Behavioral/Cognitive

Temporal and Region-Specific Requirements of αCaMKII in Spatial and Contextual Learning

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The α isoform of the calcium/calmodulin-dependent protein kinase II (αCaMKII) has been implicated extensively in molecular and cellular mechanisms underlying spatial and contextual learning in a wide variety of species. Germline deletion of *Camk2a* leads to severe deficits in spatial and contextual learning in mice. However, the temporal and region-specific requirements for αCaMKII have remained largely unexplored. Here, we generated conditional *Camk2a* mutants to examine the influence of spatially restricted and temporally controlled expression of αCaMKII. Forebrain-specific deletion of the *Camk2a* gene resulted in severe deficits in water maze and contextual fear learning, whereas mice with deletion restricted to the cerebellum learned normally. Furthermore, we found that temporally controlled deletion of the *Camk2a* gene in adult mice is as detrimental as germline deletion for learning and synaptic plasticity. Together, we confirm the requirement for αCaMKII in the forebrain, but not the cerebellum, in spatial and contextual learning. Moreover, we highlight the absolute requirement for intact αCaMKII expression at the time of learning.

Key words: CaMKII; cerebellum; fear conditioning; hippocampus; learning; spatial learning

Introduction

Mouse genetic studies using targeted mutations of the *Camk2a* gene have greatly contributed to establishing the causal link between synaptic plasticity and learning (Silva et al., 1992a; Silva et al., 1992b; Giese et al., 1998; Elgersma et al., 2002). However, the interpretation of results using germline knock-out mice requires consideration of possible neurodevelopmental influences on the phenotype of adult mice given the absence of gene expression, not only at the time of learning, but also throughout neural development. Perhaps most importantly for interpreting the extensive literature using germline *Camk2a* knock-out mice, it remains unclear how the absence of the α isoform of the calcium/calmodulin-dependent protein kinase II (αCaMKII) during the early postnatal period might adversely influence brain development, thereby leading to the observed deficits in spatial and contextual learning. In contrast, most current models of αCaMKII function in synaptic plasticity and learning posit its requirement

at the time of learning independently of any developmental influences (for review, see Elgersma et al., 2004). Therefore, distinguishing between the potential developmental influences of αCaMKII and its necessity at the time of learning remains a highly important goal.

Many distinct brain regions have robust αCaMKII expression, for which germline *Camk2a* knock-out mice have not provided a definitive experimental model to dissociate region-specific influences. In the cortex and hippocampus, αCaMKII is exclusively found in glutamatergic pyramidal neurons (Benson et al., 1992; Jones et al., 1994; Sik et al., 1998), whereas, in the striatum, it is localized to GABAergic medium-spiny neurons (Benson et al., 1992; Takeuchi et al., 2002). In addition, αCaMKII is expressed in cerebellar Purkinje cells, where it is required for intact parallel fiber-Purkinje cell long-term depression (LTD) (Hansel et al., 2006), a form of plasticity suggested to contribute not only to motor learning, but also to spatial and contextual learning (Lalonde and Strazielle, 2003; Burguière et al., 2005; Goddyn et al., 2006; Burguière et al., 2010; Galliano et al., 2013). Therefore, examining the region-specific role of αCaMKII on learning offers unique insights into the functioning of this highly abundant synaptic protein and, more generally, on the systems-level neurobiology of spatial memory.

In the present study, we engineered a conditional *Camk2a* allele to fully parameterize the contribution of αCaMKII to learning and memory, thereby permitting spatiotemporal control over deletion. We found that loss of αCaMKII in adulthood leads to similar deficits as observed in the global knock-out, which lacks αCaMKII expression from conception. Moreover, we confirm the critical importance of αCaMKII in the cortex and hippocam-
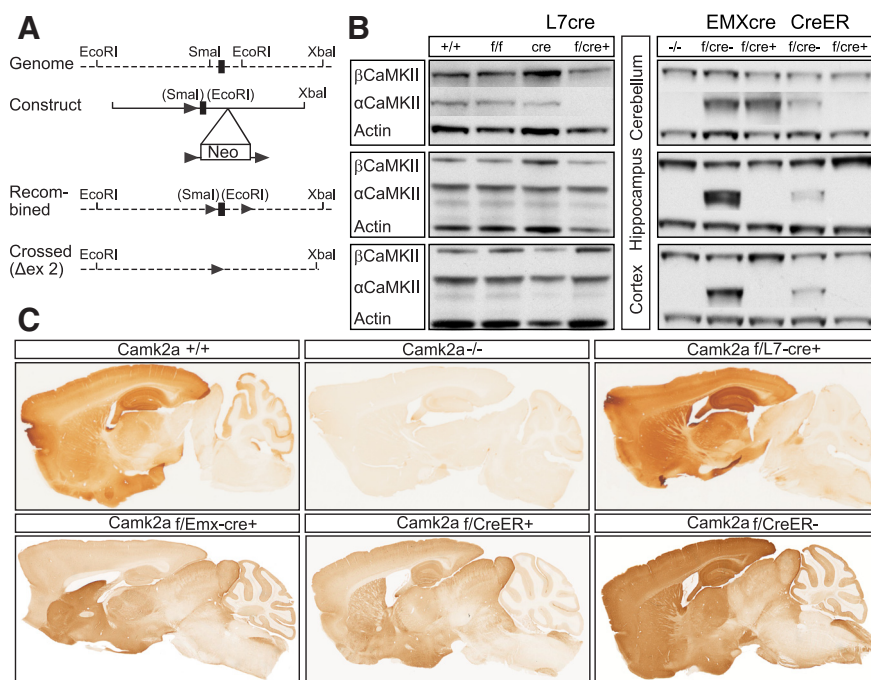
Figure 1. Generation of spatial and temporal specific *Camk2a* knock-out mice. ***A***, Schematic diagram for the generation of floxed *Camk2a* knock-out mice (Genome) Wild-type *Camk2a* locus with exon 2 depicted as a black box. (Construct) Targeting construct used for introducing the LoxP sites and neomycin resistance (Neo) gene in the *Camk2a* locus. The LoxP sites flanking exon 2 and the Neo gene are depicted as triangles. (Recombined) Mutant *Camk2a* flox locus after homologous recombination and Cre recombination in ES cells and consequential deletion of the Neo gene; (Crossed), The mutant *Camk2a* knock-out locus after crossing with Cre-positive mice and subsequent deletion of exon 2 in targeted cells. ***B***, Western blots using antibodies for αCaMKII, βCaMKII, and actin demonstrate that: (1) the αCaMKII level is specifically decreased in the cerebellum of *Camk2a*[flox/L7 - cre +] mice, but not in cortex or hippocampus (left); (2) the αCaMKII level is specifically decreased in the cortex and hippocampus of *Camk2a*[flox/Emx - cre +] mice, but not in the cerebellum (middle); and (3) αCaMKII level is decreased in all structures of *Camk2a*[flox/CreER+] mice (right). For all genotypes, Cre-negative mice are unaffected in their αCaMKII and βCaMKII expression levels. ***C***, αCaMKII stainings showing: (1) normal staining in *Camk2a*^{+/+} mice (top left); (2) no staining in *Camk2a*^{−/−} mice (top middle); (3) specific αCaMKII deletion in Purkinje cells in *Camk2a*[flox/L7 - cre +] mice (top right); (4) specific αCaMKII deletion in forebrain, but not cerebellar cells in *Camk2a*[flox/Emx - cre +] mice (bottom left); (5) specific αCaMKII deletion throughout the brain in *Camk2a*[flox/CreER+] mice (bottom middle); and (6) normal staining in *Camk2a*[flox/CreER −] mice (bottom right).

