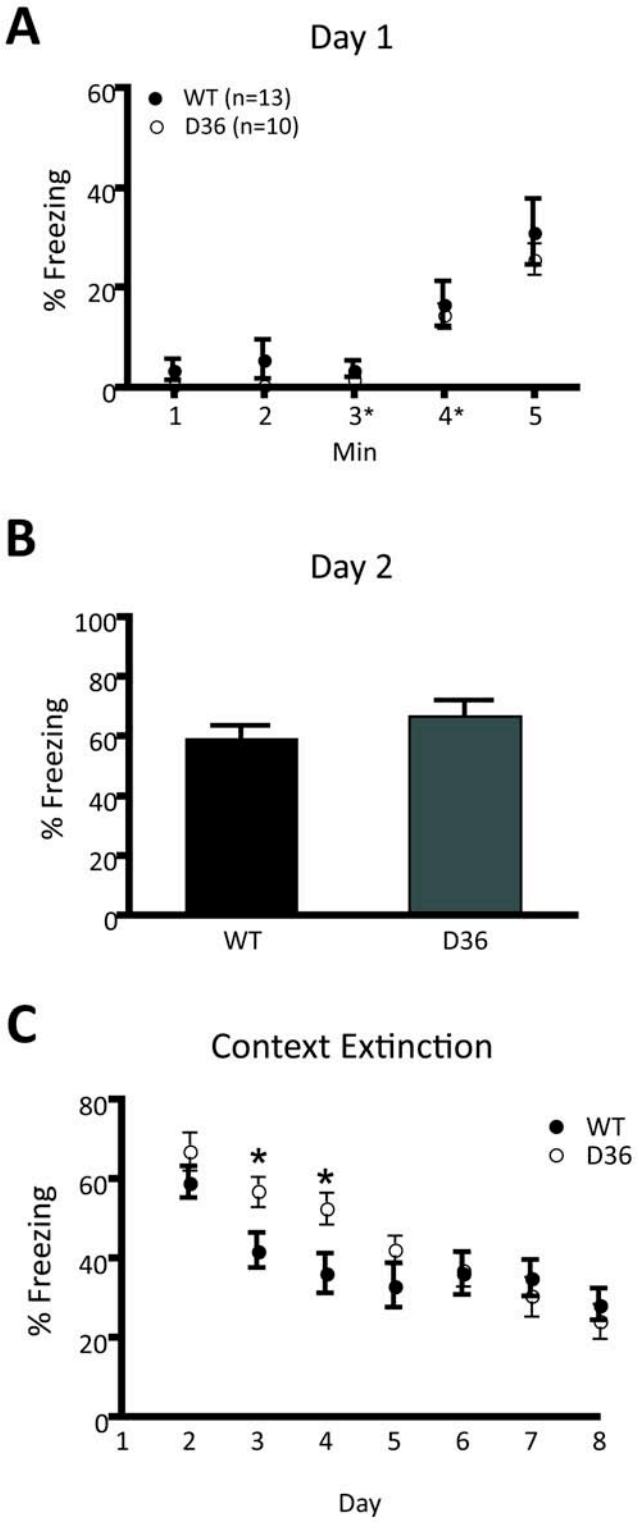

A
Day 1
WT (n=13)
D36 (n=10)
% Freezing
60
40
20
0
1
2
3*
4*
5
Min
B
Day 2
% Freezing
100
80
60
40
20
0
WT
D36
C
Context Extinction
WT
D36
% Freezing
80
60
40
20
0
*
*
1
2
3
4
5
6
7
8
Day

Figure 4-4. Extinction and reinstatement of auditory fear conditioning in AKAP5 D36 mice

A. Experimental design. D36 and litter mate WT mice were fear-conditioned by 3 tone-shock pairs on day 1 in the conditioning chamber and fear response retrieved by tone only on day 2 in the test chamber, which differed from the conditioning chamber. Fear extinction was conducted by 4 randomly given tones and freezing monitored on day 4, 9 and 16 in the test chamber. On day 17, mice received an unsignaled foot shock in the test chamber and were tested for the reinstatement on day 18 in the test chamber.

B. Freezing before and during FC on day 1. Shown are averages ± SEM of times spent frozen for each 30 sec interval between and during tone. The time course for tone and shock deliveries is graphed underneath the plot.

C. Fear retrieval on day 2. Shown are averages ± SEM of times spent frozen for three 1 min intervals before tone, the 30 sec interval during tone and a 1 min interval after tone.

D. Within-session extinction on days 2, 4, 8, and 16. Shown are averages ± SEM of times spent frozen for 30 sec intervals during tone.

E. In-between session extinction and reinstatement on day 17 as measured on 18. Shown are averages ± SEM of times spent frozen for the total testing periods on days 2,4,8,16, and 18.

