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Differential Involvement of Kinase Activity of Ca²⁺/Calmodulin-Dependent Protein Kinase II α in Hippocampus- and Amygdala-Dependent Memory Revealed by Kinase-Dead Knock-in Mouse

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1 **Differential involvement of kinase activity of**
2 **Ca²⁺/calmodulin-dependent protein kinase II α in hippocampus- and**
3 **amygdala-dependent memory revealed by kinase-dead knock-in mouse**

4

5 *Abbreviated Title: CaMKII α activity & Hippocampus- vs amygdala-memory*

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38

39

40 **Abstract**

41 Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) is a key mediator of
42 activity-dependent neuronal modifications and has been implicated in the molecular
43 mechanisms of learning and memory. Indeed, several types of CaMKII α knock-in and
44 knock-out mice revealed impairments in hippocampal synaptic plasticity and behavioral
45 learning. On the other hand, a similar role for CaMKII α has been implicated in
46 amygdala-dependent memory, but detailed analyses have not much been performed yet.
47 To better understand its involvement in amygdala-dependent memory as compared to
48 hippocampus-dependent memory, here we performed biochemical analyses and
49 behavioral memory tests using the kinase-dead CaMKII α (K42R) knock-in mouse. In
50 the Morris water maze tasks, homozygous mutants performed well in the visible
51 platform trials, while they failed to form spatial memory in the hippocampus-dependent
52 hidden platform trials. In fear conditioning, these mice were impaired but showed a
53 certain level of amygdala-dependent cued fear memory, which lasted four weeks, while
54 they showed virtually no hippocampus-dependent context discrimination. Neither
55 stronger stimulation nor repetitive stimulation compensated for their memory deficits.

56 The differential outcome of hippocampus- and amygdala-dependent memory in the
57 mutant mouse was not due to differential expression of CaMKII α between the
58 hippocampus and the amygdala, because biochemical analyses revealed that both kinase
59 activity and protein levels of CaMKII were indistinguishable between the two brain
60 regions. These results indicate that kinase activity of CaMKII α is indispensable for
61 hippocampus-dependent memory, but not necessarily for amygdala-dependent memory.
62 There may be a secondary, CaMKII α activity-independent pathway, in addition to the
63 CaMKII α activity-dependent pathway, in the acquisition of amygdala-dependent
64 memory.

65

66

67 **Significance Statement**

68 Studying molecular mechanisms of learning and memory is important to confront
69 memory-deficient and abnormal memory-associated disorders. An enzyme called
70 Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) that is abundant in the brain
71 and phosphorylates important proteins has a key role in such mechanisms. However,
72 how CaMKII α enzymatic activity is involved in hippocampus- vs. amygdala-dependent
73 memory is still not clear. Using our genetically engineered mouse that lacks kinase
74 activity but retains protein expression of CaMKII α , here we showed that kinase activity
75 of CaMKII α is indispensable for hippocampus-dependent space/context-related memory,
76 but not necessarily for amygdala-dependent fear-related memory. The role of CaMKII α
77 kinase activity in distinguishing different contexts indicates its possible involvement as
78 a measure against abnormal fear memory-associated disorders, such as post-traumatic
79 stress disorder.

80

81

82 **Introduction**

83 Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant
84 protein kinases in the central nervous system, and is thought to be a key mediator of
85 activity-dependent neuronal modifications (Hudmon and Schulman, 2002; Lisman et al.,
86 2002, 2012; Coultrap and Bayer, 2012; Hell, 2014; Herring and Nicoll, 2016a;
87 Takemoto-Kimura et al., 2017). The neuronal CaMKII holoenzyme is assembled from
88 homologous 12 subunits consisting of CaMKII α and/or CaMKII β , the two major
89 isoforms in the brain, with CaMKII α dominant in the forebrain, and with CaMKII β
90 dominant in the cerebellum. CaMKII α is especially enriched in the hippocampus, and
91 has been proposed to be the indispensable isoform to hippocampal synaptic plasticity
92 and behavioral learning (Lisman et al., 2002; Lisman, 2017; Rossetti et al., 2017;
93 Takemoto-Kimura et al., 2017).

94 Activity-dependent Ca^{2+} influx into neurons causes activation of CaMKII by binding
95 of Ca^{2+} /calmodulin. Activated CaMKII translocates to the postsynaptic sites and
96 undergoes T286(α)/T287(β)-autophosphorylation within the autoinhibitory region
97 through an inter-subunit mechanism. This autophosphorylation makes the kinase

98 persistently active and prolongs its postsynaptic association even after intracellular Ca^{2+}
99 concentration is reduced. There, the kinase phosphorylates a number of substrates
100 including AMPA-type glutamate receptors (AMPA-Rs), transmembrane AMPAR
101 regulatory proteins, synaptic Ras-GTPase activating protein, and Rho guanine
102 nucleotide exchange factors (Lee et al., 2003, 2010; Xie et al., 2007; Yang et al., 2013;
103 Arai et al., 2015; Herring and Nicoll, 2016b; Park et al., 2016), which leads to long-term
104 potentiation (LTP) of the synapse, one of the fundamental mechanisms for learning and
105 memory. When intracellular Ca^{2+} concentration is reduced, Ca^{2+} /calmodulin is detached
106 from CaMKII, and another autophosphorylation at T305/T306(α)/T306/T307(β) within
107 the calmodulin-binding region occurs through an intra-subunit mechanism. This
108 autophosphorylation prevents re-binding of Ca^{2+} /calmodulin, reduces kinase activity to
109 about a half and facilitates its dissociation from postsynaptic sites to terminate its action
110 at the synapse.

111 So far, a number of genetically engineered CaMKII α knock-out (KO) and knock-in
112 (KI) mice confirmed the direct involvement of CaMKII α in LTP, and learning and
113 memory (Silva et al., 1992; Giese et al., 1998; Elgersma et al., 2002; Yamagata et al.,

114 2009; Achterberg, et al., 2014). Among them, functionally modified KI mice, especially
115 the CaMKII α (T286A)-, (T305D)- and (K42R)-KI mice showed severe deficits not only
116 in hippocampal LTP, but also in hippocampus-dependent memory, revealing the
117 importance of persistent activation, postsynaptic association and enzymatic activity of
118 CaMKII α , respectively, in such processes (Giese et al., 1998; Elgersma et al., 2002;
119 Yamagata et al., 2009). Thus, functionally intact CaMKII α is critically involved in the
120 acquisition of hippocampus-dependent memory. A recent study using the region-specific
121 and time-specific conditional CaMKII α KO mice also confirmed its requirement in the
122 forebrain and at the time of learning (Achterberg, et al., 2014).

123 On the other hand, how CaMKII α is involved in other types of memory is still not
124 clear. Previous studies using fear conditioning in rats (Rodrigues et al, 2004) and in the
125 CaMKII α (T286A)-KI mouse (Irvine et al., 2005, 2011) indicated a similar involvement
126 of CaMKII α in amygdala-dependent memory as in hippocampus-dependent memory. A
127 recent study using a light-inducible CaMKII inhibitor also supported a role for CaMKII
128 activity in the amygdala in the acquisition of inhibitory avoidance memory (Murakoshi
129 et al, 2017). However, detailed analyses have not much been performed yet using other

130 types of CaMKII α mutant mice.

131 We previously generated the kinase-dead CaMKII α (K42R)-KI mouse to examine the
132 specific role of kinase activity of CaMKII α , separately from its other protein functions
133 (Yamagata et al., 2009). In this mouse, kinase-dead CaMKII α (K42R) could translocate
134 to postsynaptic sites in response to synaptic activation, whereas tetanic stimulation
135 could not induce LTP or dendritic spine enlargement in the hippocampus. Besides,
136 inhibitory avoidance memory was severely impaired. Thus, we could show that kinase
137 activity of CaMKII α is essential for hippocampal synaptic plasticity and behavioral
138 learning. Here we took the advantage of this KI mouse to examine how kinase activity
139 of CaMKII α is involved in amygdala-dependent memory, as compared to
140 hippocampus-dependent memory, to see if there is any mechanistic difference between
141 them.

142

143

144 **Materials and Methods**

145 *Animal experiments.* The kinase-dead CaMKII α (K42R)-KI mouse
146 (B6.Cg-*Camk2a*^{tm1.1Oyam}) was generated as previously described (Yamagata et al., 2009).
147 All animal experiments were reviewed and approved by the Institutional Animal Care
148 and Use Committee of National Institutes of Natural Sciences. All experiments were
149 conducted in accordance with the Guide for Animal Experimentation in the Institute.
150 Animals were housed in cages with *ad libitum* access to water and food and maintained
151 on a 12 h-light/dark cycle.

152 All analyses were performed using adult homozygous CaMKII α (K42R)-KI and
153 wild-type littermate control mice generated by intercrosses between heterozygous mice
154 backcrossed to C57BL/6 for more than six generations. Male or female mice were used
155 for biochemical analyses, and male mice were used for behavioral analyses. Mice that
156 showed any signs of seizure were excluded from the experiments (Yamagata et al.,
157 2009).

158

159 *Sample preparation for biochemical analyses.* Brain homogenates from the

160 hippocampus and the amygdala were prepared separately as previously described with
161 some modifications (Yamagata et al., 2009). Animals were individually decapitated
162 under carbon dioxide anesthesia, and brains were removed quickly, put in ice-cold
163 homogenization buffer within 30 s after decapitation, and left in the buffer for 30 s for
164 chilling. The hippocampus was dissected on an ice-cold Petri dish. The amygdala was
165 dissected from separate animals as follows: A two-mm thick coronal slice containing
166 most of the amygdaloid complex (0.8 to 2.8 mm posterior to the Bregma) was cut by
167 using an ice-cold Rodent Brain Matrix (ASI Instruments, Warren, MI, USA), and its
168 ventro-lateral portion against the striatum and lateral ventricle was cut bilaterally on an
169 ice-cold Petri dish. The remaining slice was checked afterwards under a
170 stereomicroscope to verify that the dissected parts corresponded to the amygdaloid
171 complex and the adjacent piriform cortex. Dissected pieces of tissue were immediately
172 frozen in liquid nitrogen and stored at -80°C until use. Frozen pieces of hippocampi
173 (collected from three to five animals) or amygdala (collected from five to seven
174 animals) were homogenized in a five-fold volume of homogenization buffer in a
175 Teflon-glass homogenizer on ice to make an independent experimental sample. The

176 homogenization buffer consisted of 20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1 mM
177 EGTA, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na₃VO₄ (ortho), 1 mM
178 dithiothreitol, 10 µg/ml each of leupeptin, antipain, pepstatin, and chymostatin, 0.1 mM
179 phenylmethylsulfonyl fluoride, and 0.1 µM calyculin A. Each sample was quickly
180 aliquoted, an aliquot was saved for the preparation of samples for SDS-PAGE, another
181 for the measurement of protein concentration, and the rest were frozen immediately and
182 stored at -80°C until additional characterization of kinase activity. Samples for
183 SDS-PAGE were prepared as previously described (Yamagata and Obata, 1998;
184 Yamagata et al., 2009). Protein concentration was determined by using BCA Protein
185 Assay Reagent (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and bovine
186 serum albumin as a standard.

