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AMPK Signaling in the Dorsal Hippocampus Negatively Regulates Contextual Fear Memory Formation

Ying Han[1,2,3,8], Yixiao Luo[4,8], Jia Sun[5], Zengbo Ding[2], Jianfeng Liu[6], Wei Yan[1,2], Min Jian[1,2], Yanxue Xue[2], Jie Shi[2], Ji-Shi Wang[*,5] and Lin Lu[*,1,2,7]

[1]Institute of Mental Health/Peking University Sixth Hospital and Key Laboratory of Mental Health, Beijing, China; [2]National Institute on Drug Dependence and Beijing Key Laboratory of Drug Dependence, Peking University, Beijing, China; [3]Department of Pharmacology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China; [4]Department of Pharmacy, Medical College, Hunan Normal University, Changsha, China; [5]Department of Hematology, Affiliated Hospital of Guiyang Medical University, Guiyang, China; [6]Department of Pharmacology and Toxicology, University of Buffalo, State University of New York, Buffalo, NY, USA; [7]Peking-Tsinghua Center for Life Sciences and PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing, China

Both the formation of long-term memory (LTM) and dendritic spine growth that serves as a physical basis for the long-term storage of information require *de novo* protein synthesis. Memory formation also critically depends on transcription. Adenosine monophosphate-activated protein kinase (AMPK) is a transcriptional regulator that has emerged as a major energy sensor that maintains cellular energy homeostasis. However, still unknown is its role in memory formation. In the present study, we found that AMPK is primarily expressed in neurons in the hippocampus, and then we demonstrated a time-dependent decrease in AMPK activity and increase in mammalian target of rapamycin complex 1 (mTORC1) activity after contextual fear conditioning in the CA1 but not CA3 area of the dorsal hippocampus. Using pharmacological methods and adenovirus gene transfer to bidirectionally regulate AMPK activity, we found that increasing AMPK activity in the CA1 impaired the formation of long-term fear memory, and decreasing AMPK activity enhanced fear memory formation. These findings were associated with changes in the phosphorylation of AMPK and p70s6 kinase (p70s6k) and expression of BDNF and membrane GluR1 and GluR2 in the CA1. Furthermore, the prior administration of an mTORC1 inhibitor blocked the enhancing effect of AMPK inhibition on fear memory formation, suggesting that this negative regulation of contextual fear memory by AMPK in the CA1 depends on the mTORC1 signaling pathway. Finally, we found that AMPK activity regulated hippocampal spine growth associated with memory formation. In summary, our results indicate that AMPK is a key negative regulator of plasticity and fear memory formation.
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INTRODUCTION

The molecular and cellular mechanisms of learning and memory are complicated and have attracted a great deal of attention. The contextual fear conditioning paradigm presents a framework with which to study the neurobiological mechanisms of contextual fear memory and mnemonic function of the hippocampus (Rudy *et al*, 2004). In this classical conditioning paradigm, an environmental context (conditioned stimulus (CS)) is paired with several deliveries of footshocks (unconditioned stimulus). Several hours later, this fear association undergoes consolidation to become a long-term memory (LTM; McGaugh, 2000; Schafe *et al*, 2001). As a result, the CS elicits conditioned fear responses when subsequently presented alone during the expression phase of fear memory (Dejean *et al*, 2015). Memory formation is well known to depend on dendritic spine growth and restructuring, which increase the efficiency of synapses (Bailey and Kandel, 1993). The formation of LTM also requires new protein synthesis, which is important for structural and functional synaptic changes that are implicated in memory formation and storage (Costa-Mattioli *et al*, 2009; Kandel, 2001; Kelleher *et al*, 2004). Although a small number of genes and proteins that are important for the formation of LTM have been identified, still unknown are the precise molecular mechanisms that are involved in the formation of LTM.

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase that is composed of one catalytic subunit and two regulatory subunits. AMPK is a major energy sensor that regulates an array of downstream target genes and maintains cellular energy homeostasis (Hardie, 2007; Mihaylova and Shaw, 2011). AMPK is activated both by canonical pathways

*Correspondence: Professor J-S Wang, Department of Hematology, Affiliated Hospital of Guiyang Medical University, Guiyang 550004, China, E-mail: jswang_yg@yahoo.com or Professor L Lu, Institute of Mental Health, Peking University, 51 Huayuan Bei Road, Beijing 100191, China, Tel: +86 10 82805308, Fax: +86 10 62032624, E-mail: linlu@bjmu.edu.cn
[8]These authors contributed equally to this work.
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(involved in increases in AMP, adenosine diphosphate, or Ca^{2+}) and non-canonical pathways (eg, those triggered by reactive oxygen species and DNA-damaging agents; Hardie, 2007). AMPK has important roles in various processes, including growth, metabolism, autophagy, cell polarity, and cell migration (Mihaylova and Shaw, 2011; Williams and Brenman, 2008). Recently, the role of AMPK in neurodegenerative disorders, such as Alzheimer's disease and other tauopathies, amyotrophic lateral sclerosis, and Huntington's disease, has attracted much attention (Ju *et al*, 2011; Mairet-Coello *et al*, 2013). Several studies showed that AMPK has a crucial role in cognitive decline and memory dysfunction, which are the most characteristic symptoms of Alzheimer's disease (DiTacchio *et al*, 2015; Du *et al*, 2015). Although inconsistent findings have been reported regarding the role of AMPK in the formation of spatial memory (Dash *et al*, 2006; Kobilo *et al*, 2014; Kobilo *et al*, 2011), these findings indicate that AMPK activity is involved in learning and memory processes. However, little is known about the role of AMPK activity in the hippocampus in contextual fear memory formation.

In the present study, we investigated the role of AMPK in the dorsal hippocampus in regulating structural plasticity and the formation of contextual fear memory using genetic and pharmacological methods in a rat model of contextual fear conditioning.

MATERIALS AND METHODS

Subjects

Male Sprague Dawley rats, weighing 240–260 g, were obtained from the Laboratory Animal Center, Peking University Health Science Center. They were housed five per cage in a temperature- (23 ± 2 °C) and humidity- (50 ± 5%) controlled animal facility with *ad libitum* access to food and water. They were kept on a reverse 12 h/12 h light/dark cycle. The behavioral experiments were conducted during the dark phase of the cycle. All of the experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Biomedical Ethics Committee of Peking University.

Surgery

Rats (weighing 280–300 g when surgery began) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Guide cannulae for viral infusions and pharmacological infusions (23 gauge; Plastics One, Roanoke, VA, USA) were bilaterally implanted 1 mm above the CA1 (anterior/posterior, −3.8 mm; medial/lateral, ±2.0 mm; dorsal/ventral, −2.5 mm) and CA3 (anterior/posterior, −3.8 mm; medial/lateral, ±3.8 mm; dorsal/ventral, −3.2 mm) areas of the hippocampus (Chai *et al*, 2014; Paxinos and Watson, 2005). The cannulae were anchored to the skull with stainless-steel screws and dental cement. A stainless-steel stylet blocker was inserted into each cannula to keep it patent and prevent infection. The rats were allowed to recover for 7 days after surgery.

Design, Construction, and Validation of Adenoviral Vectors for AMPK Subunits

Constitutively active (CA) AMPKα2 cDNA constructs (T172D mutant) and dominant-negative (DN) AMPKα2 cDNA constructs (K45R mutant) were designed and constructed according to previous studies (Jones *et al*, 2005; Mu *et al*, 2001). The CA-AMPK construct encodes residues 1–312 of AMPKα2 mutated on the threonine 172 residue to an aspartic acid (T172D). The DN-AMPK construct contains full-length AMPKα2 mutated on the lysine 45 residue to arginine (K45R). Appropriate mutagenesis was validated by sequencing. All AMPK cDNAs were subcloned into a pHBAd-CMV vector. All of the vectors contained the enhanced green fluorescence protein (eGFP) coding sequence.

Intracranial Injections of Adenoviruses and Drugs

The AMPK activator 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) was purchased from Toronto Research Chemicals (Toronto, Canada). The AMPK inhibitor compound C and mTOR inhibitor rapamycin were purchased from Sigma (St Louis, MO, USA). Compound C was dissolved in a vehicle solution that contained 80% sterile saline, 10% dimethylsulfoxide (DMSO), and 10% cremophore EL (Sigma-Aldrich). Rapamycin was dissolved in 20% DMSO. AICAR was dissolved in saline. All of the drugs were freshly prepared before delivery. The doses of the drugs (0.5 and 5 μg/μl AICAR; 0.3 and 3 μg/μl compound C; 0.5 μg/μl rapamycin) were based on previous studies (Ikegami *et al*, 2013; Ju *et al*, 2011; Parsons *et al*, 2006). The infusion volume for all of the drugs was 1.0 μl. The drugs were infused bilaterally in the CA1 or CA3 using Hamilton syringes connected to 30-gauge injectors (Plastics One) that extended 1 mm below the guide cannula over 1 min. The injection needle was kept in place for an additional 1 min to allow for drug diffusion (Lu *et al*, 2005).

The experimental procedures that were used for the virus injections were based on previous studies (Jian *et al*, 2014; Minokoshi *et al*, 2004). The rats were anesthetized with sodium pentobarbital. The Ad-GFP, Ad-CA-AMPK, and Ad-DN-AMPK adenoviruses (1×10^{11} p.f.u./ml) were delivered bilaterally over 10 min at an infusion rate of 0.1 μl/min (total volume, 1 μl per side) using Hamilton syringes connected to 30-gauge injectors (Plastics One) that reached 1 mm below the guide cannula. The injectors were left in place for an additional 5 min to allow diffusion.

Contextual Fear Memory

Contextual fear conditioning was conducted in four identical isolated shock chambers (Beijing Macro Ambition S&T Development Ltd, Beijing, China). The contextual fear conditioning procedure was based on our previous studies, with minor modifications (Chai *et al*, 2014). The rats were handled for 3 days before conditioning. On the day of fear conditioning, the rats were placed into the conditioning chamber and allowed to explore the chamber for 2 min (freezing time was defined as the baseline), after which they received an electric footshock (0.8 or 0.4 mA, 1 s). For strong contextual fear conditioning, the strength of the electric

footshock was 0.8 mA. For weak contextual fear conditioning, the strength of the electric footshock was 0.4 mA. The reason for using strong and weak contextual fear conditioning protocols was to avoid ceiling and floor effects. The 2 min/1 s procedure was repeated a total of three times, and the rats were allowed to explore the conditioning chamber for an additional 1 min. After removing the rat from the chamber, the chamber was cleaned with 75% alcohol to eliminate any residual odor. One and 24 h later, memory was tested by exposing the rats to the conditioning chamber for 5 min without shock. All of the experimental sessions were video recorded for offline analysis. Freezing behavior was defined as the lack of all movement, with the exception of respiration.