pus for intact spatial and contextual learning, but found that its expression in the cerebellum is dispensable. Together, the results of the present study show that, in contrast to cerebellar αCaMKII, the loss of forebrain αCaMKII has severe adverse effects on spatial learning in mice. Furthermore, deletion of *Camk2a* in adulthood is sufficient to fully recapitulate the learning impairments of global knock-out mice. This finding confirms the critical necessity of forebrain αCaMKII expression at the time of learning.

Materials and Methods

Animals. The following mice were used in this study: *Camk2a*^{+/+} (WT); *Camk2a*[flox] (homozygously floxed *Camk2a* mice); *Camk2a*[flox/L7 - cre −] and *Camk2a*[flox/L7 - cre+] (Purkinje cell-specific αCaMKII knock-out mice); *Camk2a*[flox/Emx - cre −] and *Camk2a*[flox/Emx - cre +] (telencephalon-restricted αCaMKII knock-out mice); *Camk2a*[flox/CreER −] and *Camk2a*[flox/CreER+] (global αCaMKII knock-out mice with temporal control over gene deletion); and germline αCaMKII knock-out mice (*Camk2a*^{−/−}). All mice were backcrossed >10 times into the C57BL/6JOlaHsd background.

Mice were group housed and maintained under a 12 h light/dark cycle (lights on at 07:00 A.M.), with *ad libitum* access to food and water. Behavioral testing was performed during the light phase. The experimenter remained blinded to the genotype throughout the experiments and data analysis. All behavioral experiments were performed using littermates. Mice were between 2 and 5 months of age. Experimental group assignments were made in consideration of sex and age matching. All

experiments were performed in accordance with the Dutch Animal Ethical Committee (DEC).

Generation of floxed Camk2a mice. Camk2a floxed mutant ES cells were generated as follows: a genomic clone of ~8 kb encoding the *Camk2a* exon 2 (aa 22–53) was isolated by screening a mouse library. The targeting construct was made by inserting a PGK neomycin cassette flanked by LoxP sites into the EcoRI site ~1.2 kbp downstream of exon 2. Another LoxP site was inserted into the SmaI site 60 bp upstream of exon 2. Targeted ES clones (61 of 130) were identified by Southern blot analysis. PCR analysis revealed that 20 of these 61 clones contained the additional 5′ LoxP site. The PGK neocassette was removed by transient expression of Cre recombinase (pBS185; Invitrogen). Therefore, a mutant allele was created in which the *Camk2a* exon 2 was flanked by LoxP sites (Fig. 1A).

Generation of L7-cre mice. Heterozygous floxed αCaMKII mice were crossed with L7/ pcp-2 Cre transgenic mice (RRID:IMSR_JAX:004146; Oberdick et al., 1990; Barski et al., 2000). Male *Camk2a*[flox/L7 - cre+] mice from this F1 offspring were crossed with female *Camk2a*[flox/L7 - cre −] mice to obtain the two experimental genotypes investigated: *Camk2a*[flox/L7 - cre −] and *Camk2a*[flox/L7 - cre+]. L7 is expressed in cerebellar Purkinje cells *in utero*. Therefore, in *Camk2a*[flox/L7 - cre+] mice, deletion of *Camk2a* is Purkinje-cell specific and occurs before the normal postnatal expression of αCaMKII (Smeyne et al., 1991).

Generation of Emx1-cre mice. Camk2a[flox] mice were crossed with Emx1-Cre transgenic mice (RRID:IMSR_RBRC01345; Iwasato et al., 2004). Male *Camk2a*[flox/Emx - cre+] mice from this F1 offspring were crossed with female *Camk2a*[flox] mice. Male *Camk2a*[flox/Emx - cre+] mice from this F2 offspring were crossed again with female *Camk2a*[flox] to obtain the two experimental genotypes: *Camk2a*[flox/Emx - cre −] and *Camk2a*[flox/Emx - cre +]. Emx1 is expressed in glutamatergic telencephalic neurons *in utero* (Simeone et al., 1992). Therefore, in *Camk2a*[flox/Emx - cre +] mice, deletion of *Camk2a* is restricted to the telencephalon and occurs before the normal postnatal expression of αCaMKII.

Generation of Cre[ER] *mice. Cre*[ER] mice were generated by engineering a fusion protein between cre recombinase and the ligand binding domain of a tamoxifen-responsive mutant human estrogen receptor, driven by the widespread CMV early enhancer/chicken β actin (CAG) promoter (RRID:IMSR_JAX:004682; Hayashi and McMahon, 2002). Only upon tamoxifen binding will the Cre[ER] protein translocate into the nucleus and catalyze loxP recombination (Van Duyne, 2001). Therefore, tamoxifen administration in adulthood allows the normal endogenous expression of αCaMKII protein throughout development. For breeding of the experimental genotypes, male *Cre*[ER+] mice were crossed with female *Camk2a*[flox] mice. The resulting F2 male *Camk2a*[flox/CreER+] mice were crossed with female *Camk2a*[flox] mice to obtain the *Camk2a*[flox/CreER+] and *Camk2a*[flox/CreER −] genotypes.

