Autophosphorylation of F-actin binding domain of CaMKIIβ is required for fear learning

Karam Kima,1, Akio Suzukia, Hiroto Kojima,b,c, Meiko Kawamurad, Ken Miya,e, Manabu Abe,d, Ikuko Yamada,f, Tamio Furusef, Shigenaru Wakana,f,2, Kenji Sakimurad, Yasunori Hayashia,c,g,h,⁎

a Brain Science Institute, RIKEN, Wako, Saitama 351-0198, Japan
b Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan
c Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan
d Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan
e Department of Molecular Neurobiology, Graduate School of Comprehensive Human Sciences, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
f Technology and Development Team for Mouse Phenotype Analysis: Japan Mouse Clinic, RIKEN Bioresource Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan
g Brain and Body System Science Institute, Saitama University, Saitama 338-8570, Japan
h School of Life Science, South China Normal University, Guangzhou 510631, China

ARTICLE INFO

Keywords:
Ca2+/calmodulin-dependent protein kinase II
Actin cytoskeleton
Synaptic plasticity
Mouse behavior battery
Learning and memory
Fear conditioning test
Phosphorylation
Knock-in mouse

ABSTRACT

CaMKII is a pivotal kinase that plays essential roles in synaptic plasticity. Apart from its signaling function, the structural function of CaMKII is becoming clear. CaMKII – F-actin interaction stabilizes actin cytoskeleton in a dendritic spine. A transient autophosphorylation at the F-actin binding region during LTP releases CaMKII from F-actin and opens a brief time-window of actin reorganization. However, the physiological relevance of this finding in learning and memory was not presented. Using a knock-in (KI) mouse carrying phosphoblock mutations in the actin-binding domain of CaMKIIβ, we demonstrate that proper regulation of CaMKII – F-actin interaction is important for fear conditioning memory tasks. The KI mice show poor performance in contextual and cued versions of fear conditioning test. These results suggest the importance of CaMKII – F-actin interactions in learning and memory.

1. Introduction

Calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII), a serine/threonine protein kinase, is one of the most abundant proteins in the brain (Chen, 2005; Dosemeci, 2007; Kennedy, Bennett, & Erondu, 1983; Peng, 2004; Sheng & Hoogenraad, 2007), and is essential for synaptic plasticity such as long-term potentiation (LTP) of synaptic transmission and learning (Coultrap & Bayer, 2012; Hell, 2014; Lisman, Yasuda, & Raghavachari, 2012; Shonesy, 2014). In response to an increase in intracellular Ca2+ concentration, CaMKII is activated by Ca2+/CaM binding, which causes autophosphorylation at Thr286 and makes CaMKII activity Ca2+-independent. Activation of CaMKII is not only necessary (Giese, 1998; Malinow, Schulman, & Tsien, 1989; Otmakhov, Griffith, & Lisman, 1997) but also sufficient for LTP induction as introduction of active CaMKII mimics and occludes LTP induction (Hayashi, 2000; Joudain, Fukunaga, & Muller, 2003; Lledo, 1995; Pettit, Perlman, & Malinow, 1994; Poncer, Esteban, & Malinow, 2002).

Consistent with the essential roles CaMKII plays during synaptic plasticity, results from genetically modified mice with alterations in the properties and/or quantity of CaMKII demonstrate clear roles of CaMKII in various types of learning and memory. It has been reported that various types of CaMKII mutants mice show deficits in hippocampus-dependent and independent learning. In mice where CaMKIIa is absent or reduced, severe impairment of spatial learning in Morris water maze and remote memory for contextual fear conditioning were reported (Elgersma, 2002; Frankland, 2004; Silva, 1992). Mice harboring a mutation blocking or mimicking phosphorylation at Thr286, showed poor performance in water maze (Bejar, 2002; Giese, 1998; Need & Giese, 2003), Barnes maze (Bach, 1995; Mayford, 1996), olfactory learning (Wiedenmayer, 2000), cued and contextual conditioning (Bejar, 2002; Mayford, 1996) and unstable place cells (Cho, 1998;...
Ca2+/calmodulin-dependent kinase activation is disabled but F-actin cAMP-dependent LTP, freezing in contextual fear conditioning and localization of spine structure (Kim, 2015; Okamoto, 2007). Normal levels of hippocampal LTP (Borgesius, 2011), and in the novel object recognition task (Bachstetter, 2014).