Open Field Test

Locomotor activity was measured using the open field test as previously described (Xue *et al*, 2015; Zhu *et al*, 2011). Briefly, the apparatus consisted of a $75 \times 75 \times 40$ cm square arena that was divided into 25 equal squares (15×15 cm) on the floor of the arena. A single rat was placed in the center of the apparatus, and the number of crossings into adjacent squares and time spent in the center area of the apparatus were counted for 5 min.

Elevated Plus Maze

The elevated plus maze was based on our previous studies (Suo *et al*, 2013; Xue *et al*, 2015). Briefly, each rat was placed in the central zone of the elevated plus maze. The rat was allowed to freely explore the maze for 5 min. The entire test was conducted under dim light conditions. The number of entries into and time (in seconds) spent on the open arms were recorded by two independent observers who were blind to the animal groups and sat quietly 2.5 m from the maze.

Tissue Sample Preparation and Subcellular Fractionation

Crude cytoplasmic and synaptosomal membrane fractions were collected based on our previous studies (Chai *et al*, 2014; Jian *et al*, 2014; Liu *et al*, 2014; Lu *et al*, 2005). After decapitation, the brains were rapidly extracted and frozen in −60 °C *N*-hexane. The brains were then transferred to a −80 °C freezer. Bilateral tissue punches (16 gauge) of the CA1 and CA3 were placed in a 1.5 ml microtube that contained ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), and protease/phosphatase inhibitor cocktail, pH 7.4). After being homogenized by an electrical disperser (Wiggenhauser, Sdn Bhd, Los Lunas, USA), the homogenate was centrifuged at 1000 *g* for 10 min at 4 °C to obtain the pellet (P1) that contained nuclei and large debris. The supernatant (S1) was again centrifuged at 10 000*g* for 30 min at 4 °C to generate a crude synaptosomal fraction (P2) and supernatant (S2). The crude synaptosomal membrane pellet (P2) was lysed hypo-osmotically and centrifuged at 25 000 *g* for 30 min at 4 °C to generate the synaptosomal membrane fraction (LP1). LP1 was resuspended in the HEPES-lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, and protease/phosphatase inhibitor cocktail, pH 7.4). The protein concentrations of all of the samples were determined using the bicinchoninic acid assay (Beyotime Biotechnology, Jiangsu, China). The samples were further diluted in HEPES-lysis buffer to equalize the protein concentrations. Four times loading buffer (16% glycerol, 20% mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue) was added to each sample (3 : 1, sample : loading buffer) before being boiled for 5 min.

Western Blot

Equal amounts of protein (10–20 μg) for each sample were loaded into SDS-polyacrylamide gel electrophoresis (10–15% acrylamide/0.27% *N,N′*-methylenebisacryalamide resolving gel) for ~40 min at 80 V in stacking gel and ~1 h at 120 V in resolving gel. Proteins were electrophoretically transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA) at 250 mA for 2.5 h. The membranes were blocked with blocking buffer (5% bovine serum albumin (BSA) in TBST) for 2 h at room temperature. They were then incubated overnight at 4 °C with anti-p-AMPK antibody, anti-AMPK antibody, anti-p-p70s6k antibody, anti-p70s6k antibody, anti-GluR1 antibody, anti-GluR2 antibody (1 : 1000; Cell Signaling Technology, Danvers, MA, USA), anti-BDNF antibody (1 : 2000; Abcam, Cambridge, UK), or anti-*β*-actin antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST plus 5% BSA. After three 5-min washes in TBST buffer, the blots were incubated for 45 min at room temperature on a shaker with the corresponding horseradish peroxidase-conjugated secondary antibody (goat anti-mouse immunoglobulin G (IgG) for *β*-actin and goat anti-rabbit IgG for the others; 1 : 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were washed three times for 5 min each in TBST, and immunostaining was visualized with a layer of Super Signal Enhanced chemiluminescence substrate (Detection Reagents 1 and 2, 1:1 ratio, Pierce Biotechnology, Rockford, IL, USA). The immunoblots were then screened using the ChemiDoc MP System (BioRad, Hercules, CA, USA) for 5–60 s. Band intensities were quantified using the Quantity One 4.4.0 software (BioRad). Phosphorylated protein levels were normalized to total protein levels.

Golgi-Cox Staining

To study the role of AMPK in regulating neuronal morphology *in vivo*, we used previously described methods, with modification (Restivo *et al*, 2009; Russo *et al*, 2009). Three days after injecting Ad-GFP, Ad-CA-AMPK, or Ad-DN-AMPK, the rats were trained for strong or weak contextual fear conditioning or maintained in their home cage. They were then anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.9% saline solution. The brains were dissected and impregnated using a Golgi-Cox solution according to a previously described method (Glaser and Van der Loos, 1981). Briefly, the brains were first immersed in the Golgi-Cox solution at room temperature for 14 days and then transferred to a 30% sucrose solution. They were then sectioned using a vibratome. Coronal sections (100 μm thick) were mounted on gelatinized slides, stained according to a previously

described method (Gibb and Kolb, 1998), coverslipped, and allowed to dry before the quantitative analysis. Spine density was measured on pyramidal neurons that were located in the CA1 region of the dorsal hippocampus. Neurons, identified using a light microscope under low magnification, were chosen by first locating the regions of interest in their respective coronal sections. For each group, we examined 3–5 neurons per animal and 5–6 animals per group, after which an average value was calculated for each animal for the statistical analysis. All of the images were captured with an Olympus BX53 microscope using a ×100 oil-immersion objective. Dendrite length was measured using the NIH ImageJ software, and the number of dendritic spines was counted by two trained observers who were blind to the experimental conditions. The average number of spines per 10 μm of dendrite was calculated.

Immunohistochemistry

Rats were perfused with 0.01 mol/l phosphate-buffered saline (PBS) and 4% paraformaldehyde (pH 7.4), followed by post-fixation overnight at 4 °C. The brains were coronally sectioned at 20 μm using a sliding microtome. Primary antibody incubation was performed on a shaker overnight at 4 °C, followed by secondary antibody incubation for 1 h at room temperature. The following antibodies were used at the indicated concentrations: anti-pAMPK (1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GFP (1 : 2000; Abcam, Cambridge, UK), anti-NeuN (1 : 2000; Abcam, Cambridge, UK), anti-GFAP (1 : 2000; Abcam, Cambridge, UK), Alexa Fluor goat anti-rabbit 488 (1 : 500; Life Technologies, Carlsbad, CA, USA), and Alexa Fluor goat anti-rabbit 594 (1 : 500; Life Technologies, Carlsbad, CA, USA). Finally, the brain slices were counterstained with DAPI and examined using an Olympus IX83 fluorescent microscope.

Histology

After the behavioral experiments, the rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused with 0.01 mol/l PBS and 4% paraformaldehyde, pH 7.4. The brains were then extracted and postfixed in 4% paraformaldehyde for 24 h. Subsequently, the brains were cryoprotected in 30% sucrose in 0.2 mol/l phosphate buffer.

Cannulae placements were assessed using Nissl staining with a section thickness of 40 μm under light microscopy. Rats with misplaced cannulae were excluded from the statistical analysis. The locations of the cannulae are shown in Figures 2b and 3b. In addition, the brains were coronally sectioned at 20 μm using a sliding microtome, and the brain slices were counterstained with DAPI and examined using an Olympus IX83 fluorescent microscope to evaluate eGFP expression in the CA1 and CA3 (Jian *et al*, 2014).

Statistical Analysis

The data are expressed as mean ± SEM. The statistical analyses were performed using repeat-measures analysis of variance (ANOVA) for all of the experiments, except for the open field test, elevated plus maze, and western blot results, which were analyzed using one-way ANOVA. The spine density results were analyzed using two-way ANOVA. *Post hoc* analyses of significant effects in the ANOVAs were performed using the Tukey test (for details, see Results section). Values of $p<0.05$ were considered statistically significant.

RESULTS

AMPK is Primarily Expressed in Neurons and its Activity is Decreased by Contextual Fear Conditioning

To address AMPK localization in the hippocampus, we double-labeled hippocampal tissue sections with the neuronal marker NeuN and an antibody against pAMPK. The immunohistochemical analysis revealed strong colocalization of pAMPK and NeuN signals in neurons throughout the hippocampus (Figure 1a). We also examined the expression of AMPK in non-neuronal cells in the CNS by double labeling sections with the astrocytic markers GFAP and pAMPK. At lower magnification, we did not observe obvious colocalization. Under higher magnification, however, we detected low levels of pAMPK in several astrocytes (Figure 1b).

We then determined whether AMPK activity is modified by fear conditioning. Six groups of rats ($n=7$ per group) were used to assess the effect of strong contextual fear conditioning on AMPK/p70s6 kinase (p70s6k) activity in the CA1 and CA3. Rats first underwent strong contextual fear conditioning. They were then decapitated 10 min, 30 min, 1, 3, and 6 h later. The rats in the naive group were subjected only to daily handling in the home cage and then decapitated. Their brains were removed for the subsequent determination of AMPK phosphorylation (Thr172) and p70s6k phosphorylation (Thr 389) in the CA1 and CA3 (Figure 1c). The phosphorylation of AMPK decreased in the CA1 10 and 30 min after strong contextual fear conditioning and returned to baseline levels 6 h after training (one-way ANOVA, $F_{5,41}=10.86$, $p<0.01$; Figure 1d), but no changes were observed in the CA3 ($p>0.1$; Figure 1e). The phosphorylation of p70s6k increased in the CA1 1 h after strong contextual fear conditioning (one-way ANOVA, $F_{5,41}=5.21$, $p<0.01$; Figure 1d), but no changes were observed in the CA3 ($p>0.1$; Figure 1e). These results indicate that contextual fear conditioning decreased AMPK activity and increased mTORC1 activity in the CA1 but not CA3.