Tamoxifen injections for Cre[ER] *experiments.* Adult deletion of *Camk2a* was achieved using four injections of tamoxifen. Tamoxifen (Sigma-Aldrich) was dissolved in sunflower oil in a dilution of 20 mg/ml. Injections were performed once daily for 4 consecutive days at a dose of 0.10 mg/g bodyweight. Behavioral testing was initiated 4 weeks after the first day of tamoxifen injection (Hayashi and McMahon, 2002). Importantly, to control for any potential influence of tamoxifen itself, all mice (both

Camk2a $^{flox/CreER+}$ and *Camk2a* $^{flox/CreER-}$) received tamoxifen injections. Notably, however, previous studies have demonstrated that, using this dosing regimen, the administration of tamoxifen has no discernible influence on emotional reactivity, neurological functioning, or learning (Vogt et al., 2008).

Morris water maze. All mice were handled for 1 min daily beginning a week before the experiment. The water maze is a circular pool with a diameter of 1.2 m that is filled with an opaque mixture of water and white paint. Water temperature was maintained at 25–26°C. The escape platform had a diameter of 11 cm and was submerged 1 cm beneath the water surface. The swimming paths of the animals were captured by a closed-circuit video camera mounted directly above the center of the pool and analyzed using Smart version 2.0 software (Panlab rib_00052). The room was illuminated by ceiling lamps set to a low level of light intensity. Visually salient and readily distinguishable distal cues were displayed on each wall of the room.

Mice were trained using two 60 s trials per day, with a 30 s intertrial interval, for 5 consecutive days. During training, mice were placed on the platform for 30 s and subsequently placed into the pool at pseudorandom starting positions. After reaching the platform, the mice were allowed to remain for 30 s before returning to their home cage. If a mouse was unable to locate the platform within 60 s, the trial was concluded and the mouse was gently placed on the platform by the experimenter and remained there for 30 s. The platform location was in a fixed position throughout all trials. For each trial, latency, distance covered, mean swim speed, and path were measured.

On day 5, a probe trial was performed 1 h after the training, in which the platform was removed to assess spatial learning. During the probe trial, mice were placed in the pool at the opposite side of the platform position and were allowed to search for 60 s. The amount of time spent in each quadrant and platform location crossings were measured for each probe trial. For statistical analysis, the time in the target quadrant was compared with the average of the other three quadrants.

Pavlovian fear conditioning. Mice were placed in a steel chamber (30 × 20 cm) with a grid floor and a clear plastic door and ceiling (Med Associates). On day 1, each mouse was placed inside the conditioning chamber for 180 s. A single foot shock (2 s, 0.4 mA) was delivered after a placement-to-shock interval of 148 s. Twenty-four hours later, mice were returned to the same context for 180 s. Behavior during training and testing was recorded using a closed-circuit video camera mounted directly in front of the chambers. Freezing behavior, defined as the cessation of all movement except respiration, was quantified using an automated video-based algorithm with detection parameters calibrated by independent manual scoring (Anagnostaras et al., 2010).

Western blots. Mice were anesthetized with isoflurane and decapitated. Cortex, hippocampus, and cerebellum were rapidly dissected and frozen in liquid nitrogen. Tissue samples were homogenized using a Dounce homogenizer in lysis buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS) containing protease and phosphatase inhibitor mixtures (Sigma-Aldrich). Protein concentrations were determined using the BCA method (Pierce) and adjusted to 1 mg/ml; 10 μg of protein was loaded per lane. The following antibodies were used: αCaMKII (1:10,000, catalog #MAB8699 RRID:AB_2067919; Millipore), βCaMKII (1:10,000, catalog #ab34703 RRID:AB_2275072; Abcam), and actin (1:20,000, catalog #MAB1501R RRID:AB_94235, Millipore). Samples were electrophoresed using 10% Bis-Tris gels (Bio-Rad) and blotted on nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBST). Blots were incubated in primary antibody, diluted in TBST with 2% dry milk, and incubated in secondary antibody (goat anti-mouse, 1:3,000, AffiniPure, catalog #115–007-003 RRID:AB_2307348, Jackson ImmunoResearch. The blots were incubated in enhanced chemiluminescence reagent (Pierce) exposed to film, and analyzed with ImageJ version 64 software.

Immunohistochemistry. Mice were deeply anesthetized with pentobarbital and perfused transcardially with a series of PBS, followed by freshly prepared 4% paraformaldehyde (PFA; Sigma). Brains were carefully removed and fixed for 2 h in 4% PFA, followed by 24 h incubation in 0.1 M phosphate buffer (PB) and 10% sucrose. Brains were then embedded in gelatin blocks (10% gelatin, 10% sucrose), postfixed in 10% formaldehyde and 30% sucrose for 3 h, and kept in 30% sucrose overnight at 4°C. The embedded brains were sectioned using a freezing microtome with a section thickness of 40 μm. Sections were processed free floating with diaminobenzidine (0.05%) as the chromogen.

Slices were rinsed, blocked in 3% H_2O_2 and PBS, rinsed again, and kept at 80°C in 10 mM sodium citrate for 20 min. After rinsing, slices were preincubated for 1 h at room temperature in 10% normal horse serum (NHS), 0.5% Triton X-100, and PBS, and kept overnight at 4°C in 10% NHS, 0.5% Triton X-100, PBS, and AffiniPure Fab Fragment (donkey anti mouse, 1:200, catalog #rid_000053, Jackson ImmunoResearch). Subsequently, sections were kept in 10% NHS, 0.5% Triton X-100, PBS, and primary antibody (αCaMKII, 1:9,000, catalog #MAB8699 RRID: AB_2067919, Millipore) for 48 h at 4°C. After rinsing, sections were kept for 2 h at room temperature in the secondary antibody (RAM-HRP, 1:200, catalog #P026002 RRID:AB_2307349, Dako) in 10% NHS, 0.5% Triton X-100, and PBS. Finally, slices were stained in 0.05 M PB, 0.05% diaminotenzidinetetrachloride, and 3% H_2O_2 for 2 min. Slices were then mounted on slides with chrome (III) potassium sulfate-dodecahydrate, dehydrated in alcohol (70%, 90%, 100%), cleared with xylene, and coverslipped with Permount (Fisher Scientific).