In addition to well-known role of CaMKII as a signaling molecule, recent studies identified a structural role of CaMKII (Kim, 2016). CaMKIIβ has a unique sequence that interacts with F-actin. The dodecameric structure of CaMKII allows it to simultaneously interact with more than one F-actin through the domain. This bundles F-actin and at the same time, prevents its association with actin regulators (Kim, 2016). This binding is preserved, in contrast to KO mice (Borgesius, 2011). This indicates that F-actin binding property of CaMKIIβ is critical. Interestingly, during LTP induction, the autophosphorylation of F-actin binding domain transiently detaches CaMKII from F-actin, thereby generating a time window of F-actin modification. Consistent with the importance of this process, blocking the phosphorylation of this site impairs LTP without changing kinase activity towards exogenous substrate (Kim, 2015).

In order to further interrogate the role of the regulation of CaMKII – F-actin interaction by autophosphorylation, we generated a KI mouse, in which the actin-binding domain of CaMKIIβ has mutations leading to the elimination of phosphorylation sites of this region critical for CaMKII detachment from F-actin. The mice showed reduced freezing level in fear conditioning tests while performance in other behavior assays was comparable to WT control mice. These results confirm that CaMKII – F-actin interaction which gates synaptic plasticity by re-organizing actin cytoskeleton in spines also plays important roles in learning and memory process in live animals, but its contribution varies depending on the type of memory and brain regions.

2. Materials and methods

2.1. Animal care

Animal experiments were conducted in accordance with the institutional guidelines of RIKEN and Niigata University.

2.2. Generation of KI animal

A genomic clone, RP23-66K24 containing the CaMKIIβ gene was isolated from a C57BL6 BAC genomic library (Advanced GenoTechs, Tsukuba, Japan). The Quick and Easy BAC modification Kit (Gene Bridges, Dresden, Germany) was used for targeting vector construction. To produce CaMKIIβ(exon13:TS/A)FLEX vector, a 450 bp DNA fragment carrying exon 13 mutation (exon13:TS/A) of the CaMKIIβ, which includes alanine substitutions into all of the serine/threonine residues, was created by PCR mutagenesis. A 530 bp DNA fragment (lox2272-exon 13:TS/A-inverted loxP) was amplified by PCR and inserted in the HindIII/EcoRI sites of a middle entry clone (pDME-1) in reverse direction. In this clone, Neo cassette (Pkg promoter-driven neo-poly(A) flanked by two FRT (flipase recognition target) elements) and following loxP were located downstream of thelox2272 sequence. A 505 bp DNA fragment (lox2272 – exon 13 of CaMKIIβ) was amplified by PCR and inserted in the PacI/KpnI sites of 5′ entry clone (pD5U-2) to produce a pD5U-2/lox2272 + exon 13. The 6.05 kb upstream and 5.1 kb downstream homology arms were retrieved from the BAC clone, and appropriately subcloned to pD5U-2/lox2272 + exon 13 and 3′ entry clone (pD3DE-2), respectively.

For targeting vector assembly, three entry clones were recombined to a destination vector plasmid (pDEST-DT) containing a Cytomegalovirus enhancer/chicken β actin (CAG) promoter-driven diphtheria toxin gene) by using Multi Gateway Three-fragment Vector construction Kit (Invitrogen, California, U.S.A).

Culture of ES cells and generation of chimeric mice (CaMKIIβ(exon13:TS/A)FLEX) were performed as described previously (Mishina & Sakimura, 2007). Homologous recombinant ES clones were identified by southern blot hybridization (Fig. 1C). Genomic DNA from wild-type (+/+ ) and CaMKIIβ(exon13:TS/A)FLEX (+/+ FLEX/+ ) ES cells were digested with respective enzymes and probed with three different probes. EcoRI-digested DNA hybridized with a 5′ probe: 17.9 kb for wild-type and 12.6 kb for FLEX allele. HindIII-digested DNA hybridized with a Neo probe: 14.7 kb for FLEX allele. XbaI-digested DNA hybridized with a 3′ probe: 13.4 kb for wild-type and 8.9 kb for FLEX allele.

Cre-mediated recombination was identified by PCR (Fig. 1D). FLEXed ES cells were electroporated with plasmids carrying improved Cre recombinase (iCre) and puromycin N-acetyltransferase (Pac) genes. After selection with puromycin, DNA from transfected cells was amplified with PCR. The primers used here were 27961F: GACAGCTTCTGTC GTGGGCGTCTTC and 28630R GATGAGACAAGAGTGAGGGTCGAC. The size of the product was estimated on agarose gel and the sequence was confirmed.

To generate germine chimera, the recombinant ES cells were microinjected into 8 cell-stage embryos of CD-1 mouse strain. The embryos were cultured to blastocysts and transferred to pseudopregnant CD-1 mouse uterus. Germine chimeras were crossed with C57BL/6N female mice to obtain the heterozygous offspring (CaMKIIβ(exon13:TS/A)FLEX+/+ ). Genotyping of mice tail DNA was determined by PCR with the following specific primers: mutF, 5′-GCCCTTCTATGGCCTTCTGAC GAG-3′; 28630R; 27961F. Positions of the primers are indicated in Fig. 1B.