Effect of Pharmacological Modulation of AMPK Activity in the CA1 and CA3 on the Formation of Contextual Fear Memory

After finding that AMPK activity was decreased 10 and 30 min after contextual fear conditioning, which occurs within the time window of memory consolidation, we hypothesized that AMPK regulates memory consolidation to affect long-term fear memory. To test this hypothesis, we determined the effect of locally infusing an AMPK activator or inhibitor (AICAR and compound C, respectively) in the CA1 or CA3 immediately after contextual fear conditioning on the formation of contextual fear memory. Three groups of rats ($n=9$–10 per group) were trained for strong contextual fear conditioning and then given different doses of AICAR (0, 0.5, and 5 μg/side) bilaterally in the CA1 immediately

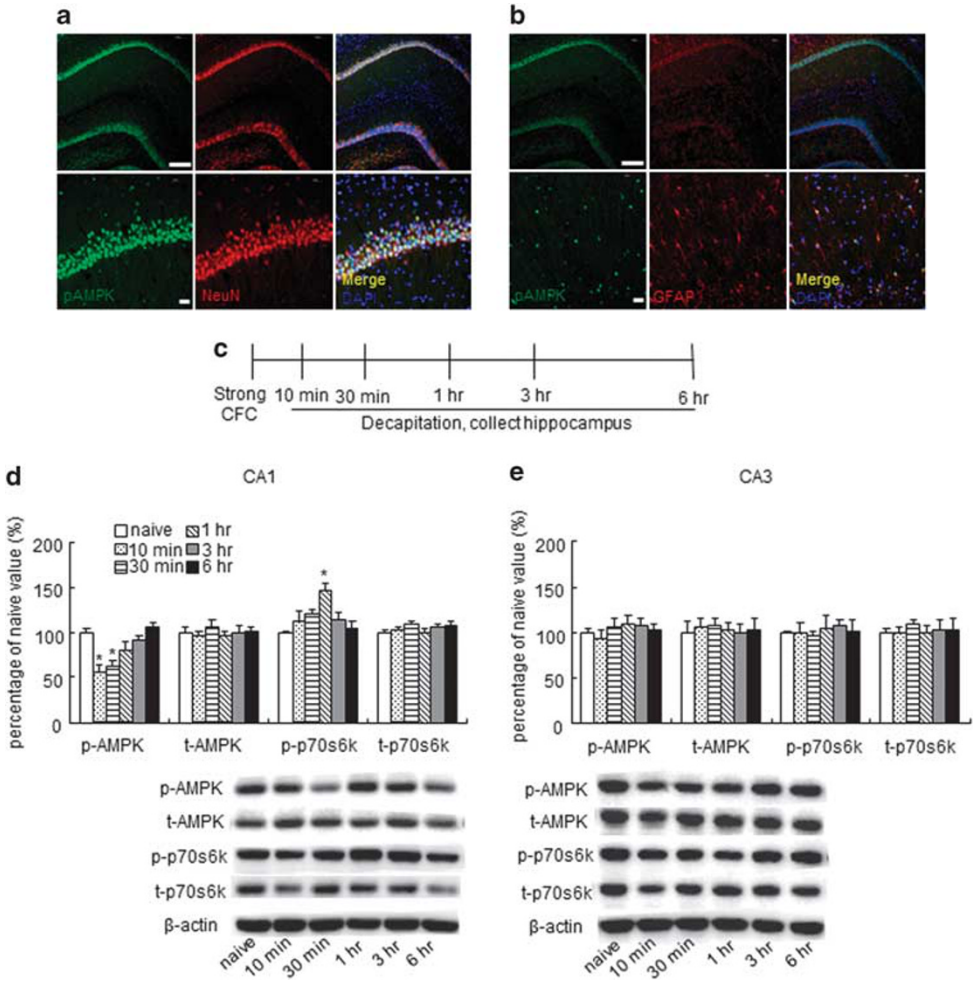

Figure 1 AMPK is primarily expressed in neurons and its activity is decreased by contextual fear conditioning. The data are expressed as mean ± SEM. (a) pAMPK-labeled cells and NeuN-labeled neurons in the hippocampus and merged image of pAMPK and NeuN double labeling, counterstained with DAPI. (b) pAMPK-labeled cells and GFAP-labeled astrocytes in the hippocampus and merged image of pAMPK and GFAP double labeling, counterstained with DAPI. Scale bar = 200 μm (low-power images) and 20 μm (high-power images). (c) Timeline of the experiment. (d and e) Phosphorylated AMPK, phosphorylated p70s6k, total AMPK, and total p70s6k protein levels and representative western blots in the CA1 and CA3 after contextual fear conditioning. The data are expressed as a percentage of phosphorylated AMPK, phosphorylated p70s6k, total AMPK, and total p70s6k relative to naive rats ($n = 7$ per group). In the CA1 but not CA3, contextual fear conditioning training decreased phosphorylated AMPK and increased phosphorylated p70s6k, which time-dependently recovered. *$p < 0.05$, compared with naive group. AMPK, adenosine monophosphate-activated protein kinase; CFC, contextual fear conditioning; p70s6k, p70s6 kinase.

after contextual fear conditioning. One and 24 h later, the rats underwent a short-term memory (STM) test and LTM test. After the end of the LTM test, all of the rats were decapitated to determine AMPK/p70s6k phosphorylation, BDNF expression, and membrane GluR1 and GluR2 expression in the CA1 (Figure 2a). The two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and AICAR dose (0, 0.5, and 5 μg/side) as the between-subjects factor, was performed to analyze freezing time, which was significantly affected by AICAR dose ($F_{2,26} = 6.15$, $p < 0.01$; Figure 2c) and test phase ($F_{2,52} = 131.94$, $p < 0.01$; Figure 2c), with an AICAR dose × test phase interaction ($F_{4,52} = 4.56$, $p < 0.01$; Figure 2c). The *post hoc* analysis showed that freezing time significantly decreased in the group of rats that received AICAR (5 μg/side) immediately after strong contextual fear conditioning in the LTM test compared with the vehicle group ($p < 0.01$). We also found that p-AMPK levels (one-way ANOVA, $F_{2,23} = 30.34$, $p < 0.01$; Figure 2e) significantly increased, and p-p70s6k (one-way ANOVA, $F_{2,23} = 11.76$, $p < 0.01$; Figure 2e), BDNF (one-way ANOVA, $F_{2,23} = 30.43$, $p < 0.01$; Figure 2e), membrane GluR1 (one-way ANOVA, $F_{2,23} = 17.55$, $p < 0.01$; Figure 2e), and GluR2 (one-way ANOVA, $F_{2,23} = 37.57$, $p < 0.01$; Figure 2e) levels significantly decreased in rats that were treated with AICAR (5 μg/side) immediately after contextual fear conditioning. The repeated-measures AVONA of freezing time in rats that received AICAR (5 μg/side) in the CA1 immediately after weak contextual fear conditioning revealed no significant effect of AICAR and no AICAR × test phase interaction (data not shown), likely attributable to a 'floor effect' in rats that underwent weak contextual fear conditioning.

We then tested the effects of microinfusing compound C into the CA1 immediately after contextual fear conditioning on STM and LTM. Another three groups of rats ($n = 9$–11 per group) were trained for weak contextual fear

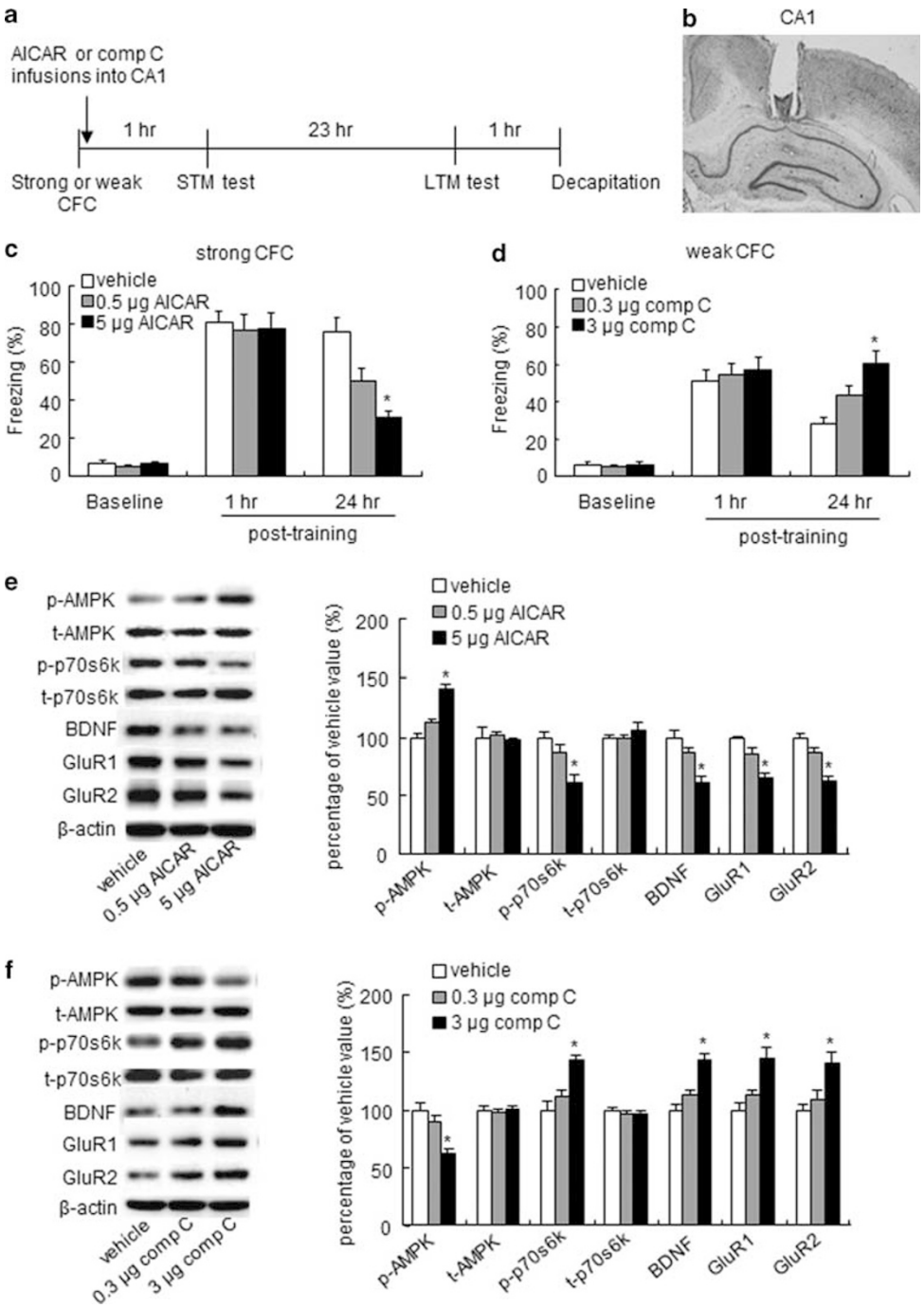

Figure 2 Pharmacological modulation of AMPK activity in the CA1 negatively regulates the formation of contextual fear memory. The data are expressed as mean ± SEM. (a) Timeline of the experiment. (b) Representative cannula placement in the CA1. (c) Percent time freezing during the baseline test, STM test (1 h after training), and LTM test (24 h after training) in rats that were injected with AICAR (0, 0.5, and 5 μg/side) in the CA1 immediately after strong contextual fear conditioning ($n = 9$–10 per group). (d) Percent time freezing during the baseline test, STM test, and LTM test in rats injected with compound C (0, 0.3, and 3 μg/side) in the CA1 immediately after weak contextual fear conditioning ($n = 9$–11 per group). (e and f) Phosphorylated AMPK, phosphorylated p70s6k, total AMPK, total p70s6k, BDNF levels, membrane GluR1 protein levels, membrane GluR2 protein levels, and representative western blots in the CA1 after the LTM test. The data are expressed as a percentage of phosphorylated AMPK and p70s6k, total AMPK and p70s6k, and BDNF and membrane GluR1 and GluR2 levels in vehicle-treated rats ($n = 8$ per group). *$p < 0.05$ compared with vehicle group. AMPK, adenosine monophosphate-activated protein kinase; CFC, contextual fear conditioning; LTM, long-term memory.