187

188 ***CaMKII kinase activity assay.*** CaMKII kinase activity assay was performed as
189 previously described (Yamagata et al., 2009). The assay was conducted in the presence
190 of 50 mM HEPES/NaOH, pH 7.5, 10 mM magnesium acetate, 1 mM EGTA, 50 µg/ml
191 BSA, 0.1% Triton X-100, 50 µM autocamtide-2 [KKALRRQETVDAL (Hanson et al.,

192 1989); synthesized by Mimotopes (Victoria, Australia)], 2 μM PKI-(5-24)-amide
193 (Peninsula Laboratories, San Carlos, CA, USA), 2 μM PKC-(19-36)-amide (Peninsula
194 Laboratories), 100 μM [γ - ^{32}P]ATP (400-800 cpm/pmol, PerkinElmer, Waltham, MA,
195 USA), and with (for the total activity) or without (for the Ca^{2+} /calmodulin-independent
196 autonomous activity) 1.5 mM CaCl_2 and 25 $\mu\text{g}/\text{ml}$ calmodulin in a final volume of 50
197 μl . The reaction was started by the addition of [γ - ^{32}P]ATP, performed for 1 min at 30°C,
198 and terminated by the addition of acetic acid (final concentration, 10%). After spotting
199 aliquots onto pieces of P81 phosphocellulose paper (Whatman, Thermo Fisher
200 Scientific) and washing the paper with 75 mM phosphoric acid for five times, the
201 retained radioactivity on the paper was measured in a beta scintillation counter
202 (Beckman Coulter, Brea, CA, USA). The amount of protein used for the kinase activity
203 assay was 0.6 μg from hippocampal or amygdala homogenates, and the reaction was
204 linear in terms of both protein concentration and incubation time. Kinase activity was
205 expressed as the amount of ATP incorporated into the substrate peptide in one min per
206 mg of protein (nmol/min/mg), and compared between the hippocampus and amygdala
207 in each genotype by using unpaired *t*-test, two-tailed, $n=6$ for each brain region from

208 each genotype (Fig. 1). When relative activity of KI samples was calculated, it was
209 expressed as a percentage against the value of control wild-type samples in the same
210 experimental group measured on the same day (Fig. 1, *right, above the columns*).

211

212 ***Immunoblot analyses.*** Quantitative immunoblot analyses were performed as
213 previously described (Yamagata et al., 2009). Equal amounts of protein from each
214 sample were subjected to SDS-PAGE, transferred to nitrocellulose transfer membranes
215 (Whatman, Thermo Fisher Scientific), and immunoblotted using one of the following
216 antibodies. Antibodies against CaMKII α (mouse monoclonal; 6G9; 1:1000; BIOMOL
217 Cat#SA-112, Enzo Life Sciences, Farmingdale, NY, USA; RRID: AB_10617228),
218 CaMKII β (mouse monoclonal; CB-beta-1; 1:200; Zymed Cat#13-9800, Thermo Fisher
219 Scientific; RRID: AB_2533045), and phospho-T286-CaMKII α (rabbit polyclonal;
220 1:500; Promega Cat#V1111, Madison, WI, USA) were used for the primary reaction.
221 For mouse antibodies, rabbit anti-mouse IgG (1:500; MP Biomedicals Cat#55480, Santa
222 Ana, CA, USA) was used in the secondary reaction. Blots were then visualized by using
223 ^{125}I -protein A ($3.5\text{-}5 \times 10^5$ cpm/ml; PerkinElmer), and by exposing to an X-ray film, as

224 previously described (Yamagata et al., 2015). The immunoreactive bands were cut out,
225 and their radioactivity was quantitated by using a gamma counter (Hitachi Aloka
226 Medical, Mitaka, Japan). The amounts of protein used were 2 μg (for anti-CaMKII α), 4
227 μg (for anti-CaMKII β), and 8 μg (for anti-phospho-T286-CaMKII α) from hippocampal
228 or amygdala homogenates. The measured immunoreactivity was in a linear range in
229 terms of protein amounts used for each antibody. The values obtained from KI samples
230 were expressed as percentages against those from control wild-type samples in the same
231 experimental group on the same blots, and analyzed by using one sample *t*-test,
232 two-tailed, $n=6$ for each brain region (Fig. 2, Table 1).

233

234 *Animals used for behavioral experiments.* Mice were 3-5 months of age at the start of
235 behavioral experiments. They were accustomed to the experimenter by careful handling
236 for more than one week before the start of experiments. Behavioral experiments were
237 conducted with the experimenter blind to the genotype of mice.

238

239 *Water maze.* Water maze procedure followed basically as described by Crawley

240 (2000). A circular pool with a diameter of 1 m was filled with opaque water colored
241 with white paint to a depth of 20 cm. Water temperature was maintained at 24-25°C.
242 The escape platform with a diameter of 11 cm was submerged 1 cm below the water
243 surface. The visible cue for the platform consisted of a black pole (10-cm tall) with a
244 ball on top colored black in its lower half (3.5 cm in diameter) standing in the center of
245 the platform. The swimming paths of animals were recorded by a black and white
246 charge-coupled device video camera mounted above the center of the pool using
247 LabVIEW and Vision software (National Instruments, Austin, TX, USA; RRID:
248 SCR_014325), and analyzed by Igor Pro 6 software (WaveMetrics, Portland, OR, USA;
249 RRID: SCR_000325). Extra-maze cues were posted above the wall of the pool as
250 spatial references.

251 Naive mice were first accustomed to the water without spatial cues and the visible
252 cue for the platform on the day before the start of water maze training. They were gently
253 released into the pool, allowed to swim for 30 s, then guided onto the submerged
254 platform and allowed to remain there for 30 s. This procedure was repeated three times
255 for each mouse.

256 In water maze training, mice were individually subjected to two blocks (30-60 min
257 apart) of four trials (30 s-inter-trial interval) per day with spatial cues. Platform location
258 was fixed for the same group of mice trained on the same days, but altered among
259 different groups of mice (7-9 mice/group). Mice were gently released into the pool with
260 the starting position changed in each trial, and given 60 s to reach the platform. After
261 climbing onto the platform, mice were allowed to remain there for 30 s. If a mouse was
262 unable to locate the platform within 60 s, the trial was concluded, and the mouse was
263 gently guided onto the platform and remained there for 30 s. After the training, mice
264 were returned to their home cage. The escape latency was calculated as the average time
265 of the 4 trials per block to reach the platform (Fig. 3A).

266 Mice were subjected to visible platform training for the first three days. Subsequently,
267 the visible cue for the platform was removed with the platform location kept intact. The
268 mice were then given hidden platform training for another six days. On days 3 and 6 of
269 hidden platform training, the platform was removed after training trials of the day, and a
270 probe trial was performed to assess spatial memory. Mice were released into the pool
271 from the opposite side of the original platform location and allowed to search for the

272 platform location for 60 s, while their swimming paths were recorded. Mice were then
273 gently guided to the original platform location and allowed to sit on the experimenter's
274 hand for 30 s. The following parameters were measured for each probe trial: (1) the
275 percentage of time spent in each imaginary quadrant of the pool (target, left, opposite
276 and right quadrants) (Fig. 3B, *left*), (2) the number of center crossings of the
277 hypothetical platform location in each quadrant (Fig. 3B, *right*), and (3) swimming
278 speed (Fig. 3D). Swimming traces were color-coded according to the time spent at a
279 certain location (1 x 1 cm square), and averaged within each genotype after aligning the
280 location of the platform by rotation using Igor Pro 6 software (WaveMetrics; RRID:
281 SCR_000325) (Fig. 3C).

282 The escape latencies for visible and hidden platform trainings were compared
283 between the genotypes by using two-way repeated measure ANOVA, followed by
284 Bonferroni's post hoc test where appropriate, n=12 for each genotype (Fig. 3A). The
285 percentage of time spent in each imaginary quadrant of the pool and the number of
286 center crossings of the hypothetical platform location in each quadrant in probe trials
287 were analyzed in each genotype by using one-way ANOVA, followed by Tukey's post

288 hoc test where appropriate (Fig. 3B). The swimming speed in probe trials was compared
289 between the genotypes by using unpaired *t*-test, two-tailed (Fig. 3D).

290

291 ***Fear conditioning.*** The assessment of electrical footshock sensitivity and fear
292 conditioning were performed in the same clear acrylic chamber (W 300 x D 250 x H
293 215 mm) equipped with a stainless steel grid floor (3-mm diameter rods, 7-mm apart)
294 (O'HARA & CO., Tokyo, Japan). The auditory signal was supplied from a loudspeaker
295 placed on top of the lid of the chamber, and footshock and tone delivery were controlled
296 by using LabVIEW software (National Instruments; RRID: SCR_014325). We
297 employed background contextual fear conditioning using a tone as a conditional
298 stimulus (CS) paired with an unconditional stimulus (US) of a footshock. Background
299 conditioning involves the hippocampus more strongly than conditioning without a tone
300 (Phillips and LeDoux, 1994), and by using this method, we can compare hippocampus-
301 vs. amygdala-dependent memory at the same time.

302 To assess electrical footshock sensitivity, naive mice were individually placed in the
303 chamber, and a series of electrical stimuli was delivered from the grid floor for 2 s

304 (Kojima et al., 2008). The current intensity started from 0.01 mA and gradually
305 increased with one-min interval. The behavioral responses to the electrical stimuli were
306 monitored, and the threshold intensity for a paw flick and/or a step back as a measure of
307 pain sensation and that for vocalization, jump and/or running as a measure of aversive
308 response were determined. The threshold intensities were compared between the
309 genotypes by using unpaired *t*-test, two-tailed, n=14 for the wild-type mouse, n=16 for
310 the CaMKII α (K42R)-KI mouse (Table 2).

311 Fear conditioning was conducted by placing naive mice individually in the chamber,
312 while other mice waited in another nearby room where the auditory signal could not be
313 heard. The fear conditioning procedure followed basically as described by Irvine et al.
314 (2005). After an initial exploratory period of 120 s, a tone (70 dB, white noise) was
315 presented for 30 s as a CS, which co-terminated with an electrical footshock (0.3 or 0.7
316 mA, 2 s) as a US (1CS-US or 1CS-US-strong). After 30 s, the mouse was returned to its
317 home cage. The chamber was cleared with 75% ethanol before each session. Under
318 more intense training conditions, mice received three or five CS-US pairings at 60 s
319 intervals (0.3-mA footshock; 3CS-US or 5CS-US). A control experiment consisting of

320 the presentation of a tone alone without a footshock for three times at 60 s intervals
321 (3CS alone) was also conducted to assess fear response to the tone alone without
322 conditioning.