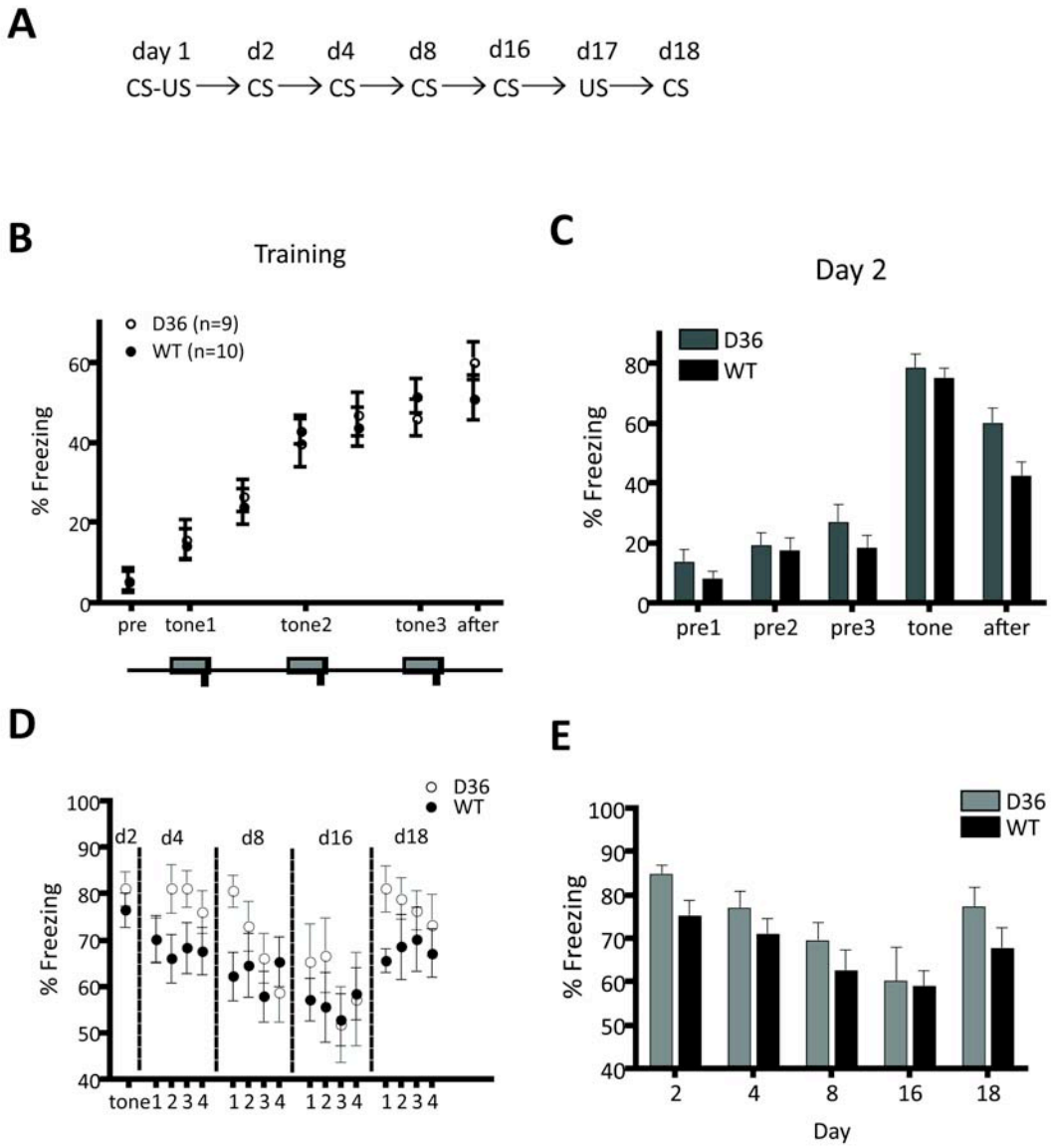

A
day 1 d2 d4 d8 d16 d17 d18
CS-US → CS → CS → CS → CS → US → CS
B
Training
D36 (n=9)
WT (n=10)
% Freezing
pre tone1 tone2 tone3 after
C
Day 2
D36
WT
% Freezing
pre1 pre2 pre3 tone after
D
D36
WT
d2 d4 d8 d16 d18
% Freezing
tone1 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
E
D36
WT
% Freezing
2 4 8 16 18
Day

Figure 4-5. Normal contextual fear in AKAP5 KO mice.

A. Contextual FC on Day 1. Animals received one footshock (0.7 mA, 1s) after 3 min and one after 4 min in the conditioning/test chamber before being transferred after a total time of 5 min into their home cages. Shown are averages ± SEM of times spent frozen for each min.

B. Contextual fear recall 24 hr after training during exposure to the conditioning chamber for 5 min. Shown are averages ± SEM of times spent frozen (in % of total chamber time).

C. Time course of contextual fear extinction. Mice were placed on 7 consecutive days each day for 5 min into the conditioning/test chamber without any footshock. Shown are averages ± SEM of times spent frozen (in % of total chamber time) for each day. The first time point is the freezing 24 hr after training shown in B.

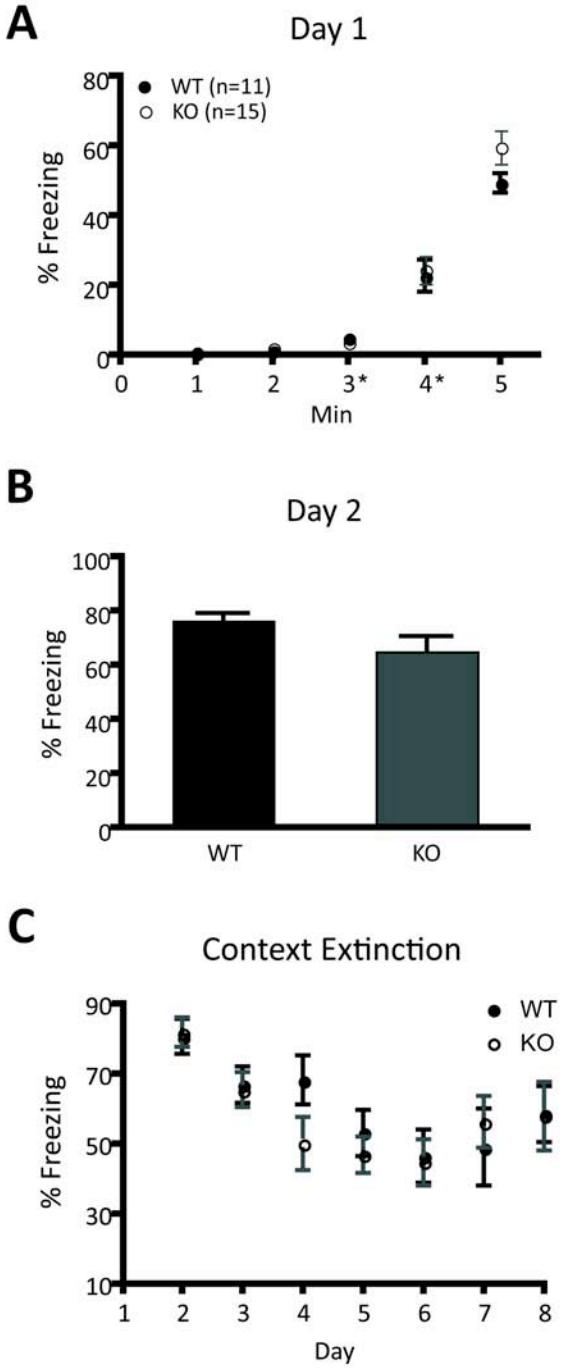
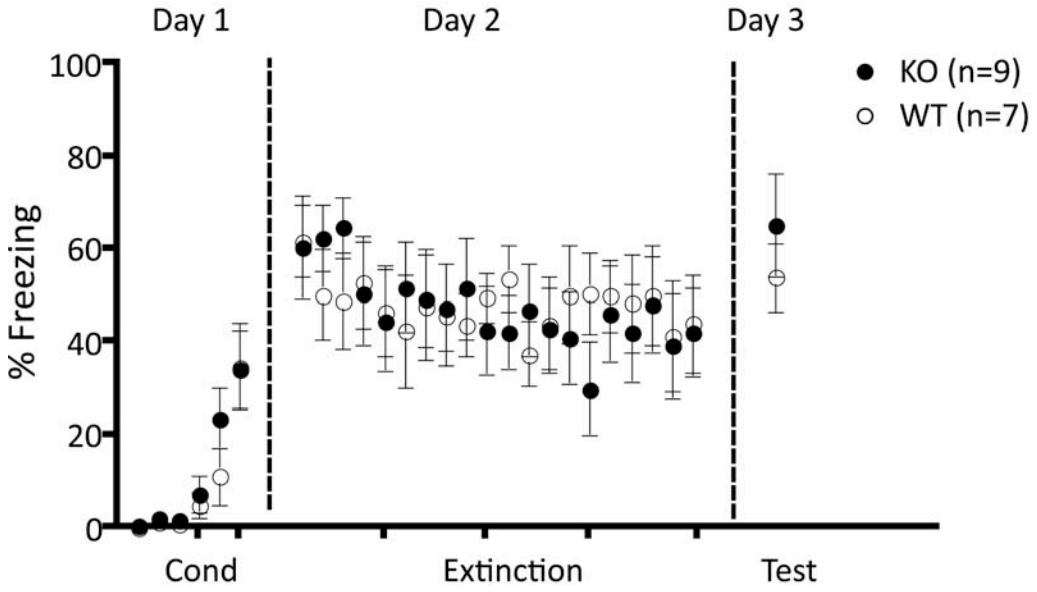