Electrophysiology. Adult mice (15–20 weeks old) were anesthetized with isoflurane and decapitated, after which the brain was taken out quickly and submerged in ice-cold oxygenated (95%) and carbonated (5%) artificial CSF (ACSF; <4.0°C) containing the following (in mM): 120 NaCl, 3.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose. Using a vibratome, 400-μm-thick sagittal slices were made and then hippocampi were dissected out. Hippocampal slices were allowed to recover in a bath of oxygenated and carbonated ACSF at room temperature for 1.5 h. At onset of the experiment, slices were placed submerged in a recording chamber that was continuously perfused at a rate of 2 ml/min with oxygenated and carbonated 30°C ACSF. All slices were recorded with platinum (Pt)/iridium (Ir) electrodes. Bipolar Pt/Ir stimulating electrodes were used to stimulate the slice with a stimulus duration of 100 μs. Stimulating and recording electrodes were placed on the CA3-CA1 Schaffer-collateral afferents and the dendrites of CA1 pyramidal cells in the stratum radiatum (150–200 μm from stratum pyramidale), respectively. Upon placement of electrodes, slices were given 30 min to rest before onset of measurements. All paired-pulse facilitation (PPF) experiments were done at one-third of slice maximum. Long-term potentiation (LTP) induced using 100 Hz tetanization (1 train of 1 s at 100 Hz) was also evoked at one-third of slice maximum. Theta burst LTP (four trains of four stimuli at 100 Hz, 200 ms apart) were evoked at two-thirds of slice maximum. Unstable recordings were excluded and determinations were made blind to genotype. The magnitude of LTP was defined as the average of the last 10 measurements of the normalized fEPSP slope.

Data analysis and statistics. Hypothesis testing was performed using SPSS version 17 software, as indicated in the "Results" section. Outcomes are expressed as mean ± SE. Pairwise comparisons were performed using either ANOVA with Tukey's *post hoc* test or two-tailed *t* tests, depending upon the experimental design and as specified in the text. Group sizes for each experiment are provided in the figure legends.

Results

Spatiotemporal deletion of αCaMKII

To investigate the temporal and region-specific contribution of αCaMKII to the learning deficits observed in global *Camk2a* $^{-/-}$ mice, we first generated a floxed allele of *Camk2a* to enable experiments using spatiotemporally controlled deletion of αCaMKII (Fig. 1A). For each of the cre lines used: *L7-cre* (cerebellar Purkinje neurons), *Emx1-cre* (glutamatergic pyramidal neurons of the cortex and hippocampus), and *CAG-CreER* (tamoxifen-inducible global deletion), we first confirmed that the protein levels and immunohistochemical distribution of αCaMKII was consistent with the expected pattern of deletion as previously reported using other floxed lines. The conditional mutants were further compared with WT and global KO mice. As
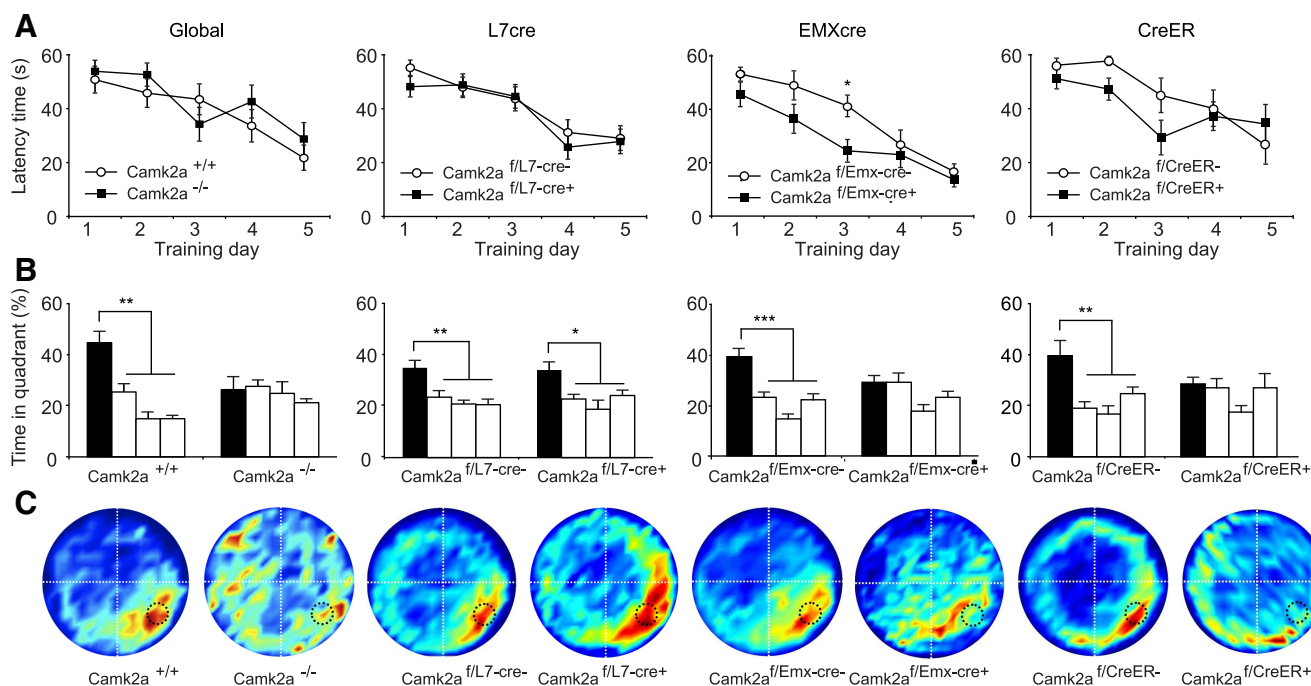
Figure 2. Water maze learning is normal in the absence of cerebellar αCaMKII. **A**, Latency times to reach the hidden platform during training are not different between the *Camk2a* mutants and their control littermates. **B**, Quantification of the time spent in each quadrant at a probe trial given after 5 d of training. Black bar indicates the target quadrant; white bars indicate adjacent right, opposite, and adjacent left quadrants, respectively. Error bars represent SEM. *Camk2a*^{+/+} mice show a clear preference for the platform quadrant, but not *Camk2a*^{−/−} mice (left). *Camk2a*[flox/L7 - cre+] mice show a clear preference for the platform quadrant (second panel), whereas *Camk2a*[flox/Emx - cre+] mice (third panel) and *Camk2a*[flox/CreER+] mice (last panel) search equally in all four quadrants. All cre-negative control littermates show a clear preference for the platform quadrant. **C**, Visual representation of all swimming tracks of each group of mice combined from the probe trial given at day 5. The color indicates the time spent at a certain location (red is high, blue is low). Note that *Camk2a*^{−/−}, *Camk2a*[flox/Emx - cre+], and *Camk2a*[flox/CreER+] mice mice search randomly, whereas the other groups search preferentially around the platform location. The numbers of mice tested were as follows: *Camk2a*^{+/+}: 9; *Camk2a*^{−/−}: 8; *Camk2a*[flox/L7 - cre −]: 15; *Camk2a*[flox/L7 - cre+]: 14; *Camk2a*[flox/Emx - cre −]: 15; *Camk2a*[flox/Emx - cre+]: 13; *Camk2a*[flox/CreER −]: 9; and *Camk2a*[flox/CreER+]: 10.