323 Twenty-four hours after conditioning, the mice were individually re-exposed to the
324 conditioning chamber without the auditory signal for 300 s to test for contextual fear
325 memory. Freezing time in the first 180 s was measured (Context). Another twenty-four
326 hours later, the mice were individually placed in a novel context consisting of a metal
327 box (W 235 x D 235 x H 145 mm) installed inside the contextual chamber to assess
328 cued fear memory. Following 180 s without a tone, the tone (70 dB, white noise) was
329 presented for 180 s. Freezing time in the first 180 s (Cued, Pre-tone) and the last 180 s
330 (Cued, Tone) was measured. Four weeks later, the mice were re-tested to assess the
331 stability of fear memory (Context; Cued, Pre-tone and Tone).

332 Freezing was defined as complete immobility other than respiration and used as an
333 index of fear (Frankland et al., 1998). Freezing time was measured manually with the
334 experimenter blind to the genotype and expressed as a percentage of time spent freezing
335 during the specified period of 180 s. Values for percent freezing time were compared

336 between the three conditions tested (Context; Cued, Pre-tone and Tone) in each
337 genotype by using one-way repeated measures ANOVA, followed by Bonferroni's post
338 hoc test where appropriate. Through this comparison, we could accurately evaluate
339 hippocampus-dependent context discrimination and amygdala-dependent cued fear
340 memory at the same time. Context discrimination is a more sensitive measure of
341 hippocampal dysfunction as reported by Frankland et al. (1998). The number of animals
342 used were: the wild-type mouse, n=15, 16, 16, 15 and 13 for 1CS-US, 1CS-US-strong,
343 3CS-US, 5CS-US and 3CS alone, respectively; the CaMKII α (K42R)-KI mouse, n=14,
344 14, 15, 14 and 13 for 1CS-US, 1CS-US-strong, 3CS-US, 5CS-US and 3CS alone,
345 respectively (Figs. 4 and 5). Tone-dependent freezing (Cued, Tone) was compared
346 between the genotypes by using unpaired *t*-test, two-tailed where appropriate. The
347 behavior of the mice during fear conditioning and tests was also videotaped.

348

349 ***Experimental design and statistical analyses.*** Details of the experimental design and
350 statistics are described in the above sections. Statistical analysis was performed by using
351 GraphPad Prism 6 software (GraphPad Software, La Jolla, CA. USA; RRID:

352 SCR_002798). Statistical significance was set at $p < 0.05$. Data are expressed as a mean

353 \pm SEM or as a scatter plot with a mean.

354

355

356 **Results**

357 **Kinase activity and protein levels of CaMKII were indistinguishable between the**
358 **hippocampus and amygdala in the wild-type mouse and in the kinase-dead**
359 **CaMKII α (K42R) knock-in mouse**

360 We first compared the kinase activity and protein levels of CaMKII between the
361 hippocampus and amygdala in the wild-type mouse and in the kinase-dead homozygous
362 CaMKII α (K42R)-KI mouse (Figs. 1 and 2, Table 1). As for CaMKII activity, no
363 significant difference was observed between the hippocampus and amygdala in the
364 wild-type mouse (total activity, $t_{(10)}=0.7433$, $p=0.4744$; autonomous activity,
365 $t_{(10)}=0.5082$, $p=0.6223$; unpaired t -test, $n=6$) (Fig. 1, *left*), indicating that the total
366 amounts of CaMKII composed of CaMKII α and CaMKII β , the two major isoforms in
367 the brain, are indistinguishable between the two brain regions. In the same way, no
368 significant difference was observed between the two brain regions in the CaMKII α
369 (K42R)-KI mouse (total activity, $t_{(10)}=1.812$, $p=0.1001$; autonomous activity,
370 $t_{(10)}=0.8094$, $p=0.4371$; unpaired t -test, $n=6$) (Fig. 1, *right*), demonstrating that the
371 remaining CaMKII activity derived from intact CaMKII β is indistinguishable between

372 the two brain regions. This also indicates that the amount of CaMKII β is basically the
373 same between the two brain regions in the CaMKII α (K42R)-KI mouse.

374 We then compared CaMKII α and CaMKII β protein levels between the genotypes in
375 the hippocampus and in the amygdala by quantitative immunoblot analyses (Fig. 2,
376 Table 1). The CaMKII β protein level in the CaMKII α (K42R)-KI mouse was
377 unchanged from that in the wild-type mouse in the both brain regions (hippocampus,
378 $t_{(5)}=0.2667$, $p=0.8004$; amygdala, $t_{(5)}=0.8194$, $p=0.4498$; one sample t -test, $n=6$; Table
379 1). Combined with activity data, the amount of CaMKII β , and also of CaMKII α , in the
380 wild-type mouse is considered to be the same between the two brain regions. In addition,
381 there seem to be no compensatory changes in the CaMKII β level in the CaMKII α
382 (K42R)-KI mouse in either brain regions.

383 It should be noted that the basic characteristics of CaMKII observed in the
384 hippocampus and the amygdala of the kinase-dead CaMKII α (K42R) KI mouse, i.e., a
385 profound decrease in CaMKII activity (Fig. 1), a moderate decrease in the
386 phospho-T286-CaMKII α level and a slight decrease in the CaMKII α protein level (Fig.
387 2, Table 1), compared to those in the wild-type mouse, are all in accordance with our

388 previous observations in the forebrain of this KI mouse (Yamagata et al., 2009).

389

390 **Impaired spatial memory and intact visually guided memory in the Morris water**
391 **maze tasks in the kinase-dead CaMKII α (K42R) knock-in mouse**

392 To examine whether hippocampus-dependent spatial memory is indeed impaired in the

393 CaMKII α (K42R)-KI mouse, we first performed the Morris water maze tasks (Fig. 3).

394 In the visible platform trials, both wild-type and CaMKII α (K42R)-KI mice showed a

395 steady shortening of escape latencies through the course of training trials, and there was

396 no significant difference between the genotypes (interaction, $F_{(5,110)}=0.8647$, $p=0.5074$;

397 genotype, $F_{(1,22)}=3.226$, $p=0.0862$; blocks of trials, $F_{(5,110)}=24.00$, $p < 0.0001$; $n=12$ for

398 each genotype; two-way repeated measure ANOVA) (Fig. 3A, *left*), indicating that

399 visual and motor abilities and motivation for escape are all intact in the CaMKII α

400 (K42R)-KI mouse. In fact, both genotypes took less than 11 s on average to reach the

401 visible platform in the second and later blocks of trials, which was significantly shorter

402 than the latency in the first block of trials (WT: 1st vs. 2nd, $p=0.01$; 1st vs. 3rd,

403 $p=0.0001$; 1st vs. 4th, 5th and 6th, $p < 0.0001$; K42R: 1st vs. 2nd, 3rd, 4th, 5th and 6th,

404 $p < 0.0001$; Bonferroni's post hoc test).

405 Next, in the following hidden platform trials with the platform location kept intact,
406 the difference between the genotypes became obvious (interaction, $F_{(11,242)} = 0.7418$,
407 $p = 0.6977$; genotype, $F_{(1,22)} = 16.53$, $p = 0.0005$; blocks of trials, $F_{(11,242)} = 1.254$, $p = 0.2522$;
408 two-way repeated measure ANOVA) (Fig. 3A, right). Wild-type mice took less than 12 s
409 on average to reach the platform throughout the trials, suggesting that they still
410 remembered the platform location, whereas CaMKII α (K42R)-KI mice took more than
411 18 s on average to reach the platform. Post hoc test revealed a significant difference in
412 the latency between the genotypes in the 5th, 6th, 8th, 9th, 11th and 12th blocks of trials
413 ($p = 0.0005$, 0.0041, 0.0415, 0.0235, 0.0087 and 0.0072, respectively, Bonferroni's post
414 hoc test).

415 In addition, probe trials performed on days 3 and 6 after training trials of the day
416 revealed that wild-type mice spent significantly longer time in the target quadrant than
417 in other quadrants (day 3, $F_{(3,44)} = 8.641$, $p = 0.0001$, one-way ANOVA; target vs. left,
418 $p = 0.0018$; target vs. opposite, $p = 0.0001$; target vs. right, $p = 0.0057$; Tukey's post hoc
419 test) (day 6, $F_{(3,44)} = 53.15$, $p < 0.0001$, one-way ANOVA; target vs. left, opposite and

420 right, $p < 0.0001$; Tukey's post hoc test) (Fig. 3B, *left*), demonstrating that spatial
421 memory was formed in wild-type mice. On the other hand, CaMKII α (K42R)-KI mice
422 spent equal time in each quadrant (day 3, $F_{(3,44)} = 1.434$, $p = 0.2457$; day 6, $F_{(3,44)} = 0.4409$,
423 $p = 0.7249$; one-way ANOVA) (Fig. 3B, *left*), demonstrating that no spatial memory was
424 formed in CaMKII α (K42R)-KI mice. Furthermore, in wild-type mice, the number of
425 center crossings of the hypothetical platform location was significantly higher in the
426 target quadrant than in other quadrants in the probe trial performed on day 6
427 ($F_{(3,44)} = 17.17$, $p < 0.0001$, one-way ANOVA; target vs. left, opposite and right, $p < 0.0001$;
428 Tukey's post hoc test), but not on day 3 ($F_{(3,44)} = 1.005$, $p = 0.3997$, one-way ANOVA)
429 (Fig. 3B, *right*), indicating that wild-type mice steadily acquired more accurate spatial
430 memory of the hidden platform location as the training proceeded. In contrast,
431 CaMKII α (K42R)-KI mice did not display any preference for locations of center
432 crossings (day 3, $F_{(3,44)} = 1.183$, $p = 0.3271$; day 6, $F_{(3,44)} = 0.03869$, $p = 0.9897$; one-way
433 ANOVA) (Fig. 3B, *right*). Averaged color-coded swimming traces of each genotype in
434 the probe trials on days 3 and 6 clearly demonstrate the preference for the target
435 location in wild-type mice and the absence of such preference in CaMKII α (K42R)-KI

436 mice (Fig. 3C). They also show the improvement of performance with further training
437 from day 3 to day 6 in wild-type mice, but not in CaMKII α (K42R)-KI mice.

438 Finally, swimming speed measured in probe trials performed on days 3 and 6 was
439 similar between the genotypes (day 3, $t_{(22)}=0.7064$, $p=0.4874$; day 6, $t_{(22)}=2.068$,
440 $p=0.0506$; unpaired t -test), indicating that swimming performance itself was normal in
441 the CaMKII α (K42R)-KI mouse (Fig. 3D). All these results clearly demonstrate that the
442 CaMKII α (K42R)-KI mouse was severely and specifically impaired in
443 hippocampus-dependent spatial memory, whereas their visually guided memory was
444 kept intact.