After obtaining exon 13:TS/A-neo flexed allele mouse, we crossed it with CAG-Cre transgenic animal (Sakai & Miyazaki, 1997) to invert exon 13:TS/A and to remove Neo cassette. The Cre-mediated recombination was confirmed by amplifying the genomic PCR, followed by the sequencing reaction. Because CAG-Cre line expresses Cre in germ line cells, the crossing was done only at one generation. After confirming the recombination, all offspring carried KI locus.

2.3. In vitro kinase assay

Hippocampus from 5 month-old KI mouse and age-matched WT was homogenized in kinase assay buffer (40 mM HEPES-NaOH [pH 8.0], 0.5 mM EGTA, 5 mM MgOAc, 0.01% Tween20) followed by the centrifugation at 16,000g, 4°C for 10 min. Supernatant was mixed with CaM (final 1 μM) and ATP (final 50 μM). Final 17 mM EDTA was added to part of the mixture and used as ‘−’ sample in Fig. 2 (no stimulation). CaMKII in the mixture was activated by adding CaCl2 (final 0.65 mM) and reaction continued for 10 min until EDTA (final 17 mM) stopped it (+ sample in Fig. 2). P-S331 and P-S371 antibodies were described in

K. Kim et al.

Neurobiology of Learning and Memory 157 (2019) 86-95
Fig. 1. Generation of knock-in mouse carrying exon 13:TS/A mutations. (A) Position of mutated residues in CaMKIIβ. (B) Strategy of knock-in. Top, original wild type allele. Middle, targeting construct. Bottom, expected genomic structure after Cre-mediated recombination. Positions of DNA probe (grey) and PCR primers (arrows) are also shown. (C) Confirmation of homologous recombination in ES cell. Genomic DNA from original ES cell line and FLEX/+ ES cells was digested with respective enzymes and probed with three different probes. The image shown here is a collage made from three southern blotting filters. The molecular weight marker was used to adjust the position of bands. (D) Confirmation of Cre-mediated recombination. The plasmid expressing iCre and Pac genes were electroporated into the FLEX/+ ES cells. The transfected cells were selected with puromycin for 24 h and further cultured. Genomic DNA was isolated and amplified with PCR using 27961F and 28630R primers. Compared with the original ES cells, the PCR product from electroporated FLEX/+ ES cells was longer by the expected length due to the insertion of loxP and lox2272 sequences. The sequence of product was confirmed (not shown).
CaMKIIβ and CaMKII antibodies were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Dallas, USA), respectively.

2.4. Tissue staining

After transcardial perfusion with 4% (w/v) paraformaldehyde (PFA)/PBS, mouse (7 months old) brain was removed and fixed in 4% PFA/PBS at 4°C for 24 h, followed by incubation in PBS for 24 h at 4°C. Parasagittal brain slices (100 μm-thick) were incubated with primary antibody (anti-CaMKIIβ, 1:100, Abcam 34703) diluted with TNB buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagents (w/v, PerkinElmer), pH 7.5) containing 0.5% Triton X-100 at 4°C for 15 weeks of age. (B) Western blotting with phosphospecific antibodies against S331 on exon 13 and S371 on exon 15, CaMKIIβ, and pan-CaMKII antibodies. Hippocampal homogenate from WT and KI were stimulated with 0.15 mM free Ca²⁺, 1 μM of calmodulin, and 50 μM of ATP. The reaction was carried out at 25°C for 10 min and stopped by adding final 17 mM EGTA. (C) Body weight of WT and KI at 15 weeks of age. (D) Brain of WT and KI animals at 7 months of age. (E) Parasagittal sections of brain of WT and KI animals stained with Hoechst H33258 (blue), anti-CaMKIIβ antibody (green) and AlexaFluor594-Phalloidin (red). Note that part of the cerebellum was torn off. (F), (G) High magnification images of hippocampus (F) and cerebellum (G) of WT and KI brain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. CaMKIIβ exon 13:TS/A knock-in animals lose phosphorylation in actin binding region but have normal body weight and brain structure. (A) Genomic PCR using 27961F and 28630R primers. FLEXed mouse was generated from FLEX/+ ES cell and further crossed with CAG-Cre mouse to invert and excise the FLEXed allele to obtain KI animal, which was further crossed to obtain KI/KI animal. The image shown here is a collage made from a single larger image, from which one lane between KI/KI and WT/KI was removed but without changing the height or intensity of the bands. The brightness is also inverted. (B) Western blotting with phosphospecific antibodies against S331 on exon 13 and S371 on exon 15, CaMKIIβ, and pan-CaMKII antibodies. Hippocampal homogenate from WT and KI were stimulated with 0.15 mM free Ca²⁺, 1 μM of calmodulin, and 50 μM of ATP. The reaction was carried out at 25°C for 10 min and stopped by adding final 17 mM EGTA. (C) Body weight of WT and KI at 15 weeks of age. (D) Brain of WT and KI animals at 7 months of age. (E) Parasagittal sections of brain of WT and KI animals stained with Hoechst H33258 (blue), anti-CaMKIIβ antibody (green) and AlexaFluor594-Phalloidin (red). Note that part of the cerebellum was torn off. (F), (G) High magnification images of hippocampus (F) and cerebellum (G) of WT and KI brain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Kim, 2015). CaMKIIβ and CaMKII antibodies were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Dallas, USA), respectively.
Table 1
Summary of behavior tests.