expected, *Camk2a*[flox/L7 - cre+] mice showed a selective loss of αCaMKII expression in cerebellar Purkinje neurons, the only cell type in the cerebellum that normally expresses αCaMKII (Walaas et al., 1988; Fig. 1*B*,*C*). In *Camk2a*[flox/Emx - cre+] mice, αCaMKII was deleted from cortex and hippocampus, but without any changes in the cerebellum. Consistent with previous reports (Iwasato et al., 2004), recombination was variable across distinct amygdala nuclei. Specifically, high levels of recombination were observed in the basolateral amygdala relative to the lateral amygdala and posterodorsal medial amygdala nucleus. Also consistent with previous findings (Iwasato et al., 2004), we observed a very low residual staining of αCaMKII throughout the telencephalon, with a randomly scattered and sparse distribution of labeled neurons. In contrast, we observed complete tamoxifen-inducible deletion of αCaMKII in adult *Camk2a*[flox/CreER+] mice. Importantly, however, *Camk2a*[flox] mice lacking cre recombinase had no detectable alteration in their αCaMKII protein levels, confirming that the integrated loxP sites were not interfering with *Camk2a* gene expression (Fig. 1*B*,*C*).

Telencephalic, but not cerebellar, αCaMKII is required for spatial learning

Using the hidden version of the Morris water maze, previous studies have demonstrated that *Camk2a*^{−/−} mice are severely impaired in spatial learning (Silva et al., 1992a; Elgersma et al., 2002). However, the temporal and region-specific requirements for endogenous αCaMKII in spatial learning have not been determined previously. Therefore, we examined the impact of temporal and region-specific deletion of αCaMKII on water maze

learning using a 5 d (2 trials/d) training protocol previously shown to result in severe impairments in *Camk2a*^{−/−} global knock-out mice in which the same exon was already deleted in germline (Elgersma et al., 2002). In both groups, latency times decreased equivalently (effect of genotype: $F_{(1,15)} = 0.46$, $p = 0.51$, repeated-measures ANOVA; Fig. 2*A*, first panel). However, consistent with previous studies, we confirmed that *Camk2a*^{−/−} mice have a severe spatial learning deficit, reflected in no significant preference for the target quadrant compared with the other quadrants ($t_{(7)} = 2.62$, $p = 0.80$ dependent samples t test; Fig. 2*B*, first panel), whereas *Camk2a*^{+/+} mice clearly showed a significant preference for the target quadrant ($t_{(8)} = 4.411$, $p = 0.002$, dependent samples t test; Fig. 2*B*, first panel).

Previous studies have suggested that the cerebellum may contribute significantly to the acquisition of spatial learning (Lalonde and Strazielle, 2003; Burguière et al., 2005; Goddyn et al., 2006; but see also Galliano et al., 2013). Therefore, to examine the specific contribution of cerebellar αCaMKII on spatial learning in our water maze learning paradigm, we tested *Camk2a*[flox/L7 - cre+] mice and their *Camk2a*[flox/L7 - cre −] control littermates on the hidden version of the Morris water maze. During training, latency times decreased equivalently in both genotypes (effect of genotype: $F_{(1,27)} = 0.408$, $p = 0.53$, repeated-measures ANOVA; Fig. 2*A*, second panel). Moreover, *Camk2a*[flox/L7 - cre+] mice were indistinguishable from their littermates in their capacity to locate the platform during the probe trial (average time in platform quadrant, *Camk2a*[flox/L7 - cre+]: $t_{(13)} = 2.868$, $p = 0.01$, paired t test; *Camk2a*[flox/L7 - cre −]: $t_{(14)} = 3.567$, $p = 0.003$, paired t test; Fig. 2*B*, second panel). Therefore, cerebellar expression of αCaMKII does

not appear to be required for spatial learning given that $Camk2a^{\text{flox/L7-cre+}}$ mice have complete deletion of αCaMKII in the cerebellum yet acquire spatial learning normally under conditions in which global $Camk2a^{-/-}$ mice are severely impaired.