A
Day 1
WT (n=11)
KO (n=15)
% Freezing
80
60
40
20
0
0
1
2
3*
4*
5
Min
B
Day 2
% Freezing
100
80
60
40
20
0
WT
KO
C
Context Extinction
WT
KO
% Freezing
90
70
50
30
10
1
2
3
4
5
6
7
8
Day

Figure 4-6. Cued fear expression and extinction were normal in AKAP5 KO mice. Both AKAP5 KO and littermated WT mice got cued fear conditioning by 3 cues that preceded each shock (tone: 5 kHz, 90 dB, 30 sec with 1 min interval; shock: 0.5mA, 1 sec) on day 1 in conditioning chamber; The extinction training trail (20x CS: 5 kHz, 90 dB, 30 sec with 5 sec interval) was given on day 2 in different chamber B. Both groups showed a decrease in fear expression on day2. On day 3 both groups recalled extinction similarly. Cond, conditioning.


Day 1
Day 2
Day 3
KO (n=9)
WT (n=7)
% Freezing
100
80
60
40
20
0
Cond
Extinction
Test

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

Summary

The stress neurotransmitter norepinephrine (NE) is synthesized in the locus coeruleus and released by diffusion into most part of the forebrain, which fosters arousal and learning, especially under novel and emotionally charged situations (Berman and Dudai, 2001; Cahill et al., 1994; Hu et al., 2007; Minzenberg et al., 2008; Nielson and Jensen, 1994; Stranger and Dolan, 2004; Stranger et al., 2003). It facilitates several forms of LTP in the hippocampal dentate gyrus and CA1 region (Gelinas and Nguyen, 2005; Hu et al., 2007; Katsuki et al., 1997; Lin et al., 2003; Thomas et al., 1996; Walling and Harley, 2004). NE acts via the β_1 and β_2 adrenergic receptors (AR) to activate the trimeric G_s protein, adenylyl cyclase, and ultimately PKA.

The β_2AR is enriched at excitatory postsynaptic sites of pyramidal neurons where it forms a signaling complex with the AMPAR GluA1 subunit, G_s, and adenylyl cyclase for highly localized GluA1 phosphorylation and regulation (Colledge et al., 2000; Davare et al., 2001; Joiner et al., 2010; Tavalin et al., 2002). AMPARs mediate most of the postsynaptic response at glutamatergic synapses during basal synaptic transmission. PKA phosphorylates GluA1 on Serine 845 (Ser845) (Mammen et al., 1997; Roche et al., 1996), which is important for GluA1 surface expression (Ehlers, 2000; Man et al., 2007; Oh et al., 2006; Sun et al., 2005; Swayze et al., 2004), activity-induced postsynaptic accumulation (Esteban et al., 2003) and various forms of synaptic plasticity (He et al., 2009; Hu et al., 2007; Lee et al., 2003; Seol et al., 2007) (but see (Lee et al., 2010b)).

PKA is physically linked to GluA1 by AKAP5 (previously AKAP150 in rodents, AKAP79 in humans, and AKAP75 in cow), the main postsynaptic AKAP (Lu et al., 2007; Lu et al., 2008; Tavalin, 2008; Weisenhaus et al., 2010). AKAP5 associates with the SH3 - GK region of the postsynaptic scaffolding proteins PSD-95 and SAP97 (Bhattacharyya et al., 2009; Colledge et al., 2000). PSD-95 interacts with its first two PDZ domains with the C-terminus of stargazin (γ2) and its homologues γ3, γ4, and γ8 ("TARPs"), which in turn associate with AMPARs for their postsynaptic targeting (Chen et al., 2000; El-Husseini et al., 2000; Schnell et al., 2002). SAP97 directly binds with its first two PDZ domains to the C-terminus of GluA1 (Leonard et al., 1998). Functionally AKAP5 links PKA (Carr et al., 1992), PKC (Dell'Acqua et al., 1998; Gomez et al., 2002; Gorski et al., 2005; Klauck et al., 1996; Tavalin, 2008), and PP2B (Coghlan et al., 1995; Oliveria et al., 2007; Oliveria et al., 2003) via SAP97 and perhaps also PSD-95 to GluA1 for dynamic phosphorylation and dephosphorylation (Roche et al., 1996; Tavalin, 2008; Tavalin et al., 2002; Wong and Scott, 2004).