<table>
<thead>
<tr>
<th>Behavior test</th>
<th>Age of animal</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light/dark transition test (7 weeks)</td>
<td>N.S.</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>Open field test (8 weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Object recognition test (9 weeks)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Crawley social interaction test (11 weeks)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Home cage activity test (12-13 weeks)</td>
<td>Figs. 3, N.S.</td>
<td></td>
</tr>
<tr>
<td>Y-maze test (14 weeks)</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Pre-pulse inhibition test (15 weeks)</td>
<td>N.S.</td>
<td>Fig. 5</td>
</tr>
<tr>
<td>Fear conditioning test (16 weeks)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Name of behavior test, age of animal when the test was conducted, and results are shown. N.S.: not significant.

2.5. Behavior test

All behavior tests were carried out in the Japan Mouse Clinic, RIKEN BioResource Center. The order of tests conducted and the age of animals when they underwent the tests are summarized in Table 1. Results of the behavioral tests are sensitive to prior experiences (Crawley, 2008). Therefore, to suppress effects of prior experiences, the tests were started from the least invasive one. 10 wt and 10 KI mice (all males) were used for behavioral assays. For all tests, the mice were transferred from their home cages to the testing room at least 30 min before the experiment.

2.5.1. Light/dark transition

Light/dark transition test was performed as described previously (Furuse, 2017). The apparatus consists of two sections with equal size (200 mm long × 200 mm wide × 250 mm high; O'Hara & Co., Ltd., Tokyo, Japan) divided by a partition with a hole (50 mm wide × 30 mm high). Illumination is provided by a fluorescent lamp on the ceiling and a 4 W fluorescent lamp mounted above the light chamber (353 lx in the light chamber and 0.1 lx in the dark chamber). The opening was controlled by a guillotine door. Each mouse was placed in the middle of the light section, after opening of guillotine door, allowed to freely move for 10 min. Movement of a mouse was recorded by a video-tracking system including CCD camera and analyzing software (Image LD4, O’Hara & Co., Ltd.). Total distance travelled, latency for entering into light chamber, number of transition and time spent in light chamber were automatically analyzed using the video-imaging system above.

2.5.2. Open-field

Open-field test was performed as described previously (Furuse, 2010) with slight modifications. Each mouse was placed in the middle of a peripheral zone of the arena facing the wall of an open-field apparatus (400 mm wide × 400 mm long × 300 mm high; O’Hara & Co., Ltd.) made of white polyvinyl chloride. The distance traveled by each animal in the open-field was recorded for 20 min with a video-imaging system (Image OF9; O’Hara & Co., Ltd.).

2.5.3. Object recognition

We performed object recognition task as previously reported (Jadavji, 2015; Leger, 2013) with modifications. Before exposing to the objects, mice spent 10 min in the open-field arena for habituation. Mice were exposed at a training session to two identical objects (tissue culture flask filled with granular dry-silica-gel, 9.5 cm high, 2.5 cm deep and 5.5 cm wide, transparent plastic with a blue bottle cap) in an open-field arena described above for 10 min. Entire the test, the objects were placed in adjacent corners. After the training session, mice were placed back to their home cage and one of the objects was replaced with new object (Lego cuboid, 8 cm high, 4 cm wide, and 4 cm deep, build in black, gray, and white bricks, LEGO) immediately after the replacement, mice were placed in the middle of a peripheral zone of the arena facing the wall and the mouse underwent a testing session for 10 min. The amount of time for exploring each object (defined by a state in which the nose tip of the mouse is within 2 cm from the object) was measured, and a discrimination index ((time novel − time familiar)/(time novel + time familiar)) was calculated.