conditioning and given different doses of compound C (0, 0.3, and 3 μg/side) bilaterally in the CA1 immediately after contextual fear conditioning. Freezing time was analyzed using a two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and compound C dose (0, 0.3, and 3 μg/side) as the between-subjects factor. The repeated-measures ANOVA of freezing time revealed significant effects of compound C dose ($F_{2,26} = 3.42$, $p < 0.05$; Figure 2d) and test phase ($F_{2,52} = 107.88$, $p < 0.01$;

Figure 2d) and a compound C dose × test phase interaction ($F_{4,52} = 3.50$, $p < 0.05$; Figure 2d). The *post hoc* analysis showed that freezing time significantly increased in the group of rats that received compound C (3 μg/side) in the LTM test compared with the vehicle group ($p < 0.05$). We also found that p-AMPK levels (one-way ANOVA, $F_{2,23} = 29.93$, $p < 0.01$; Figure 2f) significantly decreased, and p-p70s6k (one-way ANOVA, $F_{2,23} = 29.18$, $p < 0.01$; Figure 2f), BDNF (one-way ANOVA, $F_{2,23} = 25.65$, $p < 0.01$; Figure 2f), membrane GluR1 (one-way ANOVA, $F_{2,23} = 13.67$, $p < 0.01$; Figure 2f), and GluR2 (one-way ANOVA, $F_{2,23} = 9.50$, $p < 0.01$; Figure 2f) levels significantly increased in rats that were treated with compound C (3 μg/side) immediately after contextual fear conditioning. The repeated-measures ANOVA of freezing time in rats that received compound C (3 μg/side) in the CA1 immediately after strong contextual fear conditioning revealed no significant effect of compound C and no compound C × test phase interaction (data not shown), likely attributable to a 'ceiling effect' in the rats that underwent strong contextual fear conditioning.

In the CA3, no significant effects of AICAR or compound C were observed, with no AICAR × test phase or compound C × test phase interaction 1 and 24 h after contextual fear conditioning (all $p > 0.1$; Figure 3a–d).

We then examined whether AICAR or compound C affects locomotion and anxiety-like behavior. Three groups of rats ($n = 9$–10 per group) received vehicle, AICAR (5 μg/side), or compound C (3 μg/side) infusions in the CA1. One and 24 h later, all of the rats underwent the open field and elevated plus maze tests. The analysis revealed no significant effect of AICAR or compound C on the number of crossings (Figure 5b) or time spent in the central area (Figure 5c) in the open field test or time spent on the open arms (Figure 5d) in the elevated plus maze (all $p > 0.1$). These results indicate that AICAR and compound C infusions in the CA1 had no effect on locomotion or anxiety-like behavior.

Altogether, these results indicate that AMPK activity in the CA1 but not CA3 regulated the formation of long-term fear memory but had no effect on short-term fear memory.

Effect of Pharmacological Modulation of AMPK Activity before Training on the Acquisition of Contextual Fear Memory

We further tested the effects of modulating AMPK activity before training on the acquisition of contextual fear conditioning. Two groups of rats ($n = 8$–9 per group) were trained for strong contextual fear conditioning and given saline or AICAR (5 μg/side) bilaterally in the CA1 1 h before contextual fear conditioning. One and 24 h later, the rats underwent the STM test and LTM test (Figure 4a). The two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and AICAR dose (0 and 5 μg/side) as the between-subjects factor, was performed to analyze freezing time, revealing significant effects of AICAR dose ($F_{1,15} = 8.21$, $p < 0.05$; Figure 4b) and test phase ($F_{2,30} = 108.35$, $p < 0.01$; Figure 4b), and an AICAR dose × test phase interaction ($F_{2,30} = 8.75$, $p < 0.01$; Figure 4b). The *post hoc* analysis revealed that the AICAR group exhibited lower freezing time compared with the vehicle group 24 h after fear conditioning ($p < 0.01$), but not 1 h after fear conditioning ($p > 0.1$). These data indicate that AMPK activation in the CA1 disrupted the formation of long-term fear memory but had no effect on the acquisition of fear conditioning.

Another two groups of rats ($n = 10$ per group) were trained for weak contextual fear conditioning and given vehicle or compound C (3 μg/side) bilaterally in the CA1 1 h before contextual fear conditioning. The two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and compound C dose (0 and 3 μg/side) as the between-subjects factor, was performed to analyze freezing time, revealing significant effects of compound C dose ($F_{1,18} = 4.56$, $p < 0.05$; Figure 4c) and test phase ($F_{2,36} = 63.61$, $p < 0.01$; Figure 4c) and a compound C dose × test phase interaction ($F_{2,36} = 4.29$, $p < 0.05$; Figure 4c). The *post hoc* analysis showed that the rats that received compound C exhibited a higher freezing time 24 h after fear conditioning compared with the vehicle group ($p < 0.01$) but not 1 h after fear conditioning ($p > 0.1$). These data indicate that AMPK inhibition in the CA1 had no effect on the acquisition of contextual fear memory but enhanced the formation of long-term fear memory.

Effect of Modulating AMPK Activity in the CA1 by Adenovirus Transfer on the Formation of Contextual Fear Memory

After finding that the pharmacological modulation of AMPK activity in the CA1 regulated long-term fear memory formation, we further confirmed the effects of modulating AMPK activity on the formation of fear memory by expressing constitutively active (CA, T172D mutation in the α2 subunit of AMPK) or dominant-negative (DN, K45R mutation in the α2 subunit of AMPK) subunits of AMPK in the CA1 using recombinant adenovirus. We first examined the efficiency of modulating AMPK activity by adenoviral transfer in the CA1 (Figure 6a) and CA3 (Figure 3f). Three groups of rats ($n = 6$ per group) were decapitated for the western blot assay 3 days after treatment with Ad-GFP, Ad-CA-AMPK, or Ad-DN-AMPK (Figure 6b). Compared with the Ad-GFP groups, AMPK phosphorylation significantly increased 3 days after Ad-CA-AMPK microinjection (one-way ANOVA, $F_{1,11} = 28.69$, $p < 0.01$; Figure 6b), and AMPK phosphorylation significantly decreased 3 days after Ad-DN-AMPK microinjection (one-way ANOVA, $F_{1,11} = 52.01$, $p < 0.01$; Figure 6b).

Three days after infusing Ad-GFP, Ad-CA-AMPK, or Ad-DN-AMPK into the CA1, the rats were subjected to several behavioral paradigms to evaluate locomotion, anxiety, and memory formation. A one-way ANOVA (Ad-GFP, Ad-CA-AMPK, and Ad-DN-AMPK) was conducted to analyze locomotion and anxiety-like behavior in the open field test and elevated plus maze. The analysis revealed no significant effect of vector on the number of crossings (Figure 5f) or time spent in the central area (Figure 5g) in the open field test or time spent on the open arms (Figure 5h) in the elevated plus maze (all $p > 0.1$). These results indicate that increasing or decreasing AMPK activity in the CA1 had no effect on locomotion or anxiety-like behavior.

Two groups of rats ($n = 9$–11) were then microinfused with an adenovirus that expressed the GFP control or