We used two mutant mice for investigating the function of AKAP5 in the postsynaptic complex of β_2AR / AMPA receptors. In D36 mice, the last 36 residues of AKAP5 was deleted by insertion of a stop codon to test the function of AKAP5 with respect to PKA targeting but without affecting the other interactions (Lu et al., 2007; Lu et al., 2008; Weisenhaus et al., 2010). We also have full AKAP5 knockout mice (Weisenhaus et al., 2010). D36 and KO mice have very limited and specific phenotypes with normal basal synaptic transmission and normal behavior. However, LTP was nearly absent during early development (P12-14) and in adult D36 mice and LTD was impaired at P12-14 in D36 mice (Lu et al., 2007; Lu et al., 2008; Weisenhaus et al., 2010). Our

AKAP5 KO mice had normal LTP and LTD likely because the deletion of AKAP5 eliminated not only PKA but also PP2B anchoring, causing a milder phenotype than the D36 mutation (Weisenhaus et al., 2010) (but see (Tunquist et al., 2008) for deficits in LTD (but not LTP) in another AKAP5 KO mouse). General behavior (e.g., open field) and learning (e.g., Morris water maze, novel object recognition, operant conditioning) was normal in D36 mice but reversal learning of the operant task was delayed although it still occurred (Weisenhaus et al., 2010).

To investigate roles of AKAP5 in the regulation of β_2 adrenergic / glutamate receptors signaling complex, we compared the functional and physical association of β_2AR and AMPA receptors among wild type, AKAP5 KO, and AKAP5 D36 mice.

First of all, we looked at the assembly of β_2AR / GluA1 in AKAP5 D36 and KO mice by coimmunoprecipitation. In our experimental conditions we still detected the association of β_2AR and GluA1 even loss of PKA binding or complete loss of AKAP5. Moreover the PSD components analysis in D36 and KO mice did not show the difference in GluA1 and β_2AR enrichment in PSD fractions compared to wild type mice. Our current findings suggest the dispensable role of AKAP5 in the complex formation.

Although AKAP5 was not necessary for the *in vivo* association of β_2AR / GluA1 complex and β_2AR localization in postsynaptic densities, we showed that the increase in phosphorylation of GluA1 on Ser845 in acute hippocampal slices from WT mice by the highly selective β-adrenergic agonist isoproterenol (ISO) was strongly inhibited in AKAP5 D36 and KO mice. Previous studies have shown more than 50% decrease of RIIα and about 90% decrease of RIIβ immunoreactivity in the PSD fractions from D36 mice (Lu et al., 2007). The reduction of ISO-induced Ser845 phosphorylation level in

D36 mice suggests AKAP5-anchored PKA activity is required for localized GluA1 Ser845 phosphorylation in response to β_2AR stimulation (Joiner et al., 2010). It is interesting that ISO-induced increase on Ser845 phosphorylatino was inhibited in KO mice at a similar level as in D36 mice. It is possible that PP2B activity is compensatively elevated after loss of AKAP5 gene expression (Tunquist et al., 2008).

Correlated with these effects, upregulation in synaptic transmission by ISO as observed in WT slices was impaired in D36 slices. Similarly, the synaptic transmission was enhanced by NE pretreatment in 10 Hz tetanus and this facilitation was deficient in S845A/S831A double mutant mice (Hu et al., 2007). Combined with the findings that reduced Ser845 phosphorylation increase in D36 mice after ISO stimulation, we speculate that AKAP5-anchored PKA plays an important role for ISO facilitated AMPAR mediated synaptic transmission, majorly through regulating GluA1 Ser845 phosphorylation. Whether or not complete loss of AKAP5 has a different effect on the upregulation in synaptic transmission by ISO is still under investigation.

General behavior (motor activity, anxiety) as tested in these studies did not reveal any obvious alteration in D36 and KO mice. Contextual and cued fear conditioning (FC) were also normal but their extinctions were retarded in D36 mice, although ultimately extinction in WT mice was similar to D36 mice. The mechanism of fear extinction is still not clear. The potentiation of interneurons in the lateral amygdala during extinction may inhibit the fear responses through interaction with the excitatory neurons, which normally strengthen the fear memory during consolidation (Ehrlich et al., 2009). Combined with the electrophysiological and biochemical findings in D36 mice, a possible model to explain the delayed extinction in D36 mice would be: AKAP5-

anchored PKA activity is required for the synaptic plasticity of interneurons during extinction.

In conclusion, our results showed that loss of postsynaptic PKA anchoring lead to clear reduction in GluA1 Ser845 phosphorylation and deficits in LTP but surprisingly modest yet interesting impairments in memory formation and modification.

Future directions

In our coimmunoprecipitation analysis, we did not observe a loss or a reduced association of β_2AR and GluA1 in both D36 and KO mice. Results of immunostaining found about 80% of total synaptic GluA1 was colocalized with β_2AR and majority of synaptic β_2AR was colocalized with GluA1 (Joiner et al., 2010). AKAP5 was also codistributed with majority of synaptic PSD95 while PSD95 as a MAGUK family protein was colocalized with GluA1 in hippocampal neurons (Colledge et al., 2000). It will be interesting to look at the synaptic localization of β_2AR and GluA1 in D36 and KO mice by immunofluorescence staining. Although it may be predictable that β_2AR and GluA1 are still localized in synaptic sites based on our current finding, the immunostaining of other synaptic proteins will provide us a better picture of how the β_2AR/GluA1 complex forms.

PKA also phosphorylates the C-terminus of stargazin (Choi et al., 2002), and phosphorylated stargazin changes its interaction with PSD-95. We will detect whether β_2AR activation regulates the phosphorylation of stargazin. Moreover, it will be

interesting to know how this regulation plays a role in the interaction of β_2AR with PSD-95. In parallel, we could compare the changes of stargazin in D36 and KO mice.

We could test the effect of AKAP5 on the extinction of cocaine conditioned place preference (CPP). There is a possibility that NE signaling manipulates extinction of drug-related behavior (Mueller and Cahill, 2010). Infusion of β_2AR agonist clenbuterol into infralimbic prefrontal cortex facilitated extinction of cocaine seeking in a drug self-administration paradigm (LaLumiere et al., 2010). Systemic injection of α receptor antagonist yohimbine impaired extinction of a cocaine CPP (Davis et al., 2008). In contrast, propranolol accelerated extinction of a cocaine CPP and this effect was possibly due to attenuated reconsolidation (Fricks-Gleason and Marshall, 2008). NMDARs and AMPARs are also involved in cocaine-seeking behavior. A NMDAR partial agonist has been shown to facilitate extinction of cocaine CPP in rodents (Botreau et al., 2006; Thanos et al., 2009). Another study showed both NMDAR and AMPAR antagonists abolished the acquisition and expression but not the reinstatement of the cocaine-induced CPP (Maldonado et al., 2007). It will be interesting to know whether AKAP5-anchored PKA is involved in the drug addiction.