2.5.4. Crawley social interaction

We performed Crawley’s Sociability test as previously described (Yoshizaki, 2016). In brief, the apparatus comprised a rectangular, three-chambered box and a lid containing an infrared video camera (O’Hara & Co.). Each chamber was 20 × 40 × 22 cm and the dividing walls were made from clear Plexiglass, with small square openings (5 × 3 cm) allowing access into each chamber. Each mouse was habituated to the test apparatus for 10 min. After 1 week from the habituation, the social interaction test was performed. Firstly, each mouse was habituated for 10 min again. In order to examine object exploration behavior of the subject mice, an inanimate object (colors and shape were different from the objects used in the object recognition task, Lego tower, 8 cm high, 4 cm wide, 3.2 cm depth, build in red, yellow, orange, black, green, light green, blue, and white bricks, LEGO) was placed in a wire cage in one side of the chamber. The total time spent near each cage (< 4.5 cm) during a 10-min period was determined with a video-imaging system (Time CS12, O’Hara & Co.). As for social interaction test, the inanimate object was replaced with a stranger mouse (male C57BL/6N) and the time spent near each cage (< 4.5 cm) during a 10-min period was determined.

2.5.5. Home cage activity

Home-cage activity test was performed as described previously (Furuse, 2017). Each mouse was placed alone in a testing cage (22.7 × 32.9 × 13.3 cm) under a 12 h light–dark cycle (light on at 08:00 am) and had free access to both food and water. After 1 day of acclimation, spontaneous activity in the cage was measured for 5 days (starting at 08:00 am) using an infrared sensor (activity sensor, O’Hara & Co., Ltd.). Home cage parameters included activity in light phase, activity in dark phase, total activity and activity ratio between light phase and total. These were obtained by counting the number of beam interruptions during 1 min intervals.

2.5.6. Y-maze

The apparatus consisting of three arms (arm length: 40 cm, arm bottom width: 3 cm, arm upper width: 10 cm, height of wall: 12 cm high, O’Hara Co. Ltd) was used. A mouse was placed on center of the apparatus. Spontaneous locomotor activity, number of entry into each arm, and alteration ratio are measured. These parameters were recorded using video recording system (Time YM 2, O’Hara Co. Ltd).

2.5.7. Pre-pulse inhibition

We performed pre-pulse inhibition test as previously described (Yoshizaki, 2016). Load cell, mouse chamber, sound generator, and sound-proof box (33 cm in length, 43 cm in width, 33 cm in height) were purchased from O’Hara Co., Ltd. Before each testing session, mechanical responses were calibrated. Mouse was acclimated to chamber for 5 min (only 65 dB background noise was on). During this period, 110 dB/40 ms of white noise was presented for 5 times in order to acclimate mice to startle pulse. Startle response to these stimuli were excluded from the statistical analysis. Prepulse sounds (70 dB, 75 dB, 80 dB, 85 dB for 20 ms) and a startle sound (110 dB for 50 ms) were presented 10 times in pseudorandom order, with an inter-trial interval varying randomly between 10 and 20 sec and startle amplitude was measured 50 ms after presentation of the prepulse sound. Percentage PPI was calculated as ([startle amplitude without prepulse] − [startle amplitude of trial with prepulse])/([startle amplitude without prepulse] × 100).

2.5.8. Fear conditioning

We performed fear-conditioning test as previously described with slight modifications (Furuse, 2017). We used automated fear contextual and tone dependent fear conditioning apparatus, Image F24 (O’Hara Co. Ltd.) in this study. On the training day (day 1), each mouse was
placed into a shocking chamber (10 × 10 × 10 cm, white polyvinyl chloride boards, stainless steel rod floor, O’Hara & Co. Ltd) (Box A) and 120 sec later, 4 tone-shock pairs were given at 90 sec intervals. Each tone-shock pair consisted of tone (70 dB, 10 kHz) for 30 sec and a foot shock of 0.5 sec at 0.5 mA. The foot shock was presented to mice during the last 0.5 sec of the tone. On day 2, each mouse was placed back in box A for 6 min to measure contextual freezing. On day 3, each mouse was placed in a white transparent chamber (Box B), and 120 sec later, 30 sec tone was given at 90 sec intervals for 4 times. Freezing during the first 120 sec was “Pre-tone” in Box B (i.e., response to an un-conditioned context), and freezing during the tone presentations was determined as the response to the tone.