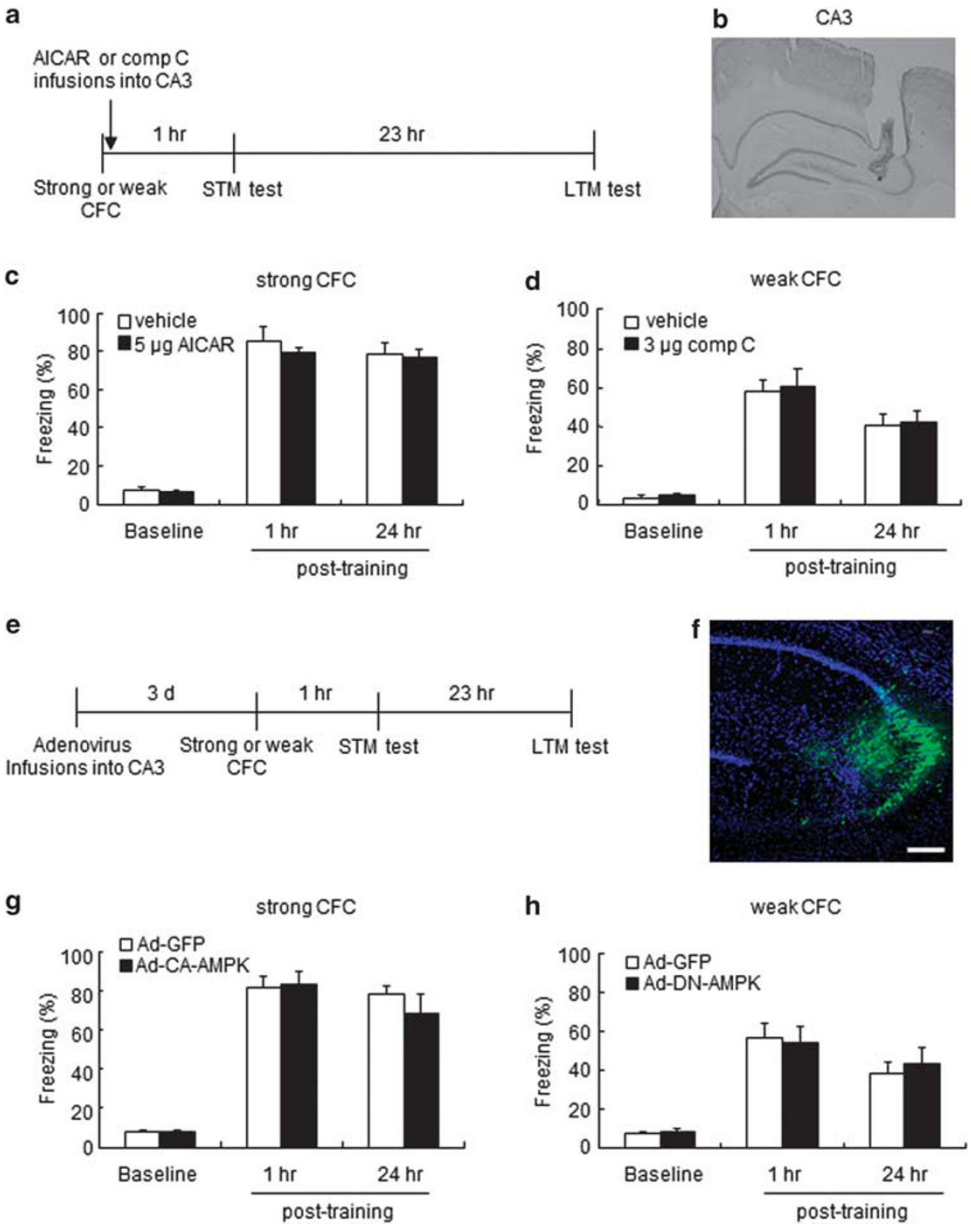

Figure 3 Modulation of AMPK activity in the CA3 has no effect on the formation of contextual fear memory. The data are expressed as mean ± SEM. (a) Timeline of the experiment. (b) Representative cannula placement in the CA3. (c) Percent time freezing during the baseline test, STM test (1 h after training), and LTM test (24 h after training) in rats that were injected with AICAR (0 and 5 μg/side) in the CA3 immediately after strong contextual fear conditioning ($n = 8$–10 per group). (d) Percent time freezing during the baseline test, STM test, and LTM test in rats injected with compound C (0 and 3 μg/side) in the CA3 immediately after weak contextual fear conditioning ($n = 8$ per group). (e) Timeline of the experiment. (f) Representative image of adenovirus-mediated transgene expression in the CA3. The figure shows representative micrographs of enhanced green fluorescent protein (eGFP; green), counterstained with DAPI, after CA3 microinjection, showing adenovirus vector-mediated eGFP expression within a defined boundary of the CA3. Scale bar = 200 μm. (g) Percent time freezing during the baseline test, STM test (1 h after training), and LTM test (24 h after training) in rats that were injected with an adenovirus that expressed GFP or the CA-AMPK vector in the CA3 3 days before strong contextual fear conditioning ($n = 8$–9 per group). (h) Percent time freezing during the baseline test, STM test, and LTM test in rats injected with an adenovirus that expressed GFP or the DN-AMPK vector in the CA3 3 days before weak contextual fear conditioning ($n = 8$–9 per group). AMPK, adenosine monophosphate-activated protein kinase; CFC, contextual fear conditioning; LTM, long-term memory; STM, short-term memory.

CA-AMPK vector in the CA1, trained for strong contextual fear conditioning, and tested 1 h (STM test) and 24 h (LTM test) later. After the end of the LTM test, all of the rats were decapitated to determine AMPK/p70s6k phosphorylation and BDNF and membrane GluR1 and GluR2 expression in the CA1 (Figure 6c). Freezing time was tested 1 and 24 h after fear conditioning and analyzed using a two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and vector (GFP and CA-AMPK) as the between-subjects factor. The repeated-measures ANOVA of freezing time revealed significant effects of vector ($F_{1,18} = 5.78$, $p < 0.05$; Figure 6d) and test phase ($F_{2,36} = 86.04$, $p < 0.01$; Figure 6d) and a vector × test phase interaction ($F_{2,36} = 3.53$, $p < 0.05$;

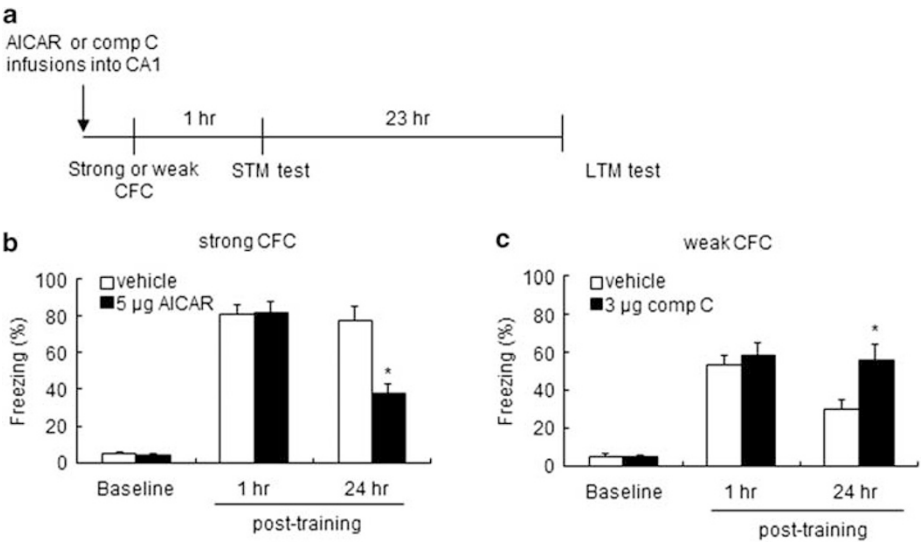

Figure 4 AMPK in the CA1 is not necessary for the acquisition of contextual fear memory. The data are expressed as mean ± SEM. (a) Timeline of the experiment. (b) Percent time freezing during the baseline test, STM test (1 h after training), and LTM test (24 h after training) in rats that were injected with AICAR (0 and 5 μg/side) in the CA1 1 h before strong contextual fear conditioning ($n = 8$–9 per group). (c) Percent time freezing during the baseline test, STM test, and LTM test in rats that were injected with compound C (0 and 3 μg/side) in the CA1 1 h before weak contextual fear conditioning ($n = 10$ per group). *$p < 0.05$, compared with vehicle group. AMPK, adenosine monophosphate-activated protein kinase; CFC, contextual fear conditioning; LTM, long-term memory; STM, short-term memory.

Figure 6d). The *post hoc* analysis showed that freezing time significantly decreased in the group of rats that received Ad-CA-AMPK infusions in the LTM test ($p < 0.01$) but not STM test ($p > 0.1$) compared with the GFP control group. We also found that p-AMPK levels (one-way ANOVA, $F_{1,15} = 80.53$, $p < 0.01$; Figure 6f) significantly increased, and p-p70s6k (one-way ANOVA, $F_{1,15} = 30.81$, $p < 0.01$; Figure 6f), BDNF (one-way ANOVA, $F_{1,15} = 15.89$, $p < 0.01$; Figure 6f), membrane GluR1 (one-way ANOVA, $F_{1,15} = 40.67$, $p < 0.01$; Figure 6f), and membrane GluR2 (one-way ANOVA, $F_{1,15} = 72.50$, $p < 0.01$; Figure 6f) levels significantly decreased in rats that received Ad-CA-AMPK infusions. These data indicate that increasing AMPK activity in the CA1 impaired the formation of long-term fear memory and inhibited mTORC1 activity and BDNF and membrane GluR1 and GluR2 expression.

Another two groups of rats ($n = 9$–11) that were microinfused with an adenovirus that expressed the GFP control or DN-AMPK vector in the CA1 were trained for weak contextual fear conditioning and tested 1 h (STM test) and 24 h (LTM test) later (Figure 6c). Freezing time was tested 1 and 24 h after fear conditioning and analyzed using a two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and vector (GFP and DN-AMPK) as the between-subjects factor. The repeated-measures ANOVA of freezing time revealed significant effects of vector ($F_{1,18} = 4.61$, $p < 0.05$; Figure 6e) and test phase ($F_{2,36} = 60.97$, $p < 0.01$; Figure 6e) and a vector × test phase interaction ($F_{2,36} = 5.43$, $p < 0.01$; Figure 6e). The *post hoc* analysis showed that freezing time significantly increased in the group of rats that received Ad-DN-AMPK infusions in the LTM test ($p < 0.01$), but not STM test ($p > 0.1$) compared with the GFP control group. We also found that p-AMPK levels (one-way ANOVA, $F_{1,15} = 23.78$, $p < 0.01$; Figure 6g) significantly decreased, and p-p70s6k (one-way ANOVA, $F_{1,15} = 7.50$, $p < 0.05$; Figure 6g), BDNF (one-way ANOVA, $F_{1,15} = 7.52$, $p < 0.05$; Figure 6g), membrane GluR1 (one-way ANOVA, $F_{1,15} = 9.33$, $p < 0.01$; Figure 6g), and GluR2 (one-way ANOVA, $F_{1,15} = 10.75$, $p < 0.01$; Figure 6g) levels significantly increased in rats that received Ad-DN-AMPK infusions. These data indicate that decreasing AMPK activity in the CA1 promoted the formation of long-term fear memory and enhanced mTORC1 activity and BDNF and membrane GluR1 and GluR2 expression.

In the CA3, no significant effects of Ad-CA-AMPK or Ad-DN-AMPK were observed, with no Ad-CA-AMPK × test phase or Ad-DN-AMPK × test phase interaction 1 and 24 h after contextual fear conditioning (all $p > 0.1$; Figure 3e–h).

Enhancement of Contextual Fear Memory by AMPK Inhibition Requires mTORC1 Activation

The results above indicate that contextual fear conditioning induced dephosphorylation of AMPK and phosphorylation of p70s6k in the CA1 (Figure 1d), and modulating AMPK activity affected the phosphorylation of p70s6k in the CA1 (Figures 2e, f and 6f, g). However, this did not indicate that the negative regulation of contextual fear memory formation by AMPK in the CA1 is mediated by mTORC1 signaling. Therefore, we tested whether the prior inhibition of mTORC1 activity blocks the enhancement of contextual fear conditioning formation that is induced by AMPK inhibition in the CA1. Four groups of rats ($n = 9$–11 per group) were used in a 2 (rapamycin dose: 0 and 0.5 μg/side) × 2 (compound C dose: 0 and 3 μg/side) factorial design. The rats received rapamycin (0 and 0.5 μg/side) infusions 1 h before and compound C (0 and 3 μg/side) infusions immediately after weak contextual fear conditioning. All of the rats underwent the STM and LTM tests 1 and 24 h later. After the end of the LTM test, all of the rats were decapitated to determine AMPK/p70s6k phosphorylation and BDNF and