445

446 **Severely impaired context discrimination, whereas impaired but partially**
447 **preserved cued fear memory in the kinase-dead CaMKII α (K42R) knock-in mouse**

448 We next examined fear-conditioned memory to compare hippocampus- and
449 amygdala-dependent memory in the CaMKII α (K42R)-KI mouse. To do that, we first
450 determined the sensitivity to an electrical footshock in the CaMKII α (K42R)-KI mouse
451 as compared to that in the wild-type mouse (Table 2). The threshold currents for

452 stepping back and/or paw flick, which are the behavioral signs of pain sensation, were
453 indistinguishable between the genotypes ($t_{(28)}=0.6530$, $p=0.5191$; unpaired t -test, $n=14$
454 for wild-type mice, $n=16$ for KI mice). On the other hand, the threshold currents for
455 vocalization, running and/or jumping, which represent aversive reactions to a footshock,
456 was slightly higher in CaMKII α (K42R)-KI mice than in wild-type mice ($t_{(28)}=4.436$,
457 $p=0.0001$; unpaired t -test). To overcome the difference, we employed the stimulation
458 intensity of 0.3 mA or higher that was far above the threshold for aversive sensation for
459 both genotypes in fear conditioning. It should be noted that in conditioning, no freezing
460 was observed during the first two minutes before the tone in either genotypes.

461 We first tried single stimulation protocol, i.e., one pairing of CS and US (ICS-US)
462 consisting of a tone lasting for 30 s that co-terminated with a footshock of 0.3 mA for 2
463 s (Fig. 4A). When tested 24 h (Context) and 48 h (Cued) after conditioning, wild-type
464 mice showed significantly longer freezing time in the contextual chamber (Context in
465 Fig. 4A, *left*), as well as in the cued chamber with tone (Cued, Tone), than in the cued
466 chamber without tone (Cued, Pre-tone) ($F_{(2,28)}=89.16$, $p<0.0001$, $n=15$, one-way
467 repeated measures ANOVA; context vs. pre-tone, $p=0.0009$; pre-tone vs. tone,

468 $p < 0.0001$; Bonferroni's post hoc test) (Fig. 4A, *left*), demonstrating the formation of
469 both context-dependent and tone-dependent fear memory in the wild-type mouse. On
470 the other hand, CaMKII α (K42R)-KI mice showed almost no freezing in the contextual
471 chamber (Context in Fig. 4A, *right*) and in the cued chamber without tone (Cued,
472 Pre-tone), whereas they showed rather small but significant freezing in the cued
473 chamber with tone (Cued, Tone) ($F_{(2,26)}=15.65$, $p < 0.0001$, $n=14$, one-way repeated
474 measures ANOVA; context vs. pre-tone, $p > 0.9999$; pre-tone vs. tone, $p = 0.0001$;
475 Bonferroni's post hoc test) (Fig. 4A, *right*), demonstrating that context-dependent fear
476 memory was not formed, but tone-dependent fear memory was formed to a certain
477 extent, although it was significantly less than that observed in wild-type mice (Cued,
478 Tone; $t_{(27)}=7.566$, $p < 0.0001$; unpaired t -test).

479 We then tried stronger 1CS-US conditioning using a footshock of 0.7 mA
480 (1CS-US-strong) in another groups of mice to examine whether stronger stimulation
481 protocol could compensate for memory deficits in the CaMKII α (K42R)-KI mouse (Fig.
482 4B). After such stronger conditioning, however, the results were basically the same:
483 Wild-type mice showed both context-dependent and tone-dependent freezing

484 ($F_{(2,30)}=46.95$, $p<0.0001$, $n=16$, one-way repeated measures ANOVA; context vs.
485 pre-tone, $p<0.0001$; pre-tone vs. tone, $p<0.0001$; Bonferroni's post hoc test) (Fig. 4B,
486 *left*), whereas CaMKII α (K42R)-KI mice did not show context-dependent freezing, but
487 showed tone-dependent freezing to a certain extent ($F_{(2,26)}=9.601$, $p=0.0008$, $n=14$,
488 one-way repeated measures ANOVA; context vs. pre-tone, $p>0.9999$; pre-tone vs. tone,
489 $p=0.0012$; Bonferroni's post hoc test) (Fig. 4B, *right*), the latter of which was still
490 significantly less than that observed in wild-type mice (Cued, Tone; $t_{(28)}=5.687$,
491 $p<0.0001$; unpaired t -test). Thus, stronger conditioning did not compensate for memory
492 deficits in the CaMKII α (K42R)-KI mouse.

493 We next performed repeated stimulation protocols consisting of three or five pairings
494 of CS-US (3CS-US or 5CS-US), using a footshock of 0.3 mA in another groups of mice
495 to examine whether repeated stimulation could compensate for memory deficits in the
496 CaMKII α (K42R)-KI mouse (Figs. 4C, D). After 3CS-US conditioning, wild-type mice
497 again showed context-dependent and tone-dependent freezing ($F_{(2,30)}=37.41$, $p<0.0001$,
498 $n=16$, one-way repeated measures ANOVA; context vs. pre-tone, $p<0.0001$; pre-tone vs.
499 tone, $p<0.0001$; Bonferroni's post hoc test) (Fig. 4C, *left*). CaMKII α (K42R)-KI mice,

500 this time, showed increased freezing in all of the three conditions tested, but still did not
501 show context-dependent freezing, whereas showed tone-dependent freezing
502 ($F_{(2,28)}=5.230$, $p=0.0117$, $n=15$, one-way repeated measures ANOVA; context vs.
503 pre-tone, $p=0.3989$; pre-tone vs. tone, $p=0.0065$; Bonferroni's post hoc test) (Fig. 4C,
504 *right*), revealing context discrimination deficits. The extent of their tone-dependent
505 freezing was comparable to that observed in wild-type mice (Cued, Tone; $t_{(29)}=1.143$,
506 $p=0.2624$; unpaired t -test). Next, after 5CS-US conditioning, wild-type mice still
507 showed context-dependent and tone-dependent freezing ($F_{(2,28)}=23.13$, $p<0.0001$, $n=15$,
508 one-way repeated measures ANOVA; context vs. pre-tone, $p=0.0067$; pre-tone vs. tone,
509 $p<0.0001$; Bonferroni's post hoc test) (Fig. 4D, *left*). CaMKII α (K42R)-KI mice, on the
510 other hand, showed generally increased freezing, and this time, freezing was neither
511 context-dependent nor tone-dependent ($F_{(2,26)}=2.655$, $p=0.0893$, $n=14$, one-way
512 repeated measures ANOVA) (Fig. 4D, *right*). Thus, repeated stimulation protocols did
513 not compensate for memory deficits, but instead revealed severe context discrimination
514 deficits, leading to generalized fear, in the CaMKII α (K42R)-KI mouse.

515 To rule out the possibility that the CaMKII α (K42R)-KI mouse may display

516 tone-dependent freezing without tone-footshock association, we exposed another groups
517 of mice to tone alone for three times without footshocks (3CS alone) and tested their
518 freezing response just as in the case after fear conditioning (Fig. 5A). Virtually no
519 freezing was observed in CaMKII α (K42R)-KI mice (n=13, Fig. 5A, *right*), as well as in
520 wild-type mice (n=13, Fig. 5A, *left*), in all of the three conditions tested (percent
521 freezing time, less than 2 % on average in any cases). Thus, tone-dependent fear
522 responses in both genotypes were indeed derived from tone-footshock association, and
523 not from tone alone.

524 So far, the results demonstrate that the CaMKII α (K42R)-KI mouse could form cued
525 fear memory at least to a certain extent, whereas they could not form contextual fear
526 memory or discriminate context difference irrespective of stimulus intensity and of
527 repetition of a tone-footshock pairing in the conditioning.

528

529 **Cued fear memory once acquired was long-lasting in the kinase-dead CaMKII α**
530 **(K42R) knock-in mouse**

531 Does the cued fear memory once formed in the CaMKII α (K42R)-KI mouse last long?

532 To solve that question, we re-tested fear-conditioned mice four weeks later (Fig. 5B-D).
533 Four weeks after strong 1CS-US conditioning, wild-type mice still displayed
534 context-dependent and tone-dependent freezing ($F_{(2,30)}=32.04$, $p<0.0001$, $n=16$,
535 one-way repeated measures ANOVA; context vs. pre-tone, $P=0.0002$; pre-tone vs. tone,
536 $P<0.0001$; Bonferroni's post hoc test) (Fig. 5B, *left*). In addition, CaMKII α (K42R)-KI
537 mice still preserved small but significant tone-dependent freezing ($F_{(2,26)}=9.253$,
538 $p=0.0009$, $n=14$, one-way repeated measures ANOVA; context vs. pre-tone, $P>0.9999$;
539 pre-tone vs. tone, $P=0.0045$; Bonferroni's post hoc test) (Fig. 5B, *right*), the extent of
540 which was significantly less than that observed in wild-type mice (Cued, Tone;
541 $t_{(28)}=7.115$, $p<0.0001$; unpaired t -test). Thus, in both genotypes, memory once formed
542 lasted long.

543 Next, four weeks after 3CS-US conditioning, wild-type mice again displayed
544 context-dependent and tone-dependent freezing ($F_{(2,30)}=39.68$, $p<0.0001$, $n=16$,
545 one-way repeated measures ANOVA; context vs. pre-tone, $p=0.0044$; pre-tone vs. tone,
546 $p<0.0001$; Bonferroni's post hoc test) (Fig. 5C, *left*). CaMKII α (K42R)-KI mice, on the
547 other hand, showed considerably decreased freezing in the three conditions tested, but

548 still displayed tone-dependent freezing without context-dependent freezing
549 ($F_{(2,28)}=15.25$, $p<0.0001$, $n=15$, one-way repeated measures ANOVA; context vs.
550 pre-tone, $p>0.9999$; pre-tone vs. tone, $p<0.0001$; Bonferroni's post hoc test) (Fig. 5C,
551 *right*). The extent of tone-dependent freezing was significantly less than that observed
552 in wild-type mice (Cued, Tone; $t_{(29)}=2.872$, $p=0.0076$; unpaired t -test). Here again, fear
553 conditioned memory once formed was long-lasting in both genotypes.