3. Result

Autophosphorylation in the F-actin-binding region of CaMKIIβ detaches CaMKII from F-actin during LTP without affecting kinase activity (Shen & Meyer, 1999; Shen, 1998). Preventing this process by phos- phoblocking mutations impairs LTP as well as structural modification of dendritic spines associated with LTP (Kim, 2015). To investigate whether detachment of CaMKIIβ from F-actin is also important during learning and memory processes in live animals, we generated a KI mouse. The F-actin binding region is encoded by four exons (exon 13–16) and we found that the region encoded by exon 13 mostly determines actin-binding ability (Kim, 2015). Therefore, we replaced 8 serines and threonines in exon 13 of mouse CaMKIIβ with alanine (Fig. 1A). Because more extensive phosphoblock mutations in CaMKIIβ-specific sequence including these sites did not affect both Ca2+/calmodulin-induced and constitutive kinase activity (Kim, 2015), we expected the phosphoblock mutations in exon 13 will not affect the kinase activity either. We first generated ES cells carrying FLExed allele by homologous recombination (Fig. 1B and C). After confirming that it can undergo proper inversion and removal of neo cassette by transient expression of Cre recombinase (Fig. 1D), we generated a FLExed mouse line from the cells. The line was crossed with CAG-Cre mouse line, which expresses Cre in germ line, and obtained CaMKIIβlox13:TS/A knock-in (KI) animals. PCR of tail biopsy DNA using primers binding to intron regions upstream and downstream of exon 13 results in larger fragment in KI mouse than WT due to loxP/lox2272 sequences when recombination occurs (Fig. 2A). We also confirm that serines and threonines in the target area are mutated to alanine in the KI mouse by sequencing RT-PCR fragments (data not shown).

To confirm whether the change in chromosome level leads to the elimination of phosphorylation of the target site, we carried out in vitro kinase assays using hippocampal lysate, and monitored the phosphorylation of serine 331 and 371, encoded by exon 13 and 15 respectively, by specific antibodies (Kim, 2015). As shown in Fig. 2B, phosphorylation at serine 331 disappeared due to alanine mutation while serine 371 phosphorylation remained intact. Upward band-shift upon Ca2+/CaM stimulation in CaMKIIβ and pan-CaMKII antibody blots indicates that sites in CaMKIIβ other than targeted sites are properly phosphorylated in KI mouse. This is consistent with our previous observation that phosphorylation of the F-actin binding region does not interfere with kinase regulation and activity using recombinant protein (Kim, 2015). These results show that CaMKIIβ in KI mice harbor alanine mutations blocking phosphorylation of this region despite CaMKII activation, which enables CaMKIIβ to maintain its interaction with F-actin.

The KI mice were apparently healthy, fertile and indistinguishable from WT. Their body weight was comparable to WT (Fig. 2C). The size and gross morphology of their brain were also comparable to those from WT mice (Fig. 2D). Also, cellular architectures as revealed by AlexaFluor 594-phalloidin and Hoechst staining was indistinguishable from WT (Fig. 2E). Distribution of CaMKIIβ at tissue level was also comparable between WT and KI (Fig. 2F and G).

We then carried out a series of behavior assays to test any potential abnormalities in anxiety level, sociability, cognition and sensorimotor gating (Table 1). They showed similar daily activity in home cage (Fig. 3). In contrast to CaMKIIβ KO or A303R mouse which showed an impairment in motor performance (Bachstetter, 2014; Kool, 2016; van Woerden, 2009), our KI mice did not show any obvious abnormalities in motor function although we cannot rule out the possibility of subtle phenotype which can be detected only with detailed tests. However, in the open field test, we found that the KI animals were more active and travelled more (Fig. 4A–D). The KI animals entered the center of the arena more (Fig. 4C) but the percentage at the center of arena was not different from wild type (Fig. 4E), indicating anxiety level, as measured by this assay, is not altered. Rather, we consider the increase in the entry to the center of the arena simply reflects increased activity.

In order to test the memory capacity of the animal, we carried out fear conditioning tests, which assesses a memory as a result of the association between an aversive stimulus and environmental cues. The KI mice show significantly less freezing than WT in both contextual and cued versions of fear conditioning tests (p = 0.0281, contextual version; p = 0.0007 [bin 5–10] and p = 0.0022 [bin 5, 9, 13, 17] of cued version (Fig. 5). However, the memory deficit was specific to these two tests. It did not show impairment in an object recognition test or Y-maze test. The results of other tests, in which KI did not show any significant difference from WT controls, are shown in supplementary figures.