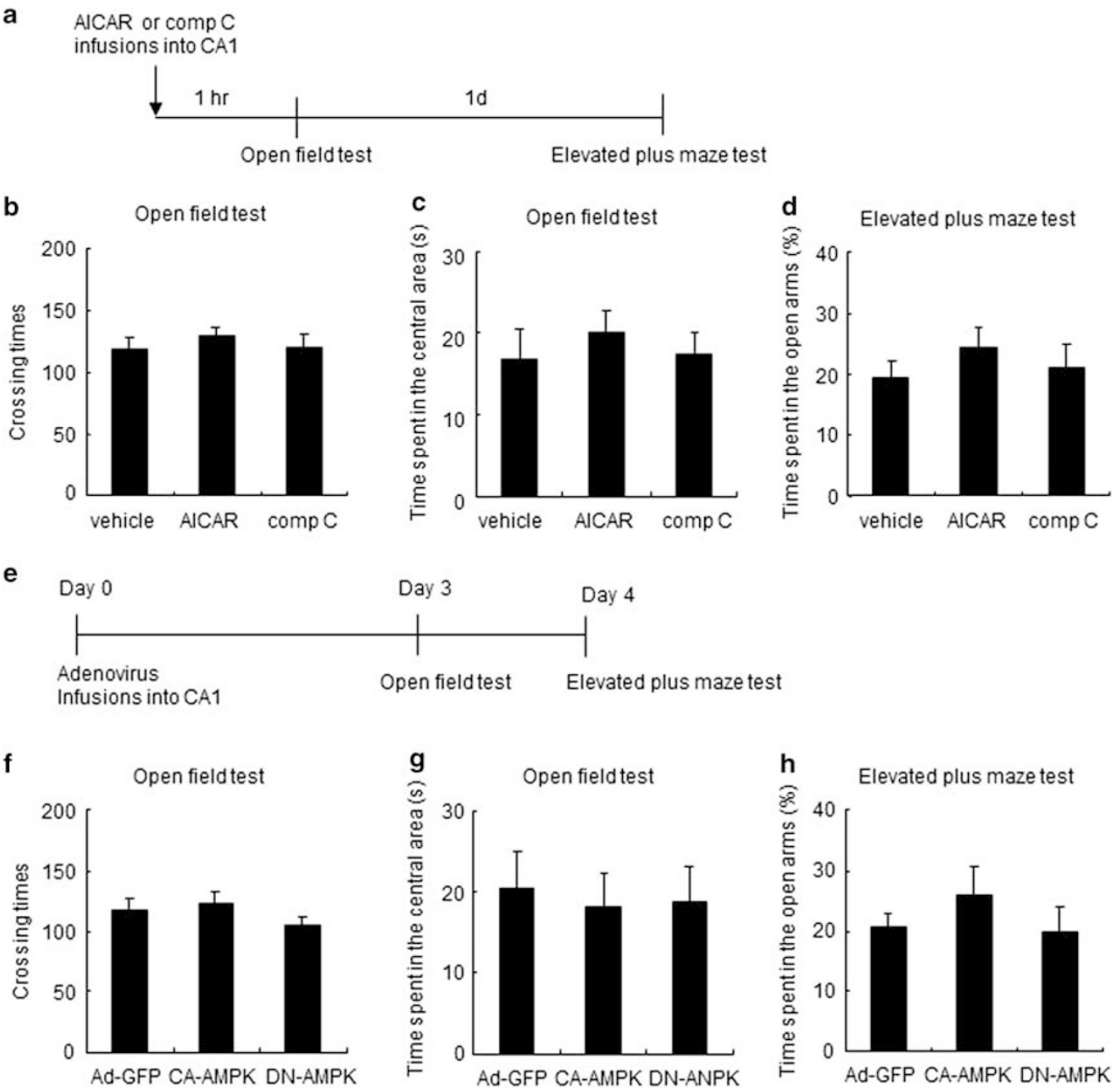

Figure 5 Modulation of AMPK activity in the CA1 has no effect on locomotor activity or anxiety-like behavior. The data are expressed as mean ± SEM. (a) Timeline of the experiment. One and 24 h after infusing vehicle, AICAR (5 μg/side), or compound C (3 μg/side) in the CA1, the rats were subjected to the open field test and elevated plus maze to evaluate locomotion and anxiety-like behavior, respectively, ($n = 9$–10 per group). (b) AICAR and compound C did not affect the number of crossings during the 5 min test in the open field. (c) AICAR and compound C did not affect the time spent in the central area of the open field (ie, a measure of anxiety). (d) AICAR and compound C did not affect the time spent in the open arms of the elevated plus maze. (e) Timeline of the experiment. Three days after infusing Ad-GFP, Ad-CA-AMPK, or Ad-DN-AMPK into the CA1, the rats were subjected to the open field test and elevated plus maze to evaluate locomotion and anxiety-like behavior ($n = 10$ per group). (f) Number of crossings during the 5 min test in the open field. (g) Time spent in the center area of the open field, a measure of anxiety. (h) Time spent in the open arms of the elevated plus maze. AMPK, adenosine monophosphate-activated protein kinase.

membrane GluR1 and GluR2 expression in the CA1 (Figure 7a). A two-way repeated-measures ANOVA, with test phase (baseline, STM test, and LTM test) as the within-subjects factor and rapamycin dose (0 and 0.5 μg/side) and compound C dose (0 and 3 μg/side) as the between-subjects factors, was performed to analyze freezing time. The ANOVA revealed a significant effect of test phase ($F_{2,76} = 88.77$, $p < 0.01$; Figure 7b), a test phase × rapamycin dose interaction ($F_{2,76} = 4.48$, $p < 0.05$; Figure 7b), and a test phase × compound C dose interaction ($F_{2,76} = 5.43$, $p < 0.01$; Figure 7b) but no test phase × rapamycin dose × compound C dose interaction ($F_{2,76} = 0.68$, $p > 0.1$; Figure 7b). The *post hoc* analysis showed that the rapamycin+compound C group exhibited a decrease in freezing in the LTM test compared with the vehicle+compound C group ($p < 0.05$), indicating that prior treatment with rapamycin blocked the enhancement of contextual fear memory formation that was induced by compound C. Rapamycin treatment alone did not significantly affect freezing time in the LTM test, which was likely attributable to a 'floor effect.' We also found that compound C decreased AMPK phosphorylation (one-way ANOVA, $F_{3,31} = 65.03$, $p < 0.01$; Figure 7c), which was not recovered by rapamycin treatment. Prior rapamycin infusion blocked the increase in p70s6k phosphorylation (one-way ANOVA, $F_{3,31} = 76.29$, $p < 0.01$; Figure 7c) and BDNF (one-way ANOVA, $F_{3,31} = 35.79$, $p < 0.01$; Figure 7c) and membrane GluR1 (one-way ANOVA, $F_{3,31} = 41.63$, $p < 0.01$; Figure 7c) and GluR2 (one-way ANOVA, $F_{3,31} = 55.18$, $p < 0.01$; Figure 7c) expression in the compound C-treated groups. Altogether, these results indicate that mTORC1

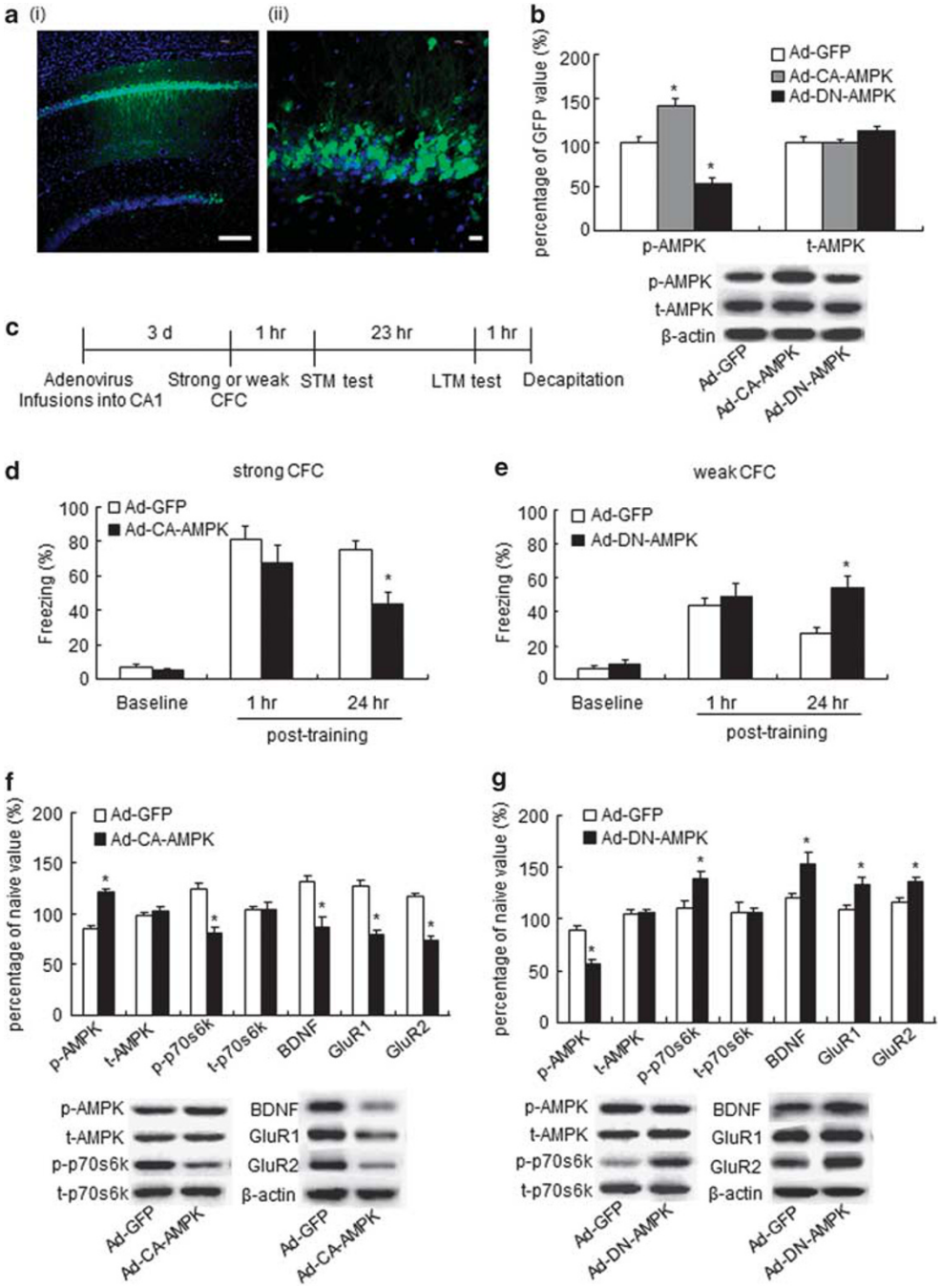

Figure 6 Modulation of AMPK activity by adenovirus transfer in the CA1 negatively regulates the formation of contextual fear memory. The data are expressed as mean ± SEM. (a) Representative image of adenovirus-mediated transgene expression in the CA1. The figure shows representative micrographs of enhanced green fluorescent protein (eGFP; green), counterstained with DAPI, after CA1 microinjection, showing adenovirus vector-mediated eGFP expression within a defined boundary of the CA1. (i) Low-power images (Scale bar = 200 μm); (ii) high-power images (Scale bar = 20 μm). (b) Western blot data for phosphorylated AMPK and total AMPK in the CA1 3 days after bilateral intra-CA1 infusion (1 μl/side) of Ad-GFP, Ad-CA-AMPK, or Ad-DN-AMPK ($n = 6$ per group). The data are expressed as a percentage of phosphorylated AMPK relative to Ad-GFP-treated rats. (c) Timeline of the experiment. (d) Percent time freezing during the baseline test, STM test (1 h after training), and LTM test (24 h after training) in rats injected with an adenovirus that expressed GFP or the CA-AMPK vector in the CA1 3 days before strong contextual fear conditioning ($n = 9$–11 per group). (e) Percent time freezing during the baseline test, STM test, and LTM test in rats injected with an adenovirus that expressed GFP or the DN-AMPK vector in the CA1 3 days before weak contextual fear conditioning ($n = 9$–11 per group). (f and g) Phosphorylated AMPK, phosphorylated p70s6k, total AMPK, total p70s6k, BDNF and membrane GluR1 and GluR2 protein levels, and representative western blots in the CA1 after the LTM test. The data are expressed as a percentage of phosphorylated AMPK, phosphorylated p70s6k, total AMPK, total p70s6k, and BDNF and membrane GluR1 and GluR2 levels relative to naive rats ($n = 8$ per group). *$p < 0.05$, compared with Ad-GFP group. AMPK, adenosine monophosphate-activated protein kinase; CFC, contextual fear conditioning; GFP, green fluorescent protein; LTM, long-term memory; p70s6k, p70s6 kinase; STM, short-term memory.