554 Finally, four weeks after 5CS-US conditioning, wild-type mice still preserved
555 tone-dependent freezing, but not context-dependent freezing any more ($F_{(2,28)}=32.57$,
556 $p<0.0001$, $n=15$, one-way repeated measures ANOVA; context vs. pre-tone, $p=0.2710$;
557 pre-tone vs. tone, $p<0.0001$; Bonferroni's post hoc test) (Fig. 5D, *left*), indicating that
558 context discrimination is more fragile than cued fear memory in the long term in the
559 wild-type mouse. CaMKII α (K42R)-KI mice, on the other hand, still showed rather
560 increased levels of freezing in the three conditions tested, while this time, they did
561 display tone-dependent freezing, which had not been observed 48 h after conditioning,
562 but without context-dependent freezing ($F_{(2,26)}=10.95$, $p=0.0004$, $n=14$, one-way
563 repeated measures ANOVA; context vs. pre-tone, $p=0.1603$; pre-tone vs. tone,

564 $p=0.0002$; Bonferroni's post hoc test) (Fig. 5D, *right*). The extent of tone-dependent
565 freezing was again less than that observed in wild-type mice (Cued, Tone; $t_{(27)}=2.583$,
566 $p=0.0155$; unpaired t -test). The results indicate that the CaMKII α (K42R)-KI mouse
567 had formed cued fear memory from the beginning after 5CS-US conditioning, but its
568 manifestation had been masked by generally increased fear when tested soon after
569 conditioning. However, when generalized fear subsided in four weeks, cued fear
570 memory became apparent.

571 All these results clearly demonstrate that cued fear memory once acquired was
572 long-lasting, not only in the wild-type mouse, but also in the CaMKII α (K42R)-KI
573 mouse.

574

575

576 **Discussion**

577 In this study, we have shown that the kinase-dead homozygous CaMKII α (K42R)-KI
578 mouse retained intact visually guided memory, but was severely impaired in
579 hippocampus-dependent spatial memory in the Morris water maze tasks (Fig. 3). In
580 addition, the CaMKII α (K42R)-KI mouse was severely impaired in
581 hippocampus-dependent context discrimination in fear memory, whereas
582 amygdala-dependent cued fear memory was impaired but preserved to some extent, and
583 once formed cued fear memory lasted as long as four weeks (Figs. 4 and 5). These
584 results indicate that hippocampus-dependent memory was severely impaired, but
585 amygdala-dependent memory was partially spared in the kinase-dead CaMKII α
586 (K42R)-KI mouse. Such difference was not due to differential expression of CaMKII α
587 between the hippocampus and the amygdala because biochemical analyses revealed that
588 both kinase activity and protein levels of CaMKII were indistinguishable between the
589 two brain regions (Figs. 1 and 2, Table 1). All these results indicate that the involvement
590 of kinase activity of CaMKII α in the mechanisms of learning and memory seems to be
591 somewhat different between the types of memory: It is indispensable for

592 hippocampus-dependent memory, whereas although important, not necessarily
593 indispensable for amygdala-dependent memory (Fig. 6).

594 The requirement of CaMKII α in the acquisition of hippocampus-dependent memory
595 has been shown by using various kinds of CaMKII α KO and KI mice (Silva et al.,
596 1992; Giese et al., 1998; Elgersma et al., 2002; Yamagata et al., 2009; Achterberg, et al.,
597 2014). In addition to the homozygous CaMKII α KO mouse (Silva et al., 1992;
598 Elgersma et al., 2002), the autophosphorylation-deficient CaMKII α (T286A)-KI mouse
599 that lacks persistent activation of CaMKII α (Giese et al., 1998; Irvin et al., 2005), and
600 the autophosphorylation-mimicking CaMKII α (T305D)-KI mouse that lacks both
601 kinase activity and postsynaptic association of CaMKII α (Elgersma et al., 2002)
602 showed profound impairments in spatial memory in the Morris water maze tasks and in
603 contextual fear memory after a single-footshock conditioning. Besides, our kinase-dead
604 CaMKII α (K42R)-KI mouse that lacks kinase activity but retains activity-dependent
605 postsynaptic translocational ability of CaMKII α was severely impaired in inhibitory
606 avoidance memory, a form of memory dependent on the hippocampus (Yamagata et al.,
607 2009). A recent study using the region-specific and time-specific conditional CaMKII α

608 KO mice also showed the necessity of CaMKII α protein in the forebrain and at the time
609 of learning for the acquisition of spatial and contextual memory (Achterberg, et al.,
610 2014). All these results showed that proper functioning of CaMKII α is essential for
611 hippocampus-dependent memory to be formed, which is in accordance with our present
612 study.

613 On the other hand, the CaMKII α (T286A)-KI mouse could acquire wild-type levels
614 of contextual and cued fear memory when multiple footshocks were delivered during
615 conditioning (Irvin et al., 2005, 2011). This is a clear contrast to our current results
616 showing that repetitive training was ineffective to overcome memory deficits, but
617 revealed context discrimination deficits and caused generalized fear response in the
618 kinase-dead CaMKII α (K42R)-KI mouse (Figs. 4C and 4D). Besides, when re-tested
619 four weeks later, generalized fear subsided considerably, and cued fear memory, but not
620 context discrimination, was observed in the kinase-dead CaMKII α (K42R)-KI mouse
621 (Fig. 5D). The differential effects of repeated training observed between the CaMKII α
622 (K42R)-KI mouse and the CaMKII α (T286A)-KI mouse could be explained by
623 biochemical differences between the two mutations or by a dominant-negative effect of

624 kinase-dead mutation. From the biochemical point of view, K42R mutation completely
625 inactivates the kinase, whereas T286A mutation does not, i.e., CaMKII α (T286A) can
626 be fully activated in the presence of Ca²⁺/calmodulin, but once Ca²⁺/calmodulin is
627 detached from it, it completely loses its activity. Repetitive stimulation in repeated
628 training given to the CaMKII α (T286A)-KI mouse may induce enough kinase activity
629 for an extended period of time, which seems to be necessary for the acquisition of
630 memory. A recent report showing that increasing the stimulation frequency could
631 prolong the activation state of CaMKII α (T286A) and enabled the induction of LTP at
632 the synaptic level supports this notion (Chang et al., 2017). On the other hand, another
633 kinase-dead mutant, CaMKII α (K42M) was shown to have a dominant-negative effect
634 in the control of synaptic strength when transfected into organotypic hippocampal slice
635 cultures (Kabakov and Lisman, 2015). Besides, viral injection of CaMKII α (K42M)
636 into the CA1 region of the hippocampus in rats caused erasure of acquired fear memory
637 in conditioned place avoidance, another hippocampus-dependent memory task in which
638 an animal receives a footshock every time it enters a shock zone (Rossetti et al., 2017).
639 These reports raise a possibility that kinase-dead mutation of CaMKII α may

640 compromise the role of intact other isoforms of CaMKII, especially that of CaMKII β in
641 the same CaMKII holoenzyme, and cause stronger memory deficits than expected. This
642 may be another reason why the kinase-dead CaMKII α (K42R)-KI mouse in the current
643 study showed severe memory deficits even after repeated training, which was in
644 contrast to the case of the autophosphorylation-deficient CaMKII α (T286A)-KI mouse.

645 As for amygdala-dependent memory, several studies implicated a similar involvement
646 of CaMKII α to the mechanism as in hippocampus-dependent memory (Rodrigues et al.,
647 2004; Irvine et al., 2005). Auditory fear conditioning in rats increased
648 T286-autophosphorylation of CaMKII α in lateral amygdala spines, and the injection of
649 a CaMKII inhibitor, KN-62 into the amygdala impaired acquisition of both auditory and
650 contextual fear memory (Rodrigues et al., 2004). In addition, as described above,
651 repeated training could compensate for deficits not only in contextual, but also in cued
652 fear memory in the CaMKII α (T286A)-KI mouse (Irvine et al., 2005, 2011). Thus,
653 CaMKII α had been thought to play a similar mechanistic role in the acquisition of
654 hippocampus- and amygdala-dependent memory. Such assumption was further
655 supported by a recent study showing that the injection of a photoactivatable CaMKII

656 inhibitory peptide, autocaimitide 2 into the mouse amygdala caused deficits in inhibitory
657 avoidance memory tested at least one hour after training (Murakoshi et al., 2017).
658 However, our present study clearly demonstrated that although small, but distinct
659 amygdala-dependent cued fear memory was observed in the kinase-dead CaMKII α
660 (K42R)-KI mouse, which was stable and long-lasting. Thus, CaMKII α activity seems to
661 be differentially involved in the two types of memory: Hippocampus-dependent
662 memory is mediated exclusively by the CaMKII α activity-dependent pathway, whereas
663 amygdala-dependent memory is mediated not only by the CaMKII α activity-dependent
664 pathway, but also by a CaMKII α activity-independent pathway, at least to some extent
665 (Fig. 6). Such differential involvement seems to be reasonable given the fact that
666 amygdala-dependent fear-related memory is essential for the survival of animals, and
667 the existence of a secondary pathway to achieve it is preferable. On the other hand,
668 hippocampus-dependent spatial memory and context discrimination contributes to the
669 efficiency of life, but is not necessarily essential for the survival of animals, and thus,
670 redundant pathway may not be necessary.

671 Then, what is the secondary, CaMKII α activity-independent pathway that may be

672 involved in the acquisition of amygdala-dependent fear memory? One possibility could
673 be the involvement of other isoforms of CaMKII, i.e., CaMKII β , CaMKII γ and
674 CaMKII δ that are derived from distinct genes. Among them, CaMKII β is suited to have
675 a complementary role to CaMKII α , because CaMKII β is the second major CaMKII
676 isoform in the forebrain and shows neuron-specific expression, besides CaMKII α . Such
677 assumption is indeed supported by the above-mentioned previous studies using CaMKII
678 inhibitors that are effective to all isoforms of CaMKII, including CaMKII α and
679 CaMKII β : Injection of KN-62 and photoactivatable autocamtide 2 into the amygdala
680 showed inhibitory effects on fear conditioned memory and inhibitory avoidance
681 memory, respectively (Rodrigues et al., 2004; Murakoshi et al., 2017). Thus, there may
682 be a synergistic role of CaMKII α and CaMKII β activity in the acquisition of
683 amygdala-dependent memory. It is interesting to note that CaMKII β was shown to play
684 a nonenzymatic role in hippocampus-dependent memory, i.e., its kinase activity is not
685 necessary, but its molecular existence itself seems to be required for proper targeting of
686 CaMKII α to the synapse (Borgesius, et al., 2011). Thus, CaMKII β may play an
687 enzymatic role, in addition to a nonenzymatic role, in the acquisition of

688 amygdala-dependent memory.

689 Another possibility could be de novo synaptogenesis, such as generation of
690 multi-innervated dendritic spines, which is analogous to the one described for the
691 formation of hippocampus-dependent contextual fear memory in the CaMKII α
692 (T286A)-KI mouse after repeated training (Radwanska et al., 2011). In the latter case,
693 multi-trial training induced long-term contextual fear memory that was accompanied by
694 PSD95 up-regulation and synaptogenesis in the CA1 region of the dorsal hippocampus.
695 A similar synaptogenetic changes in the amygdala may account for the formation of
696 long-lasting cued fear memory in the absence of CaMKII α activity after repeated
697 training, as observed in the kinase-dead CaMKII α (K42R)-KI mouse in the current
698 study. It is interesting to note that in the above-mentioned study injecting a
699 photoactivatable CaMKII inhibitory peptide, autocomtide 2 into the mouse amygdala,
700 1-h memory was indeed affected, but 24-h memory tests showed mixed results
701 (Murakoshi et al., 2017), indicating possible existence of another pathway that is
702 unrelated to CaMKII activity. Further studies are necessary to identify the exact
703 molecular mechanisms involved in the acquisition of amygdala-dependent memory,

704 other than those involving CaMKII α activity (Fig. 6).