We next examined the necessity for telencephalic αCaMKII during spatial learning using $Camk2a^{\text{flox/Emx-cre+}}$ mice and their $Camk2a^{\text{flox/Emx-cre-}}$ control littermates. Both groups of mice showed an overall similar reduction in their latency times across the training days (Fig. 2A, third panel). Although a repeated-measures ANOVA showed a significant difference between the groups (effect of genotype: $F_{(1,26)} = 6.023$, $p = 0.02$), post hoc Tukey's tests showed that a significant difference between the 2 groups was restricted to day 3 ($t_{(26)} = 3.171$, $p = 0.004$) and no differences were observed in the initial or final training days. Moreover, consistent with the requirement of telencephalic αCaMKII for spatial learning, $Camk2a^{\text{flox/Emx-cre+}}$ mice showed no significant preference for the target quadrant during the probe trial ($t_{(12)} = 1.816$, $p = 0.09$, paired t test; Fig. 2B, third panel). In contrast, the $Camk2a^{\text{flox/Emx-cre-}}$ littermates demonstrated robust spatial learning of the hidden platform location ($t_{(14)} = 4.656$, $p = 0.0004$, paired t test; Fig. 2B, third panel). There was no significant difference in thigmotaxis ($t_{(26)} = 0.107$, $p = 0.79$, unpaired t test), swim speed ($t_{(26)} = 1.882$, $p = 0.25$, unpaired t test), or path length ($t_{(26)} = 1.904$, $p = 0.26$, unpaired t test) between the two groups. Together, our results suggest that, under these commonly used experimental conditions, spatial learning is strongly dependent on αCaMKII expression in the telencephalon, but not the cerebellum.

In addition to its region-specific requirements, we also considered how developmentally regulated expression of αCaMKII influences spatial learning. αCaMKII has been well demonstrated to function critically during NMDA-dependent LTP in the telencephalon, which is widely believed to be an indispensable form of plasticity underlying spatial learning. However, given that αCaMKII expression begins in the early postnatal period, the learning impairments observed in global $Camk2a^{-/-}$ mice might result from the absence of αCaMKII during early postnatal development, rather than being required specifically at the time of learning. Therefore, we used the tamoxifen-inducible $Camk2a^{\text{flox/CreER+}}$ mice and their $Camk2a^{\text{flox/CreER-}}$ littermates to achieve temporal control over the timing of αCaMKII deletion. In particular, we reasoned that, by inducing deletion only in adulthood, we could more directly examine the necessity for αCaMKII specifically at the time of learning while controlling for any potentially confounding abnormalities that might arise from the absence of αCaMKII during early development. Therefore, adult $Camk2a^{\text{flox/CreER+}}$ mice and their $Camk2a^{\text{flox/CreER-}}$ control littermates were given daily injections of tamoxifen (0.1 mg/g, i.p.) for 4 d to induce gene deletion. As shown in Figure 1, B and C, tamoxifen-inducible deletion of αCaMKII in adulthood of $Camk2a^{\text{flox/CreER+}}$ mice was highly efficient and without any observed deletion in the absence of tamoxifen, thereby maintaining the full integrity of postnatal development.

During the training phase, $Camk2a^{\text{flox/CreER+}}$ mice and their $Camk2a^{\text{flox/CreER-}}$ littermates demonstrated a similar reduction in latency times (effect of genotype: $F_{(1,17)} = 1.982$, $p = 0.18$, repeated-measures ANOVA; Fig. 2A, last panel). However, during the probe trial, $Camk2a^{\text{flox/CreER+}}$ mice showed no significant preference for the target quadrant ($t_{(9)} = 1.593$, $p = 0.15$, paired t test; Fig. 2B, last panel), whereas their $Camk2a^{\text{flox/CreER-}}$ littermates demonstrated a search pattern that was highly localized to the location of the hidden platform ($t_{(8)} = 2.511$, $p = 0.04$, paired t test; Fig. 2B, last panel). There was no significant difference in
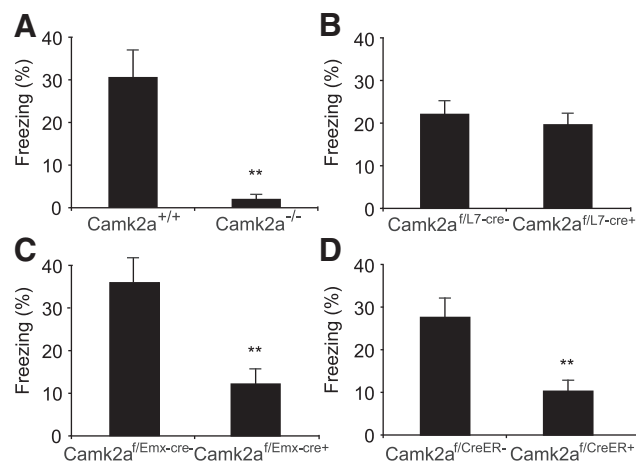
Figure 3. Contextual fear learning is unaffected in the absence of cerebellar αCaMKII. **A**, Compared with $Camk2a^{+/+}$ mice, $Camk2a^{-/-}$ mice show no contextual fear learning. **B**, $Camk2a^{\text{flox/L7-cre+}}$ mice show intact contextual fear learning compared with their $Camk2a^{\text{flox/L7-cre-}}$ control littermates. In contrast, $Camk2a^{\text{flox/Emx-cre+}}$ mice (**C**) and $Camk2a^{\text{flox/CreER+}}$ (**D**) mice show severe contextual fear learning deficits compared with their Cre-negative control littermates. Numbers of mice tested were as follows: $Camk2a^{+/+}$: 9; $Camk2a^{-/-}$: 9; $Camk2a^{\text{flox/L7-cre-}}$: 15; $Camk2a^{\text{flox/L7-cre+}}$: 17; $Camk2a^{\text{flox/Emx-cre-}}$: 17; $Camk2a^{\text{flox/Emx-cre+}}$: 21; $Camk2a^{\text{flox/CreER-}}$: 10; and $Camk2a^{\text{flox/CreER+}}$: 11. Bars represent the amount of freezing behavior, which is used as an index for recollection of the context. Error bars represent SEM.

thigmotaxis ($t_{(17)} = -0.538$, $p = 0.09$, unpaired t test), swim speed ($t_{(17)} = 0.409$, $p = 0.59$, unpaired t test), or path length ($t_{(17)} = -0.140$, $p = 0.95$, unpaired t test) between the two groups. Therefore, αCaMKII is specifically required in adulthood at the time of learning for intact acquisition of spatial information independently of its function during early postnatal development.

