Inducible protein knockout reveals temporal requirement of CaMKII reactivation for memory consolidation in the brain

Huimin Wang*†, Eiji Shimizu*†, Ya-Ping Tang*, Min Cho*, Maureen Kyin*, Wenqi Zuo*, Daphne A. Robinson‡, Peter J. Alaimo§, Chao Zhang§, Hiromi Morimoto¶, Min Zhuo‡, Ruiben Feng*‖, Kevan M. Shokat§, and Joe Z. Tsien*,**

*Department of Molecular Biology, Princeton University, Princeton, NJ 08544; ‡Department of Anesthesiology and Neurobiology, School of Medicine, Washington University, St. Louis, MO 63110; §Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143; ¶National Tritium Labeling Facility and Structural Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720; and ‖Shanghai Institute of Brain Functional Genomics, East China Normal University, Shanghai 200062, China

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By integrating convergent protein engineering and rational inhibitor design, we have developed an *in vivo* conditional protein knockout and/or manipulation technology. This method is based on the creation of a specific interaction interface between a modified protein domain and sensitized inhibitors. By introducing this system into genetically modified mice, we can readily manipulate the activity of a targeted protein, such as α-Ca^{2+}/calmodulin-dependent protein kinase II (αCAMKII), on the time scale of minutes in specific brain subregions of freely behaving mice. With this inducible and region-specific protein knockout technique, we analyzed the temporal stages of memory consolidation process and revealed the first postlearning week as the critical time window during which a precise level of CaMKII reactivation is essential for the consolidation of long-term memories in the brain.

Current-inducible and region-specific gene knockout techniques are powerful for molecular and temporal analysis of biological processes (1, 2). However, because the inactivation event occurs at the DNA level, manifestation of any phenotype depends on the turnover rate of the existing protein, which takes days or weeks. This inherently slow process has excluded precise investigation of many *in vivo* biological processes that occur within minutes and hours. Therefore, it is highly desirable to develop new types of techniques that can direct the knockout event at the protein level, rather than at the DNA level, for achieving almost instantaneous effects. Furthermore, the molecular specificity of such a knockout should surpass the conventional pharmacological inhibitors. We decided to explore methods to integrate the molecular and regional specificity of genetics with the high temporal resolution of chemical inhibition for the development of an inducible, reversible, and region-specific protein knockout technique.

Such a technique would be valuable for elucidation of molecular mechanisms underlying various temporal stages of brain function such as memory processes. The *N*-methyl-D-aspartate (NMDA) receptor has been established as a crucial molecular switch for synaptic plasticity (3, 4) and for memory formation (1, 5–8). At the molecular level, long-term memory was widely assumed to be stored in the form of synaptic structural changes resulting from a single molecular cascade triggered by learning. However, this "single cascade hypothesis" has its conceptual difficulties in accounting for long-term memory formation in the brain. For example, the time scale of a single molecular cascade (typically between hours to days) is too short for describing the hippocampus-mediated consolidation process that is known to occur over a timescale of week(s) in rodents (9–12) and years in humans (13–15). Moreover, synaptic structures in the adult brain are dynamic, and synaptic proteins such as the NMDA receptor are known to be degraded within 5 days in the brain of freely behaving animals (1). Thus, it raises fundamental concerns whether any structural changes resulting from a single molecular cascade can actually sustain such dynamical turnovers and still maintain the synaptic fidelity.

An alternative hypothesis, known as "synaptic reentry reinforcement" (SRR) has recently been proposed to account for long-term memory formation in the brain (1, 16). Inducible gene knockout experiments show that the formation of long-lasting memories is severely disrupted if the NMDA receptor in the CA1 region of the hippocampus is selectively and inducibly knocked out during the initial posttraining week(s) (1). This finding suggests that memory consolidation requires multiple rounds of postlearning synaptic modifications to reinforce synaptic changes initiated during memory acquisition (16, 17). More recently, computational analysis shows that SRR is capable of counteracting the turnover of synaptic receptors and making memory traces stronger and more stable, thus leading to memory consolidation (16, 17). It further shows that SRR can serve as a crucial mechanism for transferring the newly created memories from the hippocampus to the cortex for permanent storage (16, 17).

α-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) is a major downstream molecule in the NMDA receptor signaling process (18–23) and crucial for learning behaviors (24–27). However, fundamental questions still remain regarding whether and when CaMKII participates in memory consolidation process. In other words, is there a critical time window for CaMKII reactivation and how sensitive is memory consolidation to the numerical alterations in CaMKII reactivation level?

To investigate these questions, we used the principles of convergent protein engineering and synthetic small chemical molecule pairs for generating a highly specific inhibition interface to develop a conditional protein knockout/manipulation technique in mice. We show that, through rational designs and convergent engineering of protein/chemical pairs, this genetic technique can overcome the shortcomings of current genetic techniques (e.g., lack of precise temporal control of protein activity) and pharmacological methods (e.g., lack of the true molecular and regional specificity). Here, we describe the development of this genetic technique and its application for the elucidation of the critical time window during which the precise reactivation of αCaMKII is essential for the consolidation of long-term memories.