705 One more interesting observation in this study is that the kinase-dead CaMKII α
706 (K42R)-KI mouse displayed severe context discrimination deficits, leading to
707 generalized fear after repeated CS-US conditioning (Figs. 4C and 4D). Why fear
708 response was generalized after intense training in the KI mouse? Two factors may
709 account for this phenomenon. One is severely impaired hippocampal function caused by
710 the lack of CaMKII α kinase activity. In the CaMKII α (K42R)-KI mouse, hippocampal
711 LTP is severely impaired (Yamagata et al., 2009), which will hamper the acquisition of
712 accurate spatial memory as shown in this study (Fig. 3). Besides, the hippocampus is
713 known to play a key role in integrating discrete contextual elements processed in
714 different subcortical and cortical regions and in encoding a configured representation of
715 the context in fear conditioning (Holland and Bouton, 1999; Maren et al., 2013).
716 Normally, individual contextual elements are superseded by a representation of the
717 context formed in the hippocampus, but with hippocampal damage, each stimulus
718 elements that make up the context encoded outside the hippocampus seems to be used
719 for conditioning and associated with noxious, unconditional stimulus within the

720 amygdala. Such multiple elemental associations may occur in the CaMKII α (K42R)-KI
721 mouse after strong activation of the amygdala by repeated training. The other factor is
722 impaired amygdala function by the lack of CaMKII α kinase activity. CaMKII activity is
723 known to play an important role in NMDA receptor-dependent LTP at thalamic input
724 synapses to the lateral amygdala (Rodrigues et al., 2004; Johansen et al., 2011). A recent,
725 elegant study showed that auditory fear conditioning is mediated by input-specific LTP
726 (Kim and Cho, 2017), which enables discrimination of dangerous stimuli from safe ones
727 (Maren, 2017), and could apply to contextual conditioning as well. With the lack of
728 CaMKII α kinase activity, input specificity of LTP and of conditioning would be lost,
729 and under such a situation, strong activation of the amygdala by repeated training could
730 cause potentiation of nearby, unrelated synapses, leading to generalized fear response
731 without discrimination between dangerous and safe stimulus environments. Thus,
732 impaired hippocampal contextual configuration and impaired input-specificity in the
733 amygdala may account for fear generalization in the CaMKII α (K42R)-KI mouse after
734 intense training. It is interesting to note that hippocampal dysfunction is proposed to be
735 related to generalized fear memory in human patients with post-traumatic stress disorder

736 (PTSD) as well (Acheson et al., 2012).

737 Increased fear memory generalization is one of the characteristics of PTSD and other
738 anxiety-related disorders (Mahan and Ressler, 2012; Pitman et al., 2012). Generalized
739 fear response after repeated training in the kinase-dead CaMKII α (K42R)-KI mouse
740 makes us think if this KI mouse can serve as an animal model of PTSD. However, it
741 should be noted that when re-tested 4 w after repeated CS-US conditioning, the
742 CaMKII α (K42R)-KI mouse showed considerably reduced fear response, and fear
743 generalization was no longer observed (Fig. 5D). Natural recovery from fear
744 generalization without extinction training indicates that extinction of increased fear
745 memory seems to be preserved in the CaMKII α (K42R)-KI mouse, which does not fit
746 as a PTSD model, because in PTSD, fear generalization is sustained and difficult to be
747 extinguished (Sigmund and Wotjak, 2006; Yehuda and LeDoux, 2007). Rather,
748 generalized fear response observed in the CaMKII α (K42R)-KI mouse indicates that the
749 lack of kinase activity of CaMKII α could be one of the predispositions or risk factors
750 toward the development of PTSD. Further investigation of the kinase-dead CaMKII α
751 (K42R)-KI mouse would lead to the understanding and novel therapeutics of PTSD and

752 other abnormal fear memory-associated disorders.

753

754

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882

883

884 **Table 1. CaMKII α and CaMKII β protein levels and phospho-T286-CaMKII α level**
885 **in the hippocampus and amygdala from the kinase-dead CaMKII α (K42R)**
886 **knock-in mouse as compared to the wild-type mouse**

887

	Hippocampus	Amygdala
	(% of WT)	(% of WT)
CaMKII α	85.1 \pm 5.1*	81.0 \pm 5.1*
CaMKII β	101.8 \pm 6.7	105.6 \pm 6.8
Phospho-T286-CaMKII α	63.1 \pm 6.8**	69.5 \pm 3.2***

888 Significantly different from wild-type (WT) levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

889 (one sample t -test); Hippocampus, $n = 6$; Amygdala, $n = 6$.

890

891

892 **Table 2. Sensitivity to an electrical footshock in the kinase-dead CaMKII α (K42R)**
893 **knock-in mouse as compared to the wild-type mouse**

894

	WT	CaMKII α (K42R)-KI
Step/Paw	0.032 \pm 0.002	0.031 \pm 0.001
Vocal/Run/Jump	0.061 \pm 0.003 ^a	0.078 \pm 0.003 ^a

895 Threshold currents are expressed in mA.

896 Step/Paw, stepping back and/or paw flick, reflecting pain sensation; Vocal/Run/Jump,
897 vocalization, running and/or jumping, reflecting aversive reactions.

898 Significantly different between wild-type (WT) and CaMKII α (K42R) knock-in (KI)

899 mice: ^a, $p=0.0001$ (unpaired t-test); WT, n=14; K42R, n=16.

900

901

902 **Figure legends**

903 **Figure 1.** Kinase activity of CaMKII is basically the same between the hippocampus
904 and amygdala in the wild-type mouse and in the kinase-dead CaMKII α (K42R)
905 knock-in mouse. *Left*, Total and Ca²⁺/calmodulin-independent autonomous activity of
906 CaMKII in homogenates from the hippocampus and amygdala of wild-type (WT) mice.
907 These values reflect the summation of the activity of CaMKII α and CaMKII β , the two
908 major CaMKII isoforms in the brain. *Right*, Kinase activity of CaMKII in homogenates
909 from the hippocampus and amygdala of kinase-dead homozygous CaMKII α (K42R)-KI
910 mice. These values reflect the activity of intact CaMKII β . *Note* that figures in
911 percentages above the columns represent relative activity against the value of
912 corresponding control wild-type activity. The total activity was measured in the
913 presence of Ca²⁺/calmodulin. The Ca²⁺/calmodulin-independent autonomous activity
914 was measured in the absence of Ca²⁺/calmodulin. No significant difference was
915 observed in the activity between the hippocampus and amygdala in both genotypes (*See*
916 text in detail). Open columns, total activity; filled columns,
917 Ca²⁺/calmodulin-independent autonomous activity. Error bars indicate SEM.

918 Hippocampus, n=6; Amygdala, n=6 for each genotype.

919

920 **Figure 2.** Representative immunoblots showing the CaMKII α , CaMKII β and
921 phospho-T286-CaMKII α levels in the hippocampus and amygdala of the kinase-dead
922 CaMKII α (K42R) knock-in mouse as compared to the wild-type mouse. The amounts
923 of protein used were 2 μ g, 4 μ g and 8 μ g for the detection of CaMKII α , CaMKII β and
924 phospho-T286-CaMKII α (P-CaMKII α), respectively, from hippocampal or amygdala
925 homogenates. Autoradiography of duplicated samples from a pair of wild-type (WT)
926 and kinase-dead CaMKII α (K42R)-KI mice in the same experimental group used for
927 quantitative immunoblot analyses are shown. *See also* Table 1.

928

929 **Figure 3.** Intact visually guided memory and impaired spatial memory in the Morris
930 water maze tasks in the kinase-dead CaMKII α (K42R) knock-in mouse. **A**, Escape
931 latencies in the visible platform trials, followed by the hidden platform trials in the
932 water maze tasks in the kinase-dead CaMKII α (K42R)-KI mouse as compared to the
933 wild-type (WT) mouse. In the visible platform trials (*left*), both genotypes steadily

934 decreased escape latencies and there was no significant difference between the
935 genotypes. Both genotypes showed significantly shorter latency in the second and later
936 blocks of trials than in the first block (labeled with gray vs. black colors, $p < 0.05$,
937 Bonferroni's post hoc test). In the following hidden platform trials (*right*), latency
938 differed significantly between the genotypes: CaMKII α (K42R)-KI mice took longer
939 time to reach the platform than wild-type mice did. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
940 between the genotypes, Bonferroni's post hoc test. Open circle, wild-type mice, $n = 12$;
941 closed circle, CaMKII α (K42R)-KI mice, $n = 12$. *Note that* the platform location was
942 fixed for each mouse throughout the course of training. **B**, Probe trials performed on
943 days 3 and 6 after the training trials of the day (after 6 and 12 blocks of trials,
944 respectively). Percent time in each quadrant (*left*) and the number of center crossings of
945 hypothetical platform locations (*right*) are shown. ** $p < 0.01$, *** $p < 0.001$, ****
946 $p < 0.0001$, Tukey's post hoc test. Closed column, target quadrant; hatched column, left
947 quadrant; open column, opposite quadrant; dotted column, right quadrant. **C**, Averaged
948 swimming traces of each genotype for 60 s in the probe trials performed on days 3 and 6.
949 The color indicates the average time spent at a certain location (1 x 1 cm square) per

950 animal. The platform location was aligned in the right upper quadrant by rotation and
951 indicated by a black-lined circle. **D**, Swimming speed measured for 60 s in the probe
952 trials performed on days 3 and 6. There was no significant difference between the
953 genotypes. Open column, wild-type mice; closed column, CaMKII α (K42R)-KI mice.