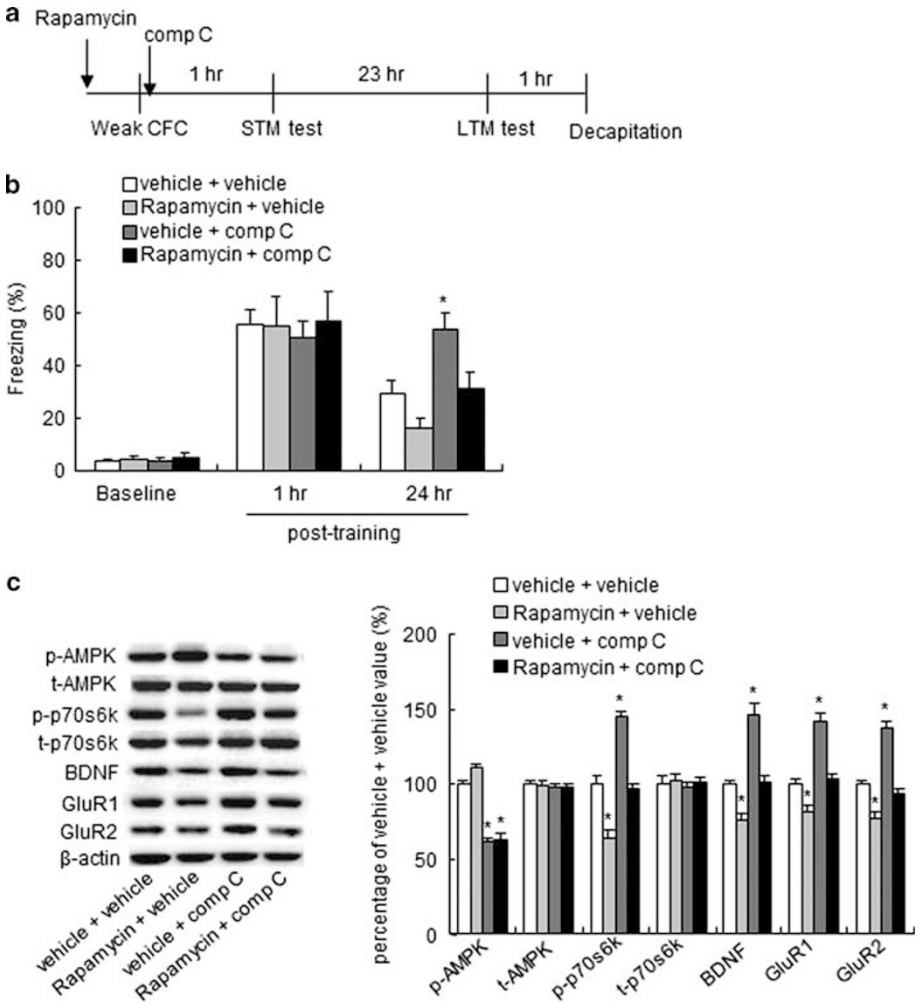

Figure 7 Enhancement of contextual fear memory by AMPK inhibition requires mTORC1 activation. The data are expressed as mean ± SEM. (a) Timeline of the experiment. (b) Percent time freezing during the baseline test, STM test, and LTM test in rats that received intra-CA1 infusions of rapamycin (0 and 0.5 μg/side) 1 h before and compound C (0 and 3 μg/side) immediately after weak contextual fear conditioning ($n = 9$–11 per group). (c) Phosphorylated AMPK, phosphorylated p70s6k, total AMPK, total p70s6k, BDNF and membrane GluR1 and GluR2 protein levels, and representative western blots in the CA1 after the LTM test. The data are expressed as a percentage of phosphorylated AMPK and p70s6k, total AMPK and p70s6k, and BDNF and membrane GluR1 and GluR2 relative to vehicle+vehicle-treated rats ($n = 8$ per group). *$p < 0.05$, compared with vehicle+vehicle group. AMPK, adenosine monophosphate-activated protein kinase; CFC, contextual fear conditioning; LTM, long-term memory; p70s6k, p70s6 kinase; STM, short-term memory.

inhibition blocked the enhancement of contextual fear memory that was induced by AMPK inhibition in the CA1.

AMPK in the CA1 Regulates Dendritic Spine Density Associated with Memory Formation

Previous studies showed that activation of the AMPK pathway suppressed axon growth and neuronal polarization and induced dendritic spine loss, whereas inhibiting AMPK pathway activity blocked Aβ42 oligomer-induced synaptotoxicity in hippocampal neurons *in vitro* and *in vivo* (Amato *et al*, 2011; Mairet-Coello *et al*, 2013). Dendritic spine growth and remodeling are crucial for memory formation (Moser *et al*, 1994; Restivo *et al*, 2009; Vetere *et al*, 2011). We investigated the effects of modulating AMPK activity in the CA1 on the learning-induced increase in spine density. Four groups of rats ($n = 5$ per group) were microinfused with an adenovirus that expressed a GFP control or CA-AMPK vector in the CA1. Three days later, the rats were trained for strong contextual fear conditioning or maintained in their home cage. One day later, the rats were perfused, and brains were removed for Golgi staining (Figure 8a). A two-way ANOVA, with condition (home cage and strong contextual fear conditioning) and vector (GFP and CA-AMPK) as the between-subjects factors, was performed to analyze total spine density. The ANOVA revealed significant effects of condition ($F_{1,16} = 17.88$, $p < 0.01$; Figure 8c) and vector ($F_{1,16} = 14.73$, $p < 0.01$; Figure 8c) and a condition × vector interaction ($F_{1,16} = 4.65$, $p < 0.05$; Figure 8c). The *post hoc* analysis showed that strong contextual fear conditioning training increased spine density in the Ad-GFP group ($p < 0.01$), which is consistent with previous studies (Moser *et al*, 1994; O'Malley *et al*, 1998; Restivo *et al*, 2009). The contextual fear conditioning induced increase in spine density was blocked by the adenovirus that expressed the CA-AMPK vector ($p < 0.01$). The CA-AMPK vector alone had no effect on spine density in rats that were confined to their home cage ($p > 0.1$).

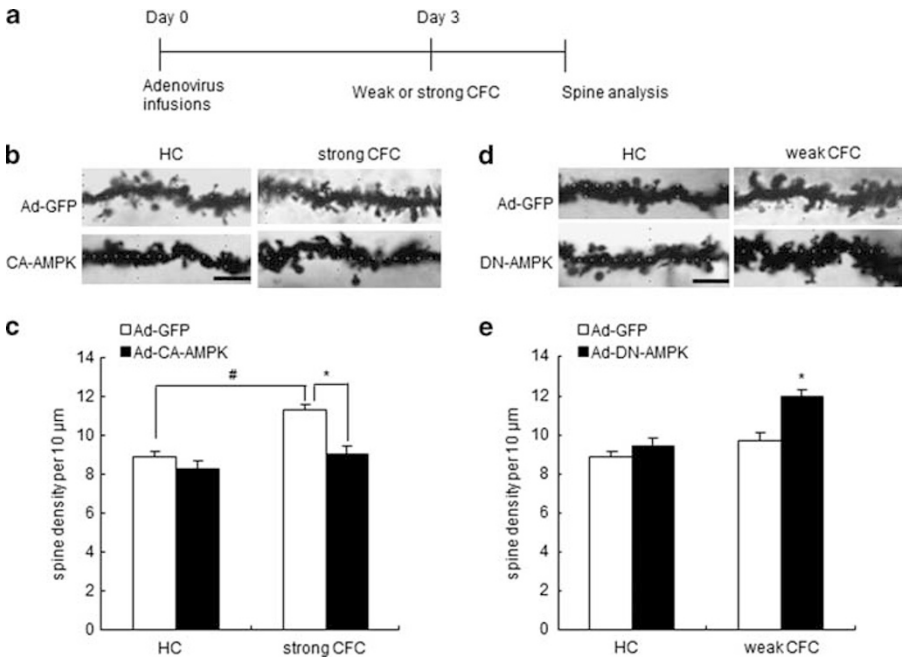

Figure 8 AMPK in the CA1 regulates dendritic spine density associated with the formation of contextual fear memory. The data are expressed as mean ± SEM. (a) Experimental protocol for rats that were microinjected with the adenovirus and trained for either strong or weak contextual fear conditioning or maintained in their home cage (HC). (b) Representative micrographs of dendritic spines in the CA1 in rats that were microinjected with an adenovirus that expressed GFP or the CA-AMPK vector and trained for strong contextual fear conditioning or maintained in their HC. Scale bar = 5 μm. (c) Spine density (per 10 μm dendritic segment) was averaged per rat in the following groups: HC-GFP, HC-CA-AMPK, strong CFC-GFP, and strong CFC-CA-AMPK ($n = 5$ rats per group). *$p < 0.05$, compared with strong CFC-GFP group; $^{\#}p < 0.05$, compared with HC-GFP group. (d) Representative micrographs of dendritic spines in the CA1 in rats that were microinjected with an adenovirus that expressed GFP or the DN-AMPK vector and trained for weak contextual fear conditioning or maintained in their home cage. Scale bar = 5 μm. (e) Spine density (per 10 μm dendritic segment) was averaged per rat in the following groups: HC-GFP, HC-DN-AMPK, weak CFC-GFP, weak CFC-DN-AMPK ($n = 5$–6 rats per group). The data are expressed as mean ± SEM. *$p < 0.05$, compared with weak CFC-GFP group. AMPK, adenosine monophosphate-activated protein kinase; HC, home cage; CFC, contextual fear conditioning; DN, dominant-negative; GFP, green fluorescent protein.