Materials and Methods

Expression Vectors for Mouse αCaMKII, Kinase Purification, and Kinase Assay. Site-directed mutagenesis was performed to make mutated αCaMKII-F89G, αCaMKII-F89A, and αCaMKII-V73A/F89A ki-

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Abbreviations: αCaMKII, α-Ca^{2+}/calmodulin-dependent protein kinase II; PP1, 4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine; NMDA, *N*-methyl-D-aspartate; SRR, synaptic reentry reinforcement; NM, naphthylmethyl; LTP, long-term potentiation; LTD, long-term depression; Tg, transgenic.

†H.W. and E.S. contributed equally to this work.

**To whom correspondence should be addressed. E-mail: jtsien@princeton.edu.

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nases (QuikChange site-directed mutagenesis kit, Stratagene). The PCR products, which contain αCaMKII-ORF, were digested with *Bam*HI and *Eco*RI and ligated into similarly digested pGEX-4T-1. Protein overexpression was carried out in BL21 *Escherichia coli* cells, and the cells were stored at 4°C overnight before centrifugation and lysis by French Press. Assays of recombinant αCaMKII phosphorylation of the specific peptide substrate AutoCamtide II were carried out in triplicate at 30°C by using CaMKII assay kit (Upstate Biotechnology, Lake Placid, NY). For detailed information, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Chemical Synthesis and Tritiation of 1-Naphthylmethyl (NM)-PP1. PP1 (4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine) and C3-devatized PP1 analogs were synthesized and purified by column chromatography (silica gel), followed by HPLC (two times) to >95% purity as described (28). Experimental procedures for the tritiation experiment were optimized by using nonlabeled materials, after modified literature procedures (29). The specific activity of the sample was determined by comparison of the UV absorbance of an unlabeled authentic sample with the liquid scintillation count of the isolated HPLC peak effluent. NM-PP1 was purified by column chromatography (silica gel), followed by HPLC (2×) as described (30). For detailed information about the tritiation of NM-PP1 and PP1 analogs that were tested, see *Supporting Materials and Methods*.

Production and Genotyping of αCaMKII-F89G Transgenic (Tg) Mice. The Tg founders were produced by pronuclear injection of the linearized DNA into B6/CBAF1 zygotes, and then intercrossed with B6/CBAF1 for all our analyses (5). For detailed information about the CaMKII F89G transgene expression vector and about genotyping, see *Supporting Materials and Methods*.

Hippocampal Homogenates CaMKII Activity Assay. Homogenates from individual hippocampus regions were assayed for CaMKII activity essentially as described (26). Briefly, tissue was homogenized, and the protein concentrations were assayed by bicinchoninic acid (BCA) protein assay (Pierce), with BSA as standard. Twenty micromolar Autocamtide-2 (CaMKII substrate; Calbiochem) was used as kinase substrate with either 1 mM $CaCl_2$ and calmodulin (5 μg/ml) (Ca^{2+}-dependent activity) or 2 mM EGTA (Ca^{2+}-independent activity). Enzyme reactions were carried out at 30°C for 1 min in a final volume of 50 μl. The reaction was initiated by the addition of 0.5–2.0 μg of hippocampal homogenates.

Northern Blot Analysis, *in Situ* Hybridization, Hippocampal Slice Recording, and Behavioral Tests. The experimental protocol was the same as described (5, 8); for detailed information, see *Supporting Materials and Methods*. All animals used in these experiments were 3–4 mo old.

Results

Engineering of αCaMKII Containing a Silent Mutation That Creates the Artificial Cavity in the ATP-Binding Pocket of the Catalytic Domain. αCaMKII, like many other kinases (31), has significant homology to its isoforms, such as β-, γ-, and δCaMKII, CaMKI, CaMKIII, CaMKIV, and CaMK kinase superfamily (19). Because of the high homology within and between the protein kinase superfamilies, traditional pharmacological inhibitors do not offer the specificity required for dissecting the role of kinases in learning behaviors. This lack of specificity is further compounded by the fact that there are almost 600 protein kinases encoded in the human genome, representing ≈2% of all gene products (32). Moreover, x-ray crystallographic studies of many kinases have revealed a well-conserved structure in the ATP binding domain (31), a structural feature hindering the development of truly specific inhibitors.

In an effort to develop a novel genetic technique (Fig. 1*a*), we turned this structural constraint into our advantage by creating a highly specific inhibitor-protein interface using chemical-genetics with high specificity. We previously showed that such a strategy works *in vitro* for v-Src, and several other kinases (33–35). To generate a "chemical-sensitive" mutant αCaMKII that can be uniquely inhibited by a rationally designed small-molecule inhibitor, we aligned αCaMKII with v-Src (also because of no crystallographic information on the catalytic domain of αCaMKII; Fig. 1*b*). We hypothesized that, in the catalytic ATP-binding site of αCaMKII, the sidechain of Phe-89 (F89) is close to the N^6 amino group of bound ATP. However, point mutation of the Phe-89 to a small side-chain amino acid, such as glycine (Gly or G) or alanine (Ala or A), should create a unique pocket in the ATP-binding site that does not occur in other CaM kinases or any other kinase in the genome. Finally, an inhibitor that is highly selective for the mutated CaMKII can then be synthesized by derivatizing a nonspecific kinase inhibitor with a bulky group that can complement the point mutation made in CaMKII. Because the rationally designed bulky inhibitor is too large to fit inside the WT ATP-binding pocket of any endogenous kinase, its specificity should be extremely high.