CHAPTER 6
MATERIALS AND METHODS

Reagents

Pharmacological agents

(-) isoproterenol bitartrate salte, atenolol, microcystin LR, and (+/-) propranolol hydrochloride were from Sigma. Ketamine was from Bioniche Pharma and xylazine from AnaSede. SQ 22536, an acdenylyl cyclase inhibitor, was from Tocris Bioscience. All the other chemicals were from Fisher or other typical suppliers.

Antibodies

The anti-GluA1 antiserum (IB 1:1000, IP 2ul) was made against the peptide MSHSSGMPLGATGL, which corresponds to the C-terminus of GluA1 (residues 894-907) (Joiner et al., 2010). The phospho-specific antibodies against Ser845 and Ser831 were produced against the synthetic peptides LIPQQ(pS)INEAIK (GluA1 residues 826-836) and TLPRN(pS)GAGASK (GluA1 residues 840-850; pS: phospho-serine) (Mammen et al., 1997). The anti-phospho Ser845 GluA1 used for basal phosphorylation analysis was purchase from Millipore. All peptides had been coupled to bovine serum albumin for immunization of rabbits, as described earlier (Davare et al., 1999). Antibody against $Ca_v1.2$ (FP1) was purified from rabbit antiserum against loop II/III of $\alpha_1 1.2$ (Davare et al., 1999). The antibody against pS1928-$Ca_v1.2$ (CH3P, IB 1:50) was affinity purified from rabbit antiserum against a peptide with residues upstream and downstream of phosphorylated Ser1928 (Davare et al., 1999; De Jongh et al., 1996). Rabbit anti-

AKAP5 antibody against rat AKAP aa428-449 (IB 1:1000) was from Upstate Co. The rabbit β_2AR (H-20) antibody was from Santa Cruz Biotech (sc-569) and made against C-terminus of β2 adrenergic receptor of human origin. The mouse anti-β_2AR antibodies (Mab5 and Mab8) were gifts from Dr. Brian Kobilka (Stanford Univ.) and made against the surface or the intracellular domain of β2 adrenergic receptor. Anti-PSD95 antibody from Neuromab (IB: 1:1000) was mouse monoclonal antibody against amino acid residues 77-299 of human PSD95/SAP90. Rabbit synaptophysin antibody (IB 1:1000) was from Biomeda Corp. Non-specific rabbit IgG control antibodies were from Santa Cruz Biotechnology.

The secondary antibodies used were horse radish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (1:10,000, from Biorad), and Protein A HRP (1:10,000, from Amersham Biosciences). ECL and ECL plus reagents were from GE healthcare and Supersignal West Femto substrate kits were from Thermo Scientific.

Generation of AKAP5 mutant mice

The AKAP5 mutant mice were generated by Dr. Stanley McKnight lab (Univeresity of Washington, Seattle) (Lu et al., 2007; Weisenhaus et al., 2010).

The AKAP5 KO mice were made by inserting a neomycin phosphotransferase (neo) cassette into the AKAP5 genomic sequence, which could be replaced after transcription. In the AKAP5 D36 mice, the last 36 amino acids containing the PKA binding region have been truncated from the full-length AKAP5 protein. To engineer

these mice, a target vector was first made from subcloning a 1.5 kb BglII/SpeI fragment including the Leu710 codon. A mutated fragment was then made by site-directed mutagenesis (QuickChange kit, Stratagene) with the overlapping oligonucleotides: 5'-CAGTATGAAACACTC**TAG**ATAGAAACAGATCTTC-3' and 5'-AGATGCTGTTTCTAT**CTA**GAGTGTTTCATACTGTT-3', which introduced a stop codon (TAG) into the sequence. This mutated D36 targeting vector was composed of an XbaI restriction site for genotyping, a neo cassette flanked by loxP sites downstream from the AKAP5 sequence.

Constructs were linearized by ClaI and electroporated into R1 ES cells. The positive ES clones were selected in G418 and confirmed by Southern Blot analysis, which should detected SacI fragments of 10 kb in wild type, 12.4 kb in KO, and 11 kb in D36 recombinant ES cells. The selected ES cells were injected into blastocysts to generate chimeric mice that were bred to C57Bl/6 mice to get germ line transmission. To remove the neomycin resistance gene, the D36 F1 offspring were crossed to a line of rosa26-Cre recombinase expressing mice. Both KO and D36 mouse lines were backcrossed to C57Bl/6 (Taconic farm) for at least 8 generations.

Biochemical assays

Protein quantification

The amount of total protein was quantified by BCA assay according to the manufacturers protocol (Thermo Scientific, Pierce, USA). The standard proteins were made from BSA (2 mg/ml) and the samples were diluted either one fifth or one fiftieth by

water. BCA reagent is a 1:50 mixture of Reagent B: Reagent A. 10 μl of each standard and sample in triplicates was loaded in the plate followed by 200 μl of BCA reagent. The plate was incubated at 37ºC for 30 mins and read the absorbance value at 546nm. The concentration of protein for each sample was calculated based on the absorbance vs. BSA concentration standard curve.

Affinity purification of antiserum

The antisera (anti-$Ca_v1.2$ (FP1), anti-pS835 GluA1, anti-pS831 GluA1, and anti-pS1928 $Ca_v1.2$ (CH3P)) were purified on pre-made affinity columns, which were composed of glutathione sepharose beads covalently linked to the antigens. Briefly, the affinity column was washed with 5-10 ml TBS for 5 times before loading the antiserum. The column was titled overnight at 4ºC and then 1 hr at RT to let antiserum interacts with antigen. The unbound antiserum was flow through the column and washed away by 5ml TBS for 10 times. The bound antiserum was eluted twice with 2.5ml 3M $MgCl_2$ and drained directly into 10ml ice-cold TBS. The eluted antiserum was concentrated by Amicon ultra centrifugal filter unit (Millipore) and then washed with TBS until the dilution factor was above 50. The antibody concentration was estimated by OD_{280} measurement. The purified antibody was stored at 4ºC in 0.02% sodium azide.