We next compared the spine density of neurons that were infected with an adenovirus that expressed GFP or a dominant-negative subunit of AMPK (DN-AMPK) in rats that were trained for weak contextual fear conditioning or maintained in their home cage ($n = 5$–6 per group; Figure 8a). A two-way ANOVA, with condition (home cage and weak contextual fear conditioning) and vector (GFP and DN-AMPK) as the between-subjects factors, was performed to analyze total spine density. The ANOVA revealed significant effects of condition ($F_{1,18} = 18.91$, $p < 0.01$; Figure 8e) and vector ($F_{1,18} = 13.43$, $p < 0.01$; Figure 8e) and a condition × vector interaction ($F_{1,18} = 4.58$, $p < 0.05$; Figure 8e). The *post hoc* analysis showed that decreasing AMPK activity by the adenovirus that expressed the DN-AMPK vector increased spine density in rats that were trained for weak contextual fear conditioning ($p < 0.01$). Weak contextual fear conditioning training did not significantly increase spine density in neurons that were infected with the control GFP vector ($p > 0.1$). The DN-AMPK vector alone had no significant effect on spine density in rats that were confined to their home cage ($p > 0.1$). Altogether, these data indicate that increasing AMPK activity inhibited learning-dependent spine growth and memory formation, whereas decreasing AMPK activity increased spine density under weak training conditions and enhanced memory formation.

DISCUSSION

In the present study, we investigated the role of AMPK signaling in the dorsal hippocampus in contextual fear memory formation. We found that AMPK is primarily expressed in neurons in the hippocampus, and we found that contextual fear conditioning induced the dephosphorylation of AMPK and phosphorylation of p70s6k in the CA1 but not CA3. Increasing AMPK activity by infusing the AMPK activator AICAR or an adenovirus that expressed the CA-AMPK vector in the CA1 impaired the formation of long-term fear memory. Decreasing AMPK activity by infusing the AMPK inhibitor compound C or an adenovirus that expressed the DN-AMPK vector in the CA1 enhanced contextual fear memory formation. These findings were associated with changes in the phosphorylation of AMPK and p70s6k and expression of BDNF and membrane GluR1 and GluR2 in the CA1. In addition, we found that increasing AMPK activity also prevented the increase in spine density that is normally associated with the formation of fear memory. Decreasing AMPK activity robustly increased spine density under weak training conditions, which is not normally sufficient to significantly increase spine density. Finally, we found that the enhancement of contextual fear memory formation by inhibiting AMPK in the CA1 was mediated by mTORC1 signaling. Altogether, our results indicate that AMPK activity in the CA1 negatively regulates

contextual fear memory formation and structural plasticity through mTORC1 signaling.

Contextual fear conditioning was associated with changes in AMPK phosphorylation in the CA1 but not CA3. Furthermore, modulating AMPK activity in the CA1 but not CA3 negatively regulated the formation of contextual fear memory. These results indicate that the effects of AMPK activity on the formation of contextual fear memory were anatomically specific. Around the time of training or during the first few hours post training, memory undergoes consolidation to become a LTM (Izquierdo *et al*, 2006; McGaugh, 1966). Short-term and long-term memories may have different molecular mechanisms. LTM but not STM depends on protein synthesis (Davis and Squire, 1984; Kandel, 2001). In the present study, we found that pre-training activation or inhibition of AMPK in the CA1 had no effect on short-term fear memory, indicating that AMPK activity in the CA1 is not required for the acquisition of contextual fear memory.

Previous studies on neurodegenerative disorders showed that the activation of AMPK in different pathological contexts has neuroprotective or deleterious outcomes in various neuronal subtypes (Mairet-Coello *et al*, 2013; Vingtdeux *et al*, 2011). The activation of AMPK was reported to increase memory dysfunction in male AβPP mice (DiTacchio *et al*, 2015). Other studies found that AMPK protected against memory impairment in a model of streptozotocin- or scopolamine-induced Alzheimer's disease in rats (Du *et al*, 2015; Kim *et al*, 2013). This inconsistency may be attributable to the specific animal models used, species, gender, or route of administration.

Under normal conditions, the effects of AMPK on spatial memory have also been inconsistent. Some studies showed that systemic administration of the AMPK agonist AICAR increased spatial memory and improved motor function in young and old female mice (Kobilo *et al*, 2014; Kobilo *et al*, 2011). Other studies found that the activation of AMPK impaired long-term spatial memory (Dash *et al*, 2006). The beneficial effects of systemic AICAR administration may be mediated by peripheral tissue, such as muscle tissue, and systemic AICAR administration is not beneficial for behavioral performance or neural plasticity in the absence of the muscle expression of AMPK, and oral AICAR administration is sufficient to increase running endurance by nearly 45% in sedentary mice (Narkar *et al*, 2008). In addition, $<1\%$ of AICAR is able to permeate the blood–brain barrier (Marangos *et al*, 1990), suggesting that the effects of systemic AICAR administration on the formation of spatial memory are likely indirect. Using a classical fear conditioning model, we found that increasing AMPK activity in the CA1 impaired the formation of long-term fear memory. Decreasing AMPK activity in the CA1 enhanced contextual fear memory formation. Our findings are consistent with previous studies, in which intra-hippocampal infusion of AICAR impaired hippocampus-dependent spatial learning (Dash *et al*, 2006). Altogether, these results indicate that AMPK in the CA1 negatively regulates the formation of contextual fear memory.

AMPK is activated by various types of metabolic stressors and drugs that alter the levels of AMP, ADP, and Ca^{2+}. AMPK can also be activated by other stimuli, independent of AMP, ADP, or Ca^{2+}, such as stimuli that are triggered by reactive oxygen species and DNA-damaging agents (Hardie, 2007). The activation of AMPK requires phosphorylation of threonine 172 within the T loop region of the catalytic α subunit. Although liver kinase B1 (also known as STK11 or Par4) mediates the majority of AMPK activation in most cell types, several lines of evidence suggest that Ca^{2+}/CaM-dependent protein kinase kinase β (CAMKKβ) is particularly involved in AMPK activation in various neuronal subtypes in response to calcium flux (Anderson *et al*, 2008; Green *et al*, 2011; Shaw *et al*, 2004). Activation of AMPK regulates cell growth and autophagy by suppressing mTORC1. The control of mTORC1 by AMPK can be regulated by direct phosphorylation of the tumor suppressor TSC2 and raptor (Mihaylova and Shaw, 2011). mTORC1 has a key role in the initiation of translation of specific classes of mRNAs via the phosphorylation of downstream effectors, such as p70s6 kinase and eIF4E binding protein. Furthermore, mTORC1 is implicated in synaptic function and memory (Costa-Mattioli *et al*, 2009; Kelleher *et al*, 2004). Previous behavioral studies showed that rapamycin treatment impaired LTM but not STM, which is consistent with the present findings, in which AMPK was found to be crucial for LTM formation but not STM (Jobim *et al*, 2012). In the present study, we also found that AMPK dephosphorylation in the CA1 after contextual fear conditioning was associated with an increase in p70s6k phosphorylation. Increasing or decreasing AMPK activity using pharmacological or gene-transfer methods consistently affected the phosphorylation of p70s6k. Moreover, we inhibited mTORC1 signaling by infusing rapamycin in the CA1 before contextual fear conditioning, which blocked the enhancement of fear memory formation that was induced by AMPK inhibition. Therefore, our data indicate that the regulation of fear memory formation by AMPK in the CA1 depends on mTORC1 signaling.

In neurons, mTORC1 is also present in the synapse and modulates the activity-dependent expression of locally translated proteins independent of mRNA synthesis. In addition, mTORC1 likely directly controls BDNF synthesis and release to dendrites (Henry *et al*, 2012). mTORC1 contributes to the dendritic translation and surface expression of GluR1, GluR2, and perhaps other synaptic proteins (Ran *et al*, 2013; Wang *et al*, 2006). Several studies indicated that BDNF upregulates protein levels of AMPAR (AMPA-type glutamate receptor) subunits in neurons and induces the distribution of AMPARs to the synapse (Caldeira *et al*, 2007; Narisawa-Saito *et al*, 2002; Reimers *et al*, 2014). The synaptic distribution of AMPARs in the hippocampus contributes to the synaptic strengthening that underlies learning and memory and is required for encoding contextual fear memories (Mitsushima *et al*, 2011). In the present study, we found that AMPK activation decreased the expression of BDNF and membrane GluR1 and GluR2. Inhibiting AMPK increased BDNF and membrane GluR1 and GluR2 expression, which were blocked by pretreatment with rapamycin. Therefore, we speculate that the modulation of fear memory formation by AMPK requires mTORC1 signaling through actions on BDNF expression and AMPAR synaptic expression.

Consistent with previous studies, we found that strong but not weak contextual fear conditioning was accompanied by an increase in dendritic spine density in CA1 pyramidal neurons (Moser *et al*, 1994; Restivo *et al*, 2009). Changes in

the density and shape of dendritic spines regulate synaptic strength associated with learning. Indeed, AMPK controls hippocampal late-phase long-term potentiation (L-LTP), which provides a cellular and molecular basis for its role in learning and memory (Potter *et al*, 2010). The fact that application of the AMPK inhibitor alone increased LTP indicated that latent AMPK activity tempers LTP maintenance under physiological conditions (Potter *et al*, 2010), which is consistent with our findings that reducing AMPK activity in the CA1 resulted in the enhanced formation of long-term contextual fear memory. We also found that the increase in spine density that is normally associated with the formation of strong fear memory was blocked by increasing AMPK activity in the CA1, whereas decreasing AMPK activity in the CA1 increased spine density under weak training conditions that are not normally sufficient to significantly increase spine density. These findings are consistent with previous studies that showed that AMPK activation induced dendritic spine loss, and inhibiting AMPK activity protected hippocampal neurons against the loss of dendritic spines and reduction of miniature excitatory postsynaptic current frequency that is induced by Aβ42 *in vitro* and *in vivo* (Mairet-Coello *et al*, 2013). The AMPK pathway also has an important role in axon initiation, neuronal polarization, and the age-related remodeling of retinal synapses (Amato *et al*, 2011; Samuel *et al*, 2014). Therefore, the regulation of synaptic and structural plasticity and LTM formation by AMPK is not only important under pathological conditions but also important under physiological conditions.

CONCLUDING REMARKS

In conclusion, we found that AMPK activity in the CA1 is involved in regulating structural plasticity and the formation of contextual fear memory in an mTORC1-dependent manner. These findings have theoretical value in elucidating the mechanisms involved in fear memory formation and possibly developing therapeutic interventions for pathologies that are related to memory dysfunction.

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