Accordingly, we replaced the Phe-89 with Gly or Ala in αCaMKII, thus generating the mutant αCaMKII-F89G and αCaMKII-F89A, respectively. In addition, we have also generated the double amino acid mutant, αCaMKII-V73A/F89A, because modeling studies suggested that V73 residue might also be in close

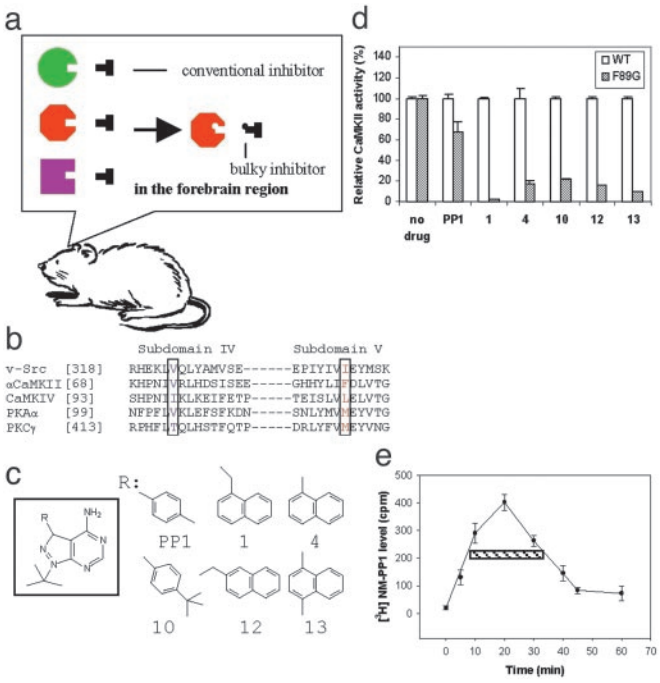

Fig. 1. (*a*) The bump-and-hole-based chemical genetic strategy for controlling genetically sensitized protein activity in freely behaving mice. The ATP-binding pocket of a protein kinase (e.g., αCaMKII) is enlarged (via silent mutation) such that a bulky inhibitor is rationally designed, synthesized, and identified to fit only the enlarged pocket but not the unmodified pocket. One can then use the bulky inhibitor to specifically and rapidly control the kinase activity at a time scale of minutes. (*b*) Partial sequence alignment of protein kinases. Two colored amino acids are the ones in close contact with N^6 amino group of bound ATP. (*c*) Chemical structures of several C^3 derivatives of PP1. Compound 1 is NM-PP1. (*d*) Specificity of PP1 analogs toward WT and αCaMKII-F89G enzymes. PP1 analogs (0.5 μM) were used. The CaMKII activities were expressed as percentages of the uninhibited WT αCaMKII (no drug). (*e*) Pharmacokinetic characterization of NM-PP1. [^{3}H]NM-PP1 was administered to mice i.p. The brain NM-PP1 level was determined from the radioactive counts of the brain homogenate. The shaded bar indicates the duration of complete inhibition (from 8 to 35 min) of αCaMKII-F89G activity by a single injection at dose of 16.57 ng per gram of body weight per mouse. Four mice per time point were used. All values are mean ± SEM.

contact with the N^6 amino group of bound ATP. Our hope was that some of these mutated αCaMKII enzymes would still accept ATP normally, but would also accept our bulky inhibitors.

We produced these recombinant αCaMKII/GST fusion proteins from *E. coli* and performed *in vitro* kinase assays using a specific αCaMKII substrate peptide. We found that the double mutant, αCaMKII-V73A/F89A, had little kinase activity (data not shown), whereas both αCaMKII-F89G and αCaMKII-F89A single mutants exhibited the kinase activities and ATP-binding affinities comparable to those of the WT αCaMKII enzyme. The specificity constant (k_{cat}/K_m) for αCaMKII-F89G is 3.8×10^4 $min^{-1} \cdot M^{-1}$, which is comparable to that of the WT αCaMKII enzyme (5.1×10^4 $min^{-1} \cdot M^{-1}$).

Screening for the Most Potent Inhibitor Targeting the Enlarged ATP Pocket of αCaMKII-F89G. We hypothesized that a mono-specific inhibitor for the mutated αCaMKII could be synthesized by derivatizing a nonspecific kinase inhibitor with a bulky group. We selected 4-amino-1-*tert*-butyl-3-phenylpyrazolo[3,4-*d*]pyramidine, a derivative of 4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine (PP1; ref. 28), as a core scaffold for making various inhibitor analogs to identify the most potent inhibitor against the αCaMKII-