4. Discussion

The most remarkable result of our study is that KI mice showed impairment in fear conditioning tests (Fig. 5). Usually, the environmental cues are a tone (‘cue’) and/or the test chamber (‘context’). Different brain regions and pathways are believed to be involved in each version, but amygdala is the common brain region in both cases where the information about the cues and aversive experience converges, and the control of fear reactions takes place (Durvari & Pare, 2014; Kim & Jung, 2006; LeDoux, 2000; Tooyeva, Fadok, & Luthi, 2015). In cued fear condition- ing, auditory inputs are transmitted to lateral amygdala (LA) via the auditory thalamus and the auditory cortex (LeDoux, Farb, & Ruggiero, 1990; Mascagni, McDonald, & Coleman, 1993; Romanski & LeDoux, 1993). On the other hand, contextual stimuli from the hippo- campus are transmitted to the basal/accessory basal amygdala (Canteras & Swanson, 1992) so both the amygdala and the hippocampus are re- quired for contextual fear conditioning (Blanchard, Blanchard, & Fial, 1970; Frankland, 1998; Kim & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Phillips & LeDoux, 1992). In both cases, conditioning to a tone in the LA and conditioning to a context in the basal/accessory basal amygdala are transmitted to the central nucleus of the amygdala and in turn, induces a fear response via the brainstem. In these terms, it is noticeable that CaMKII is highly expressed in the lateral and basolateral amygdala (Burgin, 1990; Miller & Kennedy, 1986). Accordingly, these results suggest that CaMKII is involved in learning and memory of fear conditioning, and LTP at the LA. In support of this, active CaMKIIα increases in LA synapses after fear conditioning (Rodrigues, 2004), and infusion of the CaMKII antagonist KN-62 to the LA before training disrupts both short- and long-term memory of cued and contextual fear conditioning (Rodrigues, 2004). Also, genetic manipulation of CaMKIIα restricted to the forebrain area including LA caused reversible deficits in cued and contextual fear long-term memory (Mayford, 1996; Wang, 2003). Considering the regulation of CaMKII – F-actin interaction by autophosphorylation is important in LTP induction (Kim, 2015), it is conceivable that continued binding of CaMKII to F-actin in the amygdala caused deficits in LTP, leading to impaired learning and/or memory function in fear conditioning.

Another interesting observation is that KI mice showed longer travelling distances both at the center and periphery of the open field arena with higher average speed than WT in the open field test (Fig. 4). It is not clear whether this is due to differences in their levels of spontaneous motor activity, exploratory behavior, or anxiety, however the two groups of mice spent a similar proportion of their time at the center area suggesting similar anxiety levels. However, CaMKII has also
been known to be involved in emotional behaviors such as anxiety, aggression besides learning and memory. Transgenic mice overexpressing CaMKIIα shows increased anxiety-like behaviors in open field, elevated zero maze, light-dark transition and social interaction tests (Hasegawa, 2009) whereas CaMKIIα heterozygote KO mice exhibits different characteristics, such as reductions in anxiety-like behavior, and increased defensive aggression but normal offensive aggression, as well as symptoms of psychiatric disorders such as bipolar disorder and schizophrenia (Chen, 1994; Hasegawa, 2009; Yamasaki, 2008). Therefore, CaMKIIα levels seem to positively correlate with anxiety-like behavior. CaMKIIβ levels also seem to relate to emotion, as CaMKIIβ KO mice show decreased levels of anxiety-related behavior (Bachstetter, 2014). CaMKIIβ was up-regulated in the lateral habenula of a depression model mouse, and down-regulated by antidepressants (Li, 2013). These results indicate that the maintenance of appropriate CaMKII levels is necessary for normal emotional regulation. Considering this, there is a possibility that dysregulation of CaMKII – F-actin interaction in our KI mice might cause abnormality in the performance of the open field test but more investigation would be necessary to reach a clear conclusion.

It is important to note that our KI mouse shows different phenotypes from CaMKIIβ KO mouse or those containing other types of genetic modifications. CaMKIIβ KO mouse shows deficit in novel recognition test (Bachstetter, 2014) while our KI mouse doesn’t (Supple. Fig. S2). In contrast to clear impairment in motor performance found in CaMKIIβ KO and A303R mice (Bachstetter, 2014; Kool, 2016; van Woerden, 2009), we did not observe such phenotype. Lastly, CaMKIIβ A303R mice showed normal freezing level in contextual fear conditioning (Borgesius, 2011) contrary to our KI mouse. These discrepancies can be explained by marked difference between CaMKIIβ KO or A303R mice,
and our KI mice. CaMKIIβ null mutant loses all activities of CaMKIIβ, both enzymatic and structural. In contrast, our KI mice were designed to highlight the importance of phosphorylation of the actin-binding region while maintaining the kinase activity and structural activity of unstimulated protein. Despite similarities in domain structure and function, it is becoming more and more clear that CaMKIIα and β are conducting isoform-specific functions under normal condition and during synaptic plasticity. CaMKIIβ has much higher affinity to Ca\(^{2+}\)/CaM than α (Brocke, 1999; Meyer, 1992), and it was proved that the expression of these two isoforms and the effects on synaptic strength are opposite upon neuronal activity change in dissociated hippocampal culture (Thiagarajan, Piedras-Renteria, & Tsien, 2002). Also; there are CaMKIIβ-specific functions such as targeting Arc/Arg3.1 to inactive synapse (Okuno, 2012) and synaptic localization of CaMKIIα (Borgesius, 2011). Therefore, all these CaMKIIβ-related catalytic and structural functions are disturbed in CaMKIIβ deficient mouse whereas our KI mouse has only inability to dissociate from F-actin.