Regional and temporally controlled deletion of αCaMKII on contextual fear learning
Contextual fear conditioning is a highly robust form of associative learning in which unsignaled aversive stimuli (e.g., foot shock) are administered within a previously neutral context in which a range of defensive behaviors (e.g., freezing) are subsequently elicited (Phillips and LeDoux, 1992, 1994). In addition to impaired spatial learning, $Camk2^{-/-}$ mice also have been shown to have deficits in contextual conditioning (Silva et al., 1992a). Therefore, we sought to examine the temporal and region-specific requirements for αCaMKII expression during contextual fear learning.

Consistent with previous studies, we found that $Camk2a^{-/-}$ mice with a germline deletion of αCaMKII were severely impaired in contextual fear conditioning ($t_{(16)} = 4.454$, $p = 0.0004$, unpaired t test; Fig. 3A) compared with their $Camk2a^{+/+}$ littermates. In contrast, $Camk2a^{\text{flox/L7-cre+}}$ mice with cerebellum-specific deletion of αCaMKII showed similar contextual fear learning as their $Camk2a^{\text{flox/L7-cre-}}$ control littermates ($t_{(30)} = 0.621$, $p = 0.57$, unpaired t test; Fig. 3B), suggesting that Purkinje cell expression of αCaMKII is not required for normal contextual fear conditioning.

Many previous studies have suggested that contextual fear learning requires NMDA-dependent LTP in the cortex and hippocampus (Levenson et al., 2002; Kiyama et al., 1998). However, no previous studies have examined the telencephalic region-specific requirement for αCaMKII, despite its being among the most widely hypothesized proteins underlying LTP and learning (for review, see Coultrap and Bayer, 2012). Therefore, using
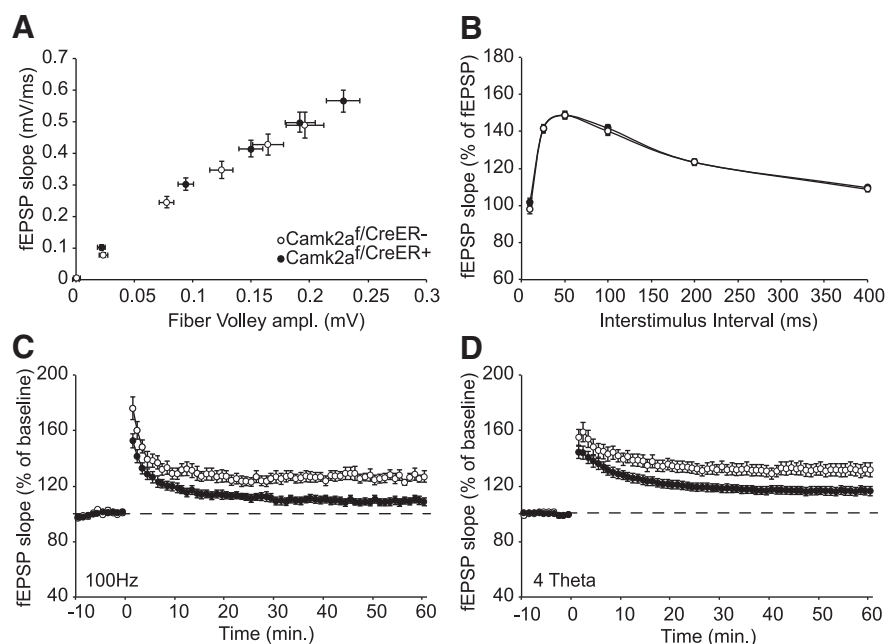
Figure 4. CA3-CA1 LTP is impaired upon deletion of αCaMKII in adulthood. *A*, $Camk2a^{flox/CreER+}$ mice show normal basal synaptic transmission ($Camk2a^{flox/CreER+}$ $n = 37$, $n = 55$; $Camk2a^{flox/CreER-}$ $n = 39$, $n = 55$ for the number of slices showing fiber volley and fEPSP, respectively). *B*, $Camk2a^{flox/CreER+}$ mice show normal PPF ($n = 30$ and $n = 31$ for $Camk2a^{flox/CreER+}$ and $Camk2a^{flox/CreER-}$ mice, respectively). *C*, $Camk2a^{flox/CreER+}$ mice show impaired 100 Hz LTP at the CA3-CA1 synapse in the hippocampus ($n = 15$ and $n = 16$ for $Camk2a^{flox/CreER+}$ and $Camk2a^{flox/CreER-}$ mice, respectively). *D*, $Camk2a^{flox/CreER+}$ mice show impaired 4 Theta LTP at the CA3-CA1 synapse in the hippocampus ($n = 31$ and $n = 23$ for $Camk2a^{flox/CreER+}$ and $Camk2a^{flox/CreER-}$ mice, respectively). Error bars indicate SEM.

$Camk2a^{flox/Emx-cre+}$ mice, we investigated whether the expression of αCaMKII specifically in the telencephalon was required for contextual conditioning. Indeed, as predicted by the predominant models of synaptic plasticity and learning, $Camk2a^{flox/Emx-cre+}$ mice showed a significant impairment in contextual fear learning compared with their littermate controls ($t_{(36)} = 3.773$, $p = 0.001$; unpaired t test; Fig. 3C).

In addition to the region-specific requirements for αCaMKII, we also examined the temporal requirement during contextual fear learning. In particular, we sought to determine whether the conditioning deficit in the global $Camk2a^{-/-}$ mice arises from a developmental abnormality or if it is due to the absence of αCaMKII in adulthood at the time of learning. Therefore, we again used $Camk2a^{flox/CreER+}$ mice to induce αCaMKII deletion selectively in adulthood while sparing the potential impact of deletion during early development. Consistent with a requirement for αCaMKII expression at the time of learning, $Camk2a^{flox/CreER+}$ mice with temporally restricted adult deletion showed a severe impairment of contextual learning ($t_{(19)} = 4.950$, $p = 0.0001$, unpaired t test; Fig. 3D).