Forebrain homogenization

The homozygous AKAP mutant or wild type mice were decapitated without anesthetization and the forebrains were quickly taken out on ice. The forebrains were

either frozen by liquid N_2 and stored at -80ºC for later use, or used for immediate biochemical studies or slices treatment.

The forebrains were dropped into 3-5 ml of homogenization buffer containing of 1% Triton X-100, 10mM Tris-HCl pH 7.5, 150mM NaCl, 10mM EGTA, 10mM EDTA, in the presence of protease inhibitors (10 μg/mL leupeptin/aprotinin, 1 μM pepstatin A, 200 μM PMSF). For maintaining endogenous phosphorylation levels, several phosphatase inhibitors (2 μM microcystin-LR, 25 mM NaF, 25 mM NaPPi, 1mM PNPP) were added if the phosphorylated proteins would be tested. The forebrain samples were homogenized by a tissue grinder (Craftman 8 in. drill press) for 40 sec on ice and centrifuged for 30 min at 50,000g (Beckman TL-100 benchtop ultracentrifuge). The supernatants were used for biochemical analysis.

Immunoprecipitation and coimmunoprecipitation

Total forebrain samples or acute slices were extracted with a tenfold excess (volume/weight) of Buffer A (150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl, pH 7.4, and protease inhibitors) containing 1% Triton X-100 with a glass-teflon homogenizer. Samples were cleared from non-solubilized material by ultracentrifugation (250,000 x g for 30 min) before immunoprecipitation with anti-GluA1, H20 against the β_2AR, or an equivalent amount of non-specific rabbit IgG (typically 2 μg; overnight at 4^{o}C) and subsequent immunoblotting as described (Leonard and Hell, 1997; Leonard et al., 1999). Proteins were separated by SDS-PAGE, transferred over night onto polyvinyldifluoride (PVDF) membranes, incubated with primary antibodies for 1 hr, washed, incubated with HRP-protein A for 1 hr, and washed for 4 hrs

before detecting ECL or ECL plus signals by film. Multiple exposures with increasing time periods were obtained to ensure that signals were in the linear range, as described (Davare and Hell, 2003; Hall et al., 2006).

Acute cortical slices preparation

The forebrains from 8-10 weeks old C57BL/6 or AKAP mutant mice were isolated and transversely sectioned by a vibratome (Leica VT 1000A) at 350 μm in the artificial cerebrospinal fluid (ACSF) (127mM NaCl, 26mM $NaHCO_2$, 1.2mM KH_2PO_4, 1.9mM KCl, 1.1mM $CaCl_2$, 2mM $MgSO_4$, 10mM D-Glucose, 290-300mOsm/kg, equilibrated in 95% O_2 / 5% CO_2). These acute cortical slices were recovered and equilibrated in oxygenated ACSF for 30 min at 37ºC followed by 30 min at 22ºC. (Hai Qian prepared for the slices for the studies.)

Acute cortical slices treatment

The individual acute slice was transferred to each well in a submersion chamber containing the oxygenated solution of 127mM NaCl, 26mM $NaHCO_2$, 1.2mM KH_2PO_4, 1.9mM KCl, 2.2mM $CaCl_2$, 1mM $MgSO_4$, 10mM D-Glucose, 290-300mOsm/kg, and treated with either vehicle control, or isoproterenol (10 μM) for 15 minutes. Slices were extracted with ice-cold triton buffer (1% Triton X-100, 10mM Tris-HCl pH7.4, 150mM NaCl, 10mM EGTA, 10mM EDTA, 200μM PMSF, 1μg/ml pepstatin A, 20μg/ml aprotinin, 10μg/ml leupeptine, 2μM microcystin, 25mM NaF, 25mM NaPPi, 1mM PNPP) and centrifuged for 30 min at 50,000g. The supernatant were precipitated either by the antibody against GluA1 C-terminus or nonspecific rabbit IgG for overnight at 4ºC.

The precipitated proteins were separated by SDS-PAGE and probed by antibody against phospho-serine 845 on GluA1. The same blot was stripped and re-probed sequentially by anti-phospho-serine 831 and anti-GluA1 antibodies.

Postsynaptic density (PSD) preparation

Mice were decapitated and whole forebrains were dissected and quickly frozen at -80ºC for later use.

For making PSD fractions (Carlin et al., 1980; Lu et al., 2007), 4 forebrains per genotype were homogenized in 10 ml of fresh made ice-cold buffer A containing 0.32M sucrose, 1 mM Tris pH 7.4, 1 mM $MgCl_2$, 10 μg/mL leupeptin/aprotinin, 1 μM pepstatin A, 200 μM PMSF, 2 μM microcystin-LR. The lysates were centrifuged at 1400g, 10 min at 4ºC (Sorvall Legend XTR centrifuge, Thermo Scientific) and the pellets were washed and rehomogenized in equal volume of buffer A followed by centrifugation (10 min, 710g, 4ºC). The combined supernatants (Lys) were centrifuged at low speed (10 min, 710g, 4ºC) and then followed by a high-speed centrifugation (10 min, 13,800g, 4ºC by Bechman Ti50) to obtain the P2 fraction (P2). The P2 pellet was resuspended in a 3 ml of buffer B, which is equivalent to buffer A but without $MgCl_2$, and layered on top of a 0.85/1/1.25M sucrose gradient. After the centrifugation (2hr, 82,500g, 4ºC by SW41), the synaptosome enriched interface (Syn) was collected between the 1M and 1.25M sucrose layers and extracted with an equal volume of Triton X-100 buffer (1% Triton X-100, 12 mM Tris pH 8.0, 125 mM KCl, 10 μg/ml leupeptin/aprotinin, 1 μM pepstatin A, 200 μM PMSF, 2 μM microcystin-LR) for 15 min at 4ºC. The Triton-insoluble pellet was spun down by centrifugation (30 min, 35,000g, 4ºC by Bechman Ti70) and

resuspended in 1 ml buffer B followed by layering on top of a 1/1.5/2M sucrose gradient. The sample-sucrose gradient mixture was centrifuged at 225,000 g, 2 hr at 4ºC (SW41) and the PSD-enriched fraction (PSD) was collected between the 1.5/2M sucrose layers. For higher purification, this fraction could be extracted with an equal volume of Triton buffer followed by the centrifugation (1h, 200,000g, 4ºC by Bechman Ti70) and the pellet was resuspended in the Tris buffer (40mM Tris, pH 8.0, 10 μg/ml Leupeptin/aprotinin, 1 μM pepstatin A, 200 μM PMSF, 2 μM microcystin-LR).