954

955 **Figure 4.** No context discrimination, whereas partially preserved cued fear memory
956 after fear conditioning in the kinase-dead CaMKII α (K42R) knock-in mouse. **A**, Fear
957 conditioned memory after a single-stimulation protocol with a 30 s-tone that
958 co-terminated with a 0.3 mA-footshock (1CS-US). Contextual fear memory (Context)
959 was tested in the same conditioning chamber 24 h after conditioning. Cued fear memory
960 (Cued) was tested in a different context without (Pre-tone) or with tone (Tone) 48 h after
961 conditioning. Ordinate indicates percent freezing time for the period of 180 s, and the
962 line in each column expresses the mean. *Left*, Wild-type (WT) mice showed
963 significantly longer freezing time in the contextual chamber than in the cued chamber
964 without tone, demonstrating context-dependent fear memory. In addition, they showed
965 significantly longer freezing time in the cued chamber with tone than without tone,

966 demonstrating tone-dependent fear memory. n=15. *Right*, CaMKII α (K42R)-KI mice
967 showed virtually no freezing in the contextual chamber, as well as in the cued chamber
968 without tone, and there was no significant difference in freezing time between the two
969 conditions. On the other hand, they showed rather small, but significant freezing in the
970 cued chamber with tone, indicating that cued fear memory was formed at least to a
971 certain extent. n=14. **B**, Fear conditioned memory after a stronger single-stimulation
972 protocol with a 0.7 mA-footshock (1CS-US-strong). Basically similar results were
973 observed as in **A**. WT, n=16 (*left*). K42R, n=14 (*right*). **C**, Fear conditioned memory
974 after a repeated-stimulation protocol with three pairings of a tone and a 0.3
975 mA-footshock (3CS-US). *Left*, Wild-type mice showed both context-dependent and
976 tone-dependent freezing. n=16. *Right*, CaMKII α (K42R)-KI mice showed increased
977 freezing in all of the three conditions tested, but they still did not show
978 context-dependent freezing, whereas showed tone-dependent freezing, revealing context
979 discrimination deficits. n=15. **D**, Fear conditioned memory after a repeated stimulation
980 protocol with five pairings of a tone and a 0.3 mA-footshock (5CS-US). *Left*, Wild-type
981 mice again showed both context-dependent and tone-dependent freezing. n=15. *Right*,

982 CaMKII α (K42R)-KI mice showed generally increased freezing, and there was no
983 significant difference in percent freezing time between the three conditions tested,
984 revealing severe context discrimination deficits, leading to generalized fear. n=14. **
985 $p<0.01$, *** $p<0.001$, **** $p<0.0001$, Bonferroni's post hoc test.

986

987 **Figure 5.** No fear response to tone alone without tone-footshock association, and
988 long-lasting cued fear memory in the kinase-dead CaMKII α (K42R) knock-in mouse. **A**,
989 Fear response after repeated exposure to tone alone for three times without footshocks
990 (3CS alone). Both wild-type (WT) (*left*) and CaMKII α (K42R)-KI mice (*right*) showed
991 almost no freezing in the three conditions tested, demonstrating the specificity of fear
992 response to tone-footshock association in both genotypes (**Figure 4**). WT, n=13. K42R,
993 n=13. **B**, Long-term memory examined 4 w after strong 1CS-US conditioning. The mice
994 in **Fig. 4B** were re-tested 4 w later. *Left*, Wild-type mice still showed context-dependent
995 and tone-dependent freezing, demonstrating the acquired fear memory was long-lasting.
996 n=16. *Right*, CaMKII α (K42R)-KI mice also retained tone-dependent freezing,
997 demonstrating the acquired fear memory was long-lasting. n=14. **C**, Long-term memory

998 examined 4 w after 3CS-US conditioning. The mice in **Fig. 4C** were re-tested 4 w later.
999 Here again, both wild-type (*left*) and CaMKII α (K42R)-KI mice (*right*) retained once
1000 acquired fear memory. WT, n=16. K42R, n=15. **D**, Long-term memory examined 4 w
1001 after 5CS-US conditioning. The mice in **Fig. 4D** were re-tested 4 w later. *Left*,
1002 Wild-type mice still showed tone-dependent freezing, but not context-dependent
1003 freezing. n=15. *Right*, CaMKII α (K42R)-KI mice revealed tone-dependent freezing this
1004 time, indicating that cued fear memory had been acquired from the beginning after
1005 5CS-US conditioning, but had been masked due to generalized fear when tested soon
1006 after conditioning (**Fig. 4D**, *right*). However, when generally increased fear subsided in
1007 four weeks, tone-dependent freezing became apparent. n=14. ** $p < 0.01$, *** $p < 0.001$,
1008 **** $p < 0.0001$, Bonferroni's post hoc test.

1009

1010 **Figure 6.** Schematic illustration showing differential involvement of kinase activity of
1011 CaMKII α in hippocampus- and amygdala-dependent memory. Hippocampus-dependent
1012 memory is mediated exclusively by the CaMKII α activity-dependent pathway, whereas
1013 amygdala-dependent memory is mediated not only by the CaMKII α activity-dependent

1014 pathway, but also by a CaMKII α activity-independent pathway, the candidate of which
1015 may include CaMKII β activity or de novo synaptogenesis, such as generation of
1016 multi-innervated dendritic spines (*See* text in detail).
1017