LTP is impaired in mice with adult deletion of αCaMKII
In addition to deficits in the Morris water maze and contextual fear conditioning, $Camk2a^{-/-}$ mice also show an impairment in LTP thought to underlie these hippocampal-dependent forms of learning (Silva et al., 1992b; Elgersma et al., 2002). Therefore, given the spatial and contextual learning deficits in $Camk2a^{flox/CreER+}$ mice, we also performed field recordings in acute hippocampal slices to determine whether adult-restricted deletion also impaired synaptic plasticity. Efficacy of synaptic transmission was unaffected in the absence of αCaMKII (Fig. 4A). Fiber volley amplitude was not significantly different in $Camk2a^{flox/CreER+}$ mice compared

with their control littermates (effect of genotype: $F_{(1,74)} = 2.37$, $p = 0.13$, repeated-measures ANOVA; Fig. 4A). Postsynaptic fEPSP slope was also independent of αCaMKII, which is consistent with previous studies using $Camk2a^{-/-}$ mice (effect of genotype: $F_{(1,108)} = 2.97$, $p = 0.09$, repeated-measures ANOVA; Fig. 4A). Moreover, PPF was also unchanged in $Camk2a^{flox/CreER+}$ mice (effect of genotype: $F_{(1,59)} = 0.26$, $p = 0.61$, repeated-measures ANOVA; Fig. 4B).

To determine whether the LTP impairment observed in $Camk2a^{-/-}$ mice is due to a critical role for αCaMKII during development and/or whether αCaMKII is necessary for the induction of LTP in adulthood, we used two independent protocols to induce LTP (100 Hz and theta-burst protocols) given their distinct cellular mechanisms yet shared impairment in $Camk2a^{-/-}$ mice. Both stimulation protocols induced robust LTP in control littermates ($Camk2a^{flox/CreER-}$; Fig. 4C,D). However, $Camk2a^{flox/CreER+}$ mice with adult-specific deletion of αCaMKII showed impaired LTP in both the 100 Hz (effect of genotype: $F_{(1,29)} = 16.88$, $p = 0.0003$, repeated-measures ANOVA; Fig. 4C) and theta-burst protocol (effect of genotype: $F_{(1,52)} = 7.85$, $p = 0.007$, repeated-measures ANOVA; Fig. 4D). Together, these findings confirm that αCaMKII is specifically required in adulthood for both learning and synaptic plasticity.

Discussion
In the present study, we used conditional $Camk2a$ mutant mice to determine the influence of spatially restricted and temporally controlled deletion of αCaMKII. The present series of experiments demonstrate that αCaMKII is required during adulthood in the telencephalon, but not the cerebellum, for both spatial and contextual learning and hippocampal LTP. Importantly, these results are entirely consistent with previous research showing that global germline deletion of αCaMKII leads to deficits in hippocampal-dependent learning and LTP (Silva et al., 1992a; Silva et al., 1992b; Elgersma et al., 2004). However, no previous experiments had ever been able to control for the possibility that germline deletion of αCaMKII might have led to abnormalities in early brain development, rather than its requirement in adulthood at the time of learning. Moreover, given evidence in the literature regarding the influence of cerebellar Purkinje cell plasticity in spatial and contextual learning (Lalonde and Strazielle, 2003; Burguière et al., 2005; Goddyn et al., 2006), we also examined region-specific $Camk2a$ deletion.

Previous studies using inducible systems (Mayford et al., 1996; Wang et al., 2003) investigated the necessity of αCaMKII during plasticity and learning using overexpression of a mutated temporally controlled and region-specific $Camk2a$ transgene. Although these studies show that enhanced levels of αCaMKII in the forebrain, independently of its activity status, can interfere with learning and memory consolidation, they do not prove the requirement of endogenous αCaMKII for plasticity and learning. Temporal overexpression of the αCaMKII-T286D transgene,

which is thought to have a dominant-negative effect due to additional phosphorylation on the TT305/6 sites (Pi et al., 2010), caused changes in the frequency threshold for LTP induction and disruption of several learning paradigms, which is reversed when turning off the transgene (Mayford et al., 1996). These experiments indicate a critical role for CaMKII signaling during learning. However, because αCaMKII forms holoenzymes with βCaMKII, these studies do not rule out that the phenotype is caused by deregulation of the entire holoenzyme (rather than just αCaMKII) due to the dominant-negative effect of the αCaMKII-T286D protein (Pi et al., 2010). Indeed, the dominant-negative mutation αCaMKII-T305D results in a much stronger deficit than $Camk2a^{-/-}$ mice (Elgersma et al., 2002).

Temporal overexpression of αCaMKII-F89G (of which the activity can be regulated by specific inhibitors) in adult mice showed that increased CaMKII expression during training and the next 4 weeks had no effects on long-term contextual and cued-fear memories as long as this level was not changed during consolidation or upon recall (Wang et al., 2003). Therefore, these studies indicated that a constant level of CaMKII during the phases of acquisition, consolidation, or recall was critical for normal learning, but did not address the requirement for endogenous αCaMKII for learning. In contrast, administering tatCN21 (a fusion peptide that inhibits stimulated and autonomous CaMKII activity) did not impair contextual fear memory expression when given 1 h before testing (Buard et al., 2010). Importantly, however, when tatCN21 was administered 1 h before training, acquisition was significantly impaired, confirming the necessity of CaMKII for intact learning and in full agreement with our findings.

Notably, we observed that mice with deletion restricted to the cerebellum demonstrated normal spatial and contextual learning, consistent with a recent study examining multiple lines of mice, each with distinct impairments in cerebellar transmission and plasticity, which found no evidence of cognitive impairments, including spatial and contextual learning (Galliano et al., 2013). Future studies should be aimed at further dissecting the regional and cell-type specific contributions of αCaMKII during learning, including the hippocampal subfields and amygdala nuclei. Moreover, it will also be important to explore the differential contributions of αCaMKII during the various phases of memory, including acquisition, consolidation, reconsolidation, maintenance, and retrieval.

We have confirmed that the well characterized learning deficits in $Camk2a^{-/-}$ mice are not merely due to absence of the protein during the developmental period. In the present study, mice with temporally restricted deletion of αCaMKII in adulthood showed equivalent spatial and contextual learning deficits as observed in the global $Camk2a^{-/-}$ mutants that lack αCaMKII expression throughout postnatal development. Therefore, we conclude that temporally controlled deletion of $Camk2a$ in adult mice is as detrimental as germline deletion for spatial and contextual learning.

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