During the procedure, 50-100 μL of samples were collected from each fraction and quantified using BCA assay. 10 μg protein of each fraction were separated by SDS-PAGE and probed with specific antibodies for PSD constituents.

SDS-PAGE and immunoblotting

Samples from forebrain extracts or slices were incubated with sodium dodecylsulfate (SDS) sample buffer (1X final, 3% SDS, 10% sucrose, 90mM Tris-HCl, 20mM DTT, 60μM bromophenol blue) for 5 min at 95ºC. The samples from immunoprecipitated resins were extracted with 20 μL of 3X SDS sample buffer. After extraction, samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 3% stacking, 7.5% or 10% resolving). The gels were transferred to polyvinylidene fluoride (PVDF) membrane at 50 volts for 10 hrs at 4ºC. Membranes were first blocked with 10% milk for one hour and then incubated with primary antibody for 2 hrs at room temperature or overnight at 4ºC. After three times of washes with 10% milk, membranes were incubated with HRP-conjugated secondary antibodies for 1 hr at RT. The blots were washed extensively with TBS and visualized by film

autophotography after ECL or ECL plus chemiluminescent substrate were applied (Amersham Biosciences). Supersignal West Femto substrate kits (Thermo Scientific) were used to enhance the very weak signals. When the sequential immunoblotting was needed, membranes were stripped with stripping buffer (50mM Tris, pH 7.5, 1% SDS, 10mM fresh added DTT) for 30 min at RT and washed with TBS for 30 min.

Electrophysiological Studies

Preparation and treatment of cortical slices

Mice were typically 8-16 weeks old. They were decapitated and brains placed into ice-cold artificial cerebrospinal fluid (ACSF, containing in mM: 127 NaCl, 26 $NaHCO_2$, 1.2 KH_2PO_4, 1.9 KCl, 2.2 $CaCl_2$, 1 Mg SO_4, and 10 D-glucose). ACSF was saturated with 95% O_2, 5% CO_2. About 1/3 of the brain was trimmed off at the rostral and then also the caudal end. 350 μm thick slices were then prepared with a vibratome (Leica VT 1000A). Slices were kept in oxygenated ACSF for 1hr at 30ºC and for 1-5 hrs at 24ºC before used for experiments. (Dr. Hai Qian prepared for the slices for the studies.

Field EPSP Recording

Brain slices were placed in a recording chamber and perfused with ACSF saturated with 95% O_2 and 5% CO_2 (2 ml/min) at 30ºC. Field excitatory postsynaptic potentials (fEPSPs) were evoked in the hippocampal CA1 area by stimulating the Schaffer collateral pathway with a bipolar tungsten electrode every 15 sec. The stimulus intensity was adjusted to induce 50% of the maximal response. The fEPSP was recorded

with a glass electrode filled with ACSF. Signals were amplified by an Axopatch 2B amplifier, digitized by a Digidata 1320A and recorded by Clampex 10 (Molecular Devices, CA). The tetanic stimulation was a train of pulses given at the frequency of 5 Hz for 3 min. For quantitative comparisons, the averages of fEPSP initial slopes were calculated from the 5 min immediately preceding the onset of perfusion with ISO, the 5 min immediately before the onset of the tetanus, and 30 min starting 15 min after the tetanus. (Dr. Hai Qian performed this study.)

Behavioral assays

Subjects

AKAP5 knockout (KO) or D36 mice and their wildtype siblings were obtained from heterozygous KO or D36 mice. For the behavioral tests mice were 4-12 months old. All the animals were housed one to five per cage and maintained on a 12:12 light/dark cycle with free access to food and water. All the tests were conducted following protocols approved by University of Iowa and University of California Davis Institutional Animal Care and Use Committee. All the tests were conducted in a noise, light and temperature controlled room between 9am and 1pm.

Open field test

To measure the animal's locomotor activity and anxiety behavior, individual mouse was placed in a corner of the square open field box (36'' by 36'') and recorded by a video camera above the box for 30 minutes. The chamber was cleaned by 70% ethanol

between each test. The behavior of the animal was measured and analyzed by an automated system (Med Associates) as 'counts of different types of movement (jump, vertical or ambulatory activities', 'average of ambulatory distance traveled' within each 10 min period, or 'time spent in the center (vs. periphery)'. The floor of the arena was divided by grid lines and arbitrarily defined certain area as 'center' (80%) and 'periphery' (20%).

Dark-light test:

The chamber (36'' by 36'') was divided into two equal compartments by a dark box with a hole, allowing the mouse to transit freely between the two compartments. The mouse was placed into one corner of the light area and allowed to freely explore the chamber for 30 mins. The transitions and time spent in both compartments were recorded. Mice were returned to the home cage after the test done.

Contextual fear conditioning

Mice were tested in standard operant chambers (Med Associates, Burlington, Vermont). The floor of the chamber is made of 12 stainless steel rods, which are wired to a shock generator. A video camera is positioned to the front of the chamber to record the mouse's behavior. Mice were transferred to the testing room at least 30 minutes before the test. On day 1, after a 3 min pre-exposure period mice were given 2 trials of a 0.75mA, 1 sec foot-shock with 1 min interval. The time of lack of movement (except the movement associated with aspiration) was measured as freezing time (in second). On the second and subsequent days, mice were put in the same chambers and observed the

freezing without any shock for 5 minutes. Mice were moved back to their home cage after the test done. The chamber floor was cleaned with a 0.01% bleach solution between each test.

Cued fear conditioning

The handling of the animals and the operation for the equipment were the same as for context fear conditioning. Mice were fear conditioned by 3 tone-shock pairs (tone: 5 kHz, 90 dB, 30s; shock: 0.7 mA, 1s) on day 1 and retrieved the fear by the same tone without foot shock on day 2 in a chamber of different shape, floor, and smell. The extinction of fear was monitored during 4 randomly given tone (5 kHz, 90 dB, 30s) on day 4, 9 and 16. On day 17, mice received an unsignaled foot shock (0.7mA, 1 S) and were tested the fear response for 4 randomly given tone (5 kHz, 90 dB, 30s) on day 18. (Jason Ulrich helped the experiments on day 17 and 18).