In addition to losing kinase activity, A303R mutant also loses Ca\(^{2+}\)/calmodulin-mediated transient detachment from F-actin due to the lack of calmodulin binding (Kim, 2015). In contrast, our phoso-block CaMKIIβ is expected to still maintain this Ca\(^{2+}\)/calmodulin-mediated, phosphorylation-independent detachment during LTP induction (Kim, 2015). On the other hand, phosphorylation of actin-binding domain may be still mediated by other kinase in A303R mutant. Because phosphorylation can be mediated by the neighboring subunit of the same oligomer (Kim, 2015), this domain of CaMKIIβ A303R can be phosphorylated by active α subunit. Also, the actin-binding domain has consensus phosphorylation sites for PKC, PKA, cdc2, CKI, p38, GSK3 and cdk5. Indeed, PKC was reported to phosphorylate this domain (Sugawara, 2017). Therefore, it is possible that CaMKIIβ A303R is still phosphorylated by other kinases. These differences can explain the observed difference in phenotypes between KO, A303R KI animal, and our exon 13 ST/A KI animals.

Further questions remain to be answered. First, why do KI mice show deficits only in fear conditioning? Although CaMKII is one of the most important molecules in LTP, it may not be reasonable to assume that CaMKII is necessary for all types of learning and memory. Despite broad expression of CaMKIIα throughout the brain, the effect of CaMKIIα mutations vary depending on brain regions and type of learning tasks (Bach, 1995; Eilersma, 2002; Giese, 1998; Silva, 1992; Wiedenmayer, 2000). Similarly, each brain region has a different ratio of α to β, and the contribution of CaMKII – F-actin interaction is likely to be different between brain regions and learning tasks. Therefore, we speculate several different possibilities. Depending on the synapses or learning paradigm, the contribution of actin-binding domain autophosphorylation may not be high when there is enough Ca\(^{2+}\) influx which triggers initial detachment of CaMKIIβ. Alternatively, CaMKII proportion of different subtype of CaMKII may change the sensitivity to the modulation of function. CaMKIIα can rescue the loss of kinase activity but not the loss of F-actin binding. Such differential expression among different synapses may explain the difference in the behavior phenotypes. Lastly, the difference in the contribution of other actin bundling proteins such as α-actinin among various synapses may contribute to the difference in the behavior.

Also, it should be considered that our KI mouse has alanine mutations only in the region encoded by exon 13 of actin-binding domain, leaving those in exon 15 intact due to technical difficulties in targeting two exons separated by ~4.5 kb. We chose exon 13 because phosphomimic aspartic acid mutations within this region totally abolished actin bundling, but similar mutations in exon 15 caused only ~50% loss (Kim, 2015). Serine 371 in exon 15 was actually phosphorylated in the hippocampus of the KI mouse (Fig. 2). Therefore, it is expected that F-actin binding of CaMKII in our KI mouse brain is not perfect, and this may dilute the effect of the genetic modification. A KI mouse in which exon 15 also has mutations may be able to show clearer and broader effects in learning and memory tests.
Kawano for help with histological analysis. This work was supported by a Grant-in-Aid for Scientific Research for Young Researchers 22700356 (K.K.), RIKEN, Grant-in-Aid for Scientific Research (A) 20240032, Grant-in-Scientific Research on Innovation Areas “Constructive understanding of multi-scale dynamism of neuropsychiatric disorders” 18H05434 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Human Frontier Science Foundation, Takeda Science Foundation, Uehara Memorial Foundation, Naito Memorial Foundation, and Opto-Science and Technology Research Foundation (Y.H.).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2018.12.003.

References


Jadavji, N. M., et al. (2015). MTHFR deficiency or reduced intake of folate or choline in pregnant mice results in impaired short-term memory and increased apoptosis in the hippocampus of wild-type offspring. Neuroscience, 300, 1–9.


Kim, K., et al. (2015). A temporary gating of actin remodeling during synaptic plasticity consists of the interplay between the kinase and structural functions of CaMKII. *Neuron, 87*(4), 813–826.