Figure 1

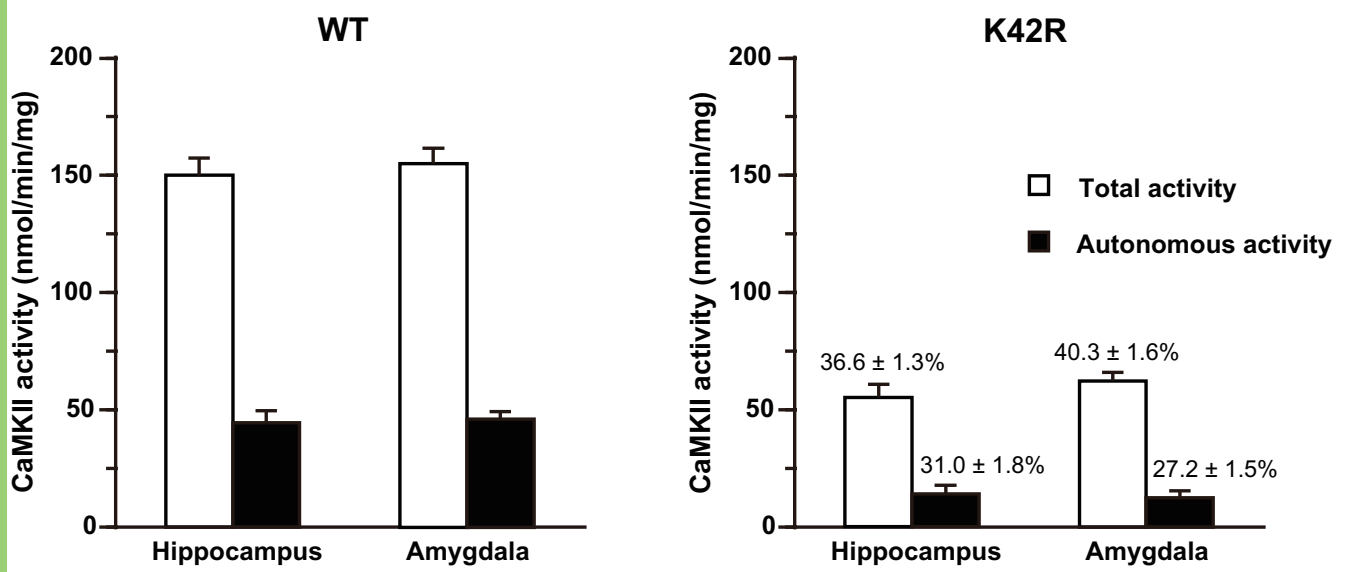


Figure 2

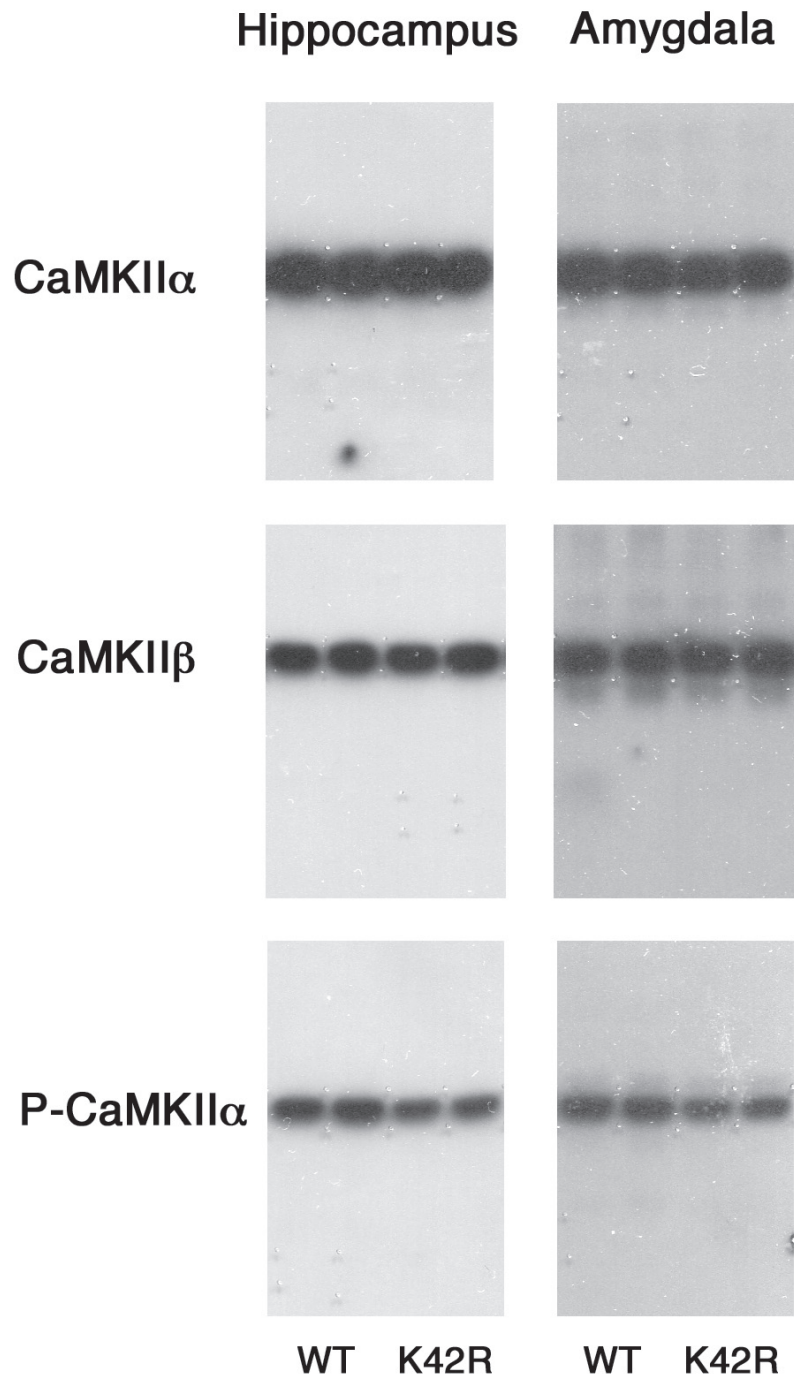


Figure 3

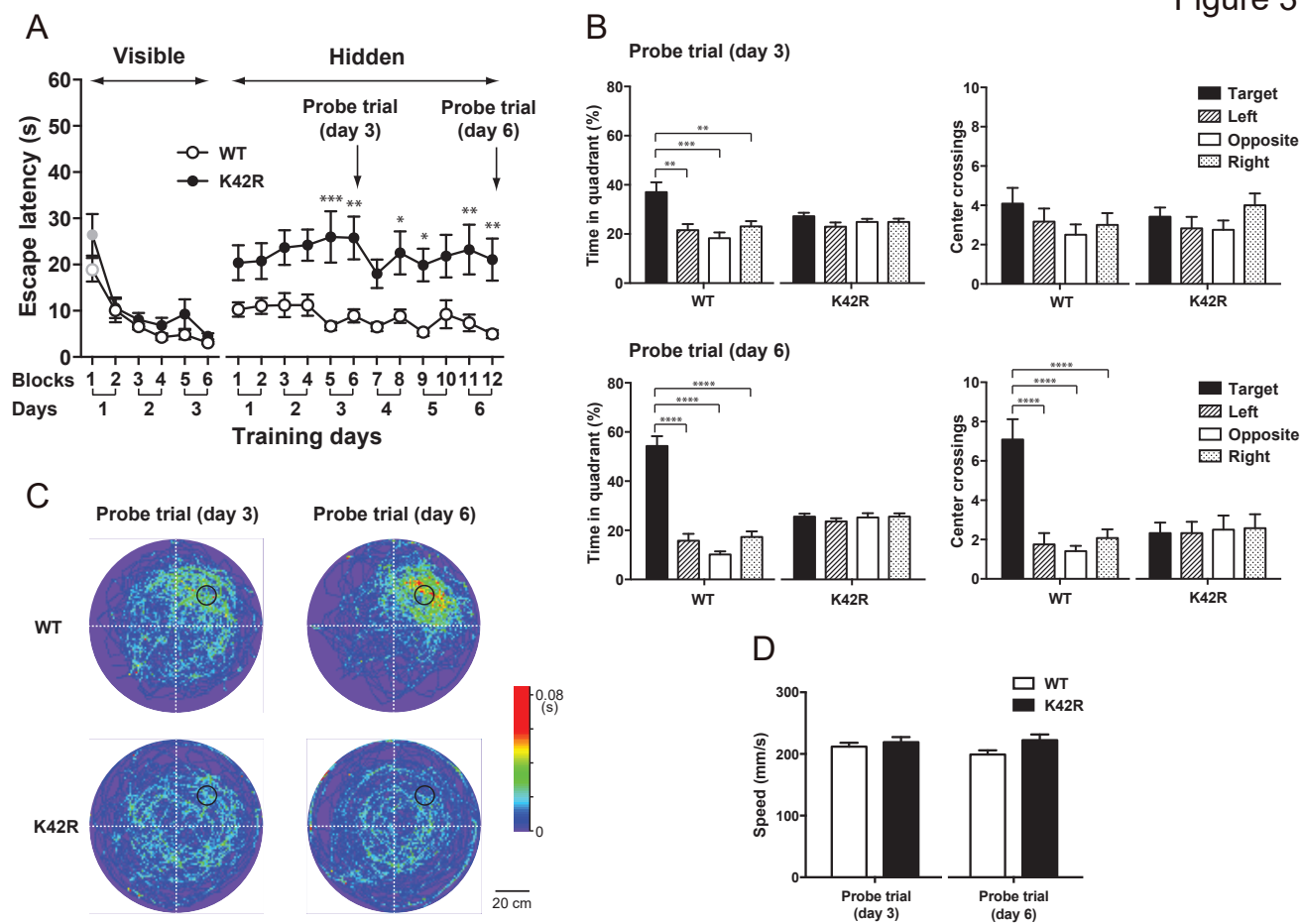


Figure 4

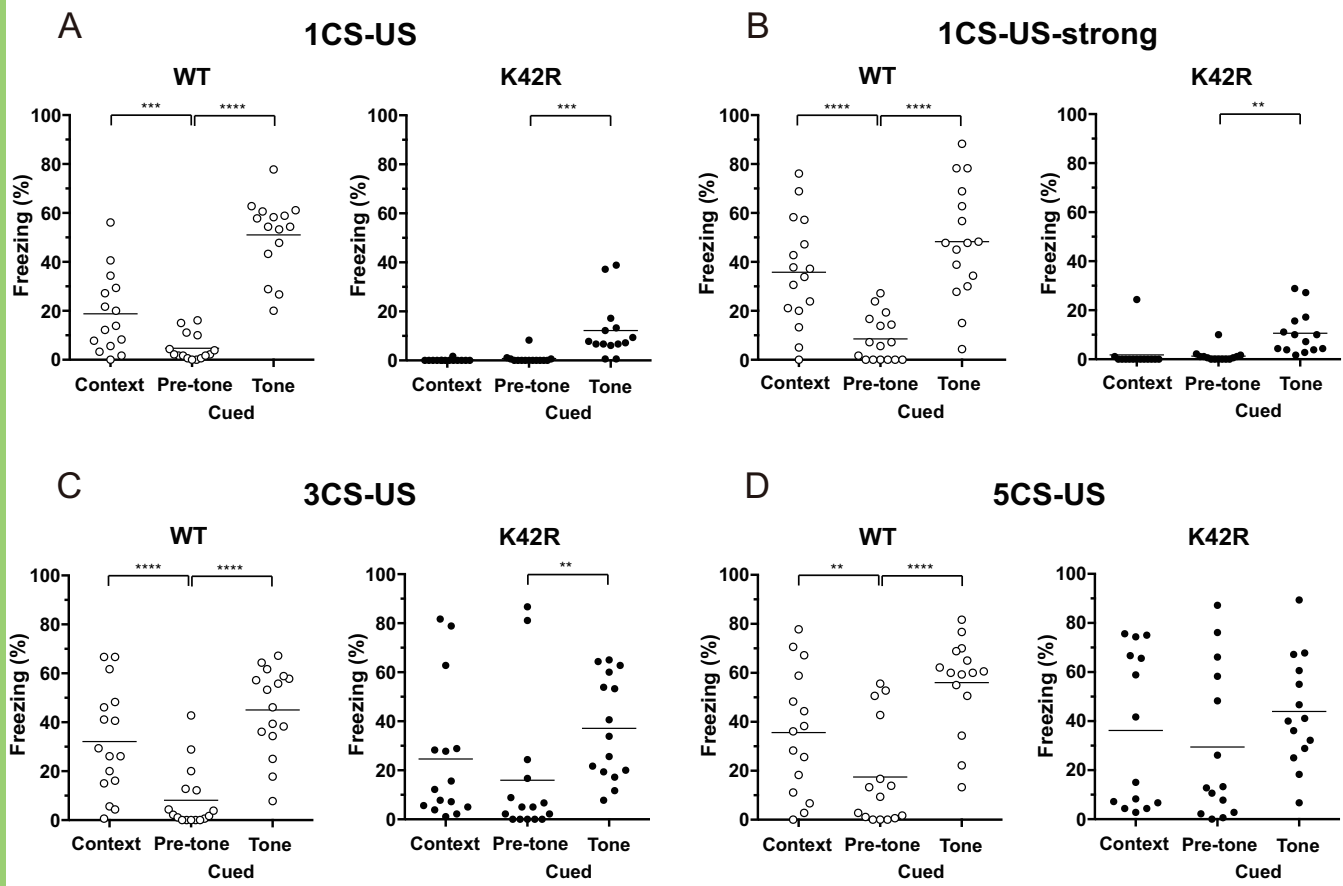


Figure 5

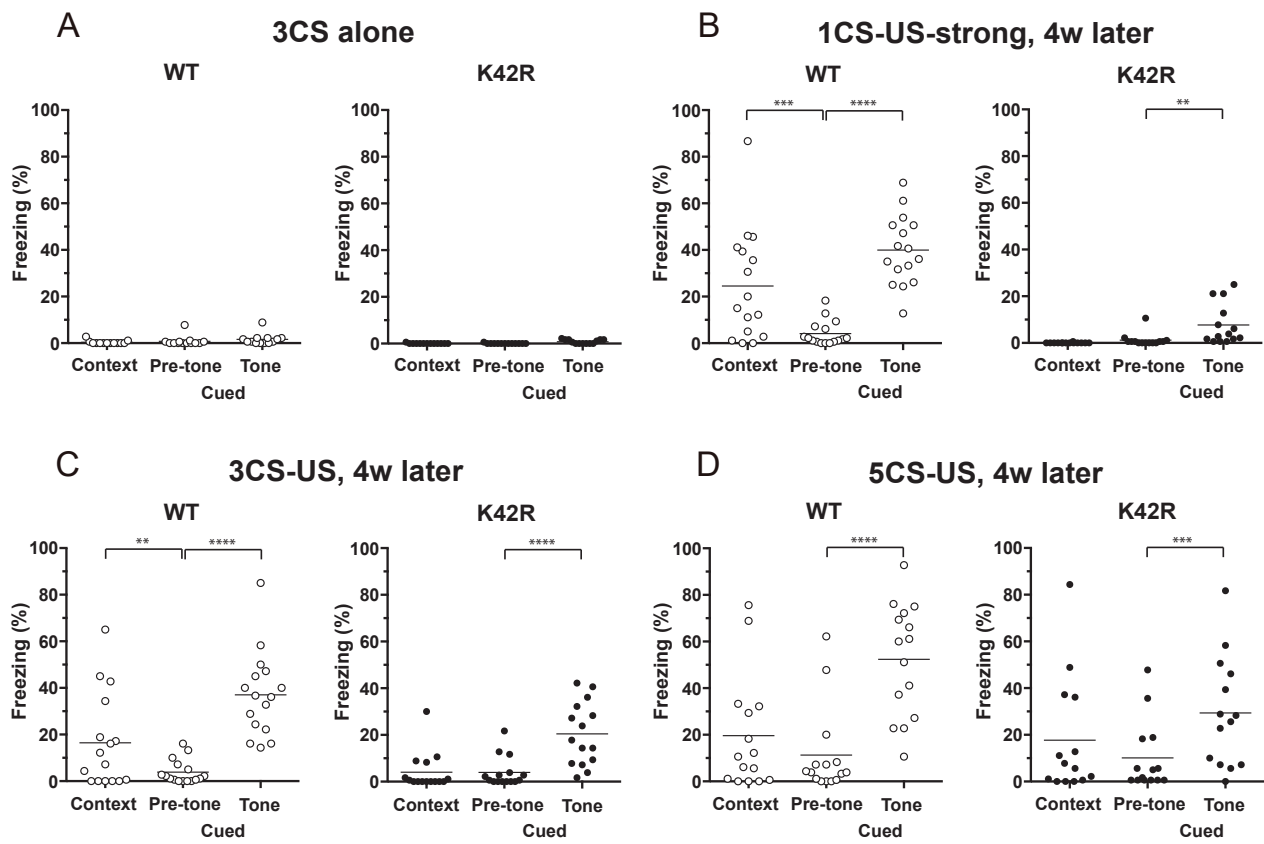


Figure 6