Monitoring of heart rate

Mice were anesthetized intramuscularly with a mixture of ketamine (80 mg/kg) and xylazine (8 mg/kg). The criteria for adequacy of anesthesia include: 1) lack of eye blink reflex; 2) no whisker movement; 3) lack of paw withdrawal in response to pinching; and 4) no irregular or sudden changes in heart rate and breathing frequency. An electrocardiogram (ECG) telemetry device (TA10EA F-20, Data Sciences International, St Paul, MN) was used for monitoring heart rate. For recording ECGs at lead II configuration, the negative lead of the transmitter was sutured to the upper right pectoralis muscle near the shoulder, and the positive lead was sutured to the left lateral

side of the xiphoid process. Continuous ECG signals were recorded at 5 kHz with Dataquest A.R.T. (Data Sciences International). Heart rate responses to ISO (8 μg/kg) were determined before and after administration of atenolol (2 mg/kg). All drugs were administrated intravenously into the jugular vein with a U-100 insulin syringe at a volume of 4 ml/kg.

Auditory brainstem response (ABR) test

Mice were anesthetized with intraperitoneal injection of xylazine hydrochloride (10mg/kg) and ketamine hydrochloride (90mg/kg). Needle electrodes were placed subcutaneously at the vertex (reference electrode), beneath the pinna of the left ear (recording electrode), and beneath the right ear (ground). ABRs were evoked with broadband click stimuli (biphasic 40 μs/phase, 30 ms interpulse interval). Acoustic stimuli were generated by a 16-bit digital-to-analog converter at a sampling rate of 100,000 samples/s. The converter was controlled by custom software written using LABVIEW® programming environment. Acoustic clicks were produced by driving an earphone (Beyerdynamic model DT-48) via an attenuator (Hewlett–Packard model 350C), and a custom-designed impedance-matching transformer. An Etymotic 3A micro speaker was placed in the left external auditory canal. Responses were routed to a battery-powered instrumentation amplifier (gain: 1000), then through a custom-built 2-pole low-pass filter. Signal averaging was performed across 500 sweeps for suprathreshold stimuli and 1000 sweeps for stimuli at or near threshold. Maximum acoustic response was first assayed with a 74 dB stimulus. Hearing threshold was then determined by attenuating stimulus intensity in 10 dB increments. The lowest stimulus

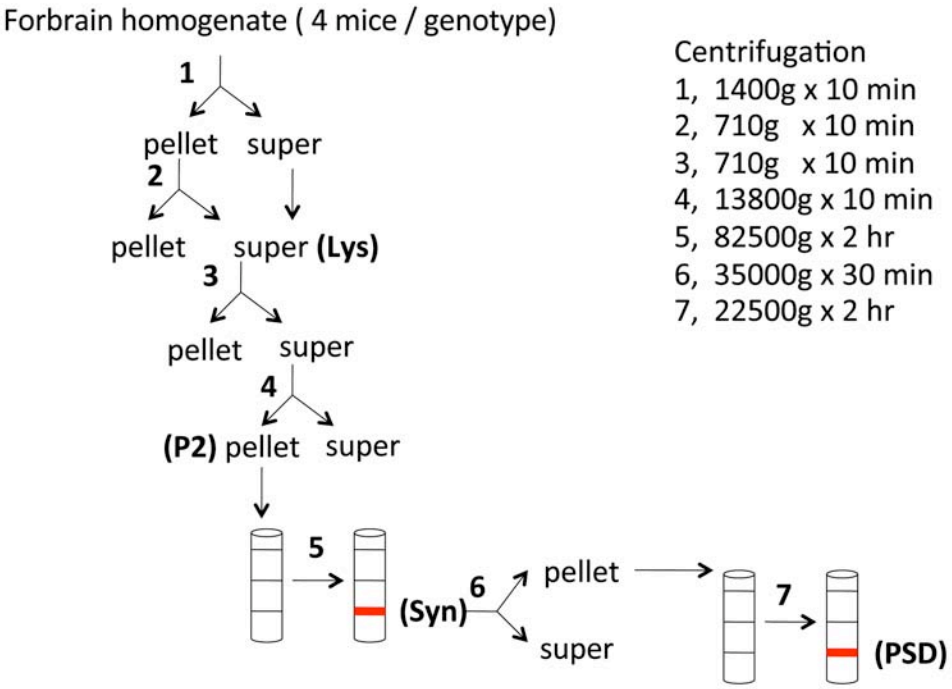
intensity to induce a reliable V1 ABR waveform was chosen as hearing threshold (Yamasoba et al., 2006). Only mice with normal hearing were tested for auditory fear conditioning after a 3-4 day recovery period. (Jonathan C. Kopelovich and Erin Bailey performed this ABR test.)

Data analysis

Immunosignals were quantified using Photoshop or ImageJ (NIH). The band signal was determined by drawing a box around the band and measured the density by 'analyze gels' function. All data were analyzed by Excel (Microsoft Corp.) and Prism 4.0 (GraphPad Software, Inc.). Data were shown as means ± SEM. Student's t test was used for two groups comparison. One- or two-way analysis of variance (ANOVA) was used for comparisons of more than two groups. If the result of one way ANOVA was significant, Tukey's post hoc comparison test was used to analyze. Two way ANOVA was used for fear extinction analysis and F value was given for consideration. $p < 0.05$ was considered statistically significant.

Figure 6-1. Procedure for making the postsynaptic density (PSD) fraction

The process for making postsynaptic fractions was shown by differential centrifugation (Lys, P2), sucrose gradient centrifugation (enriched for synaptosomes, Syn), and extraction with Triton X-100 (PSD). The numbers indicate the centrifugation and the speed and time for each centrifugation were shown in the inset.


Forbrain homogenate (4 mice / genotype)
1
pellet
super
2
pellet
super (Lys)
3
pellet
super
4
(P2) pellet
super
5
(Syn)
6
pellet
super
7
(PSD)
Centrifugation
1, 1400g x 10 min
2, 710g x 10 min
3, 710g x 10 min
4, 13800g x 10 min
5, 82500g x 2 hr
6, 35000g x 30 min
7, 22500g x 2 hr

=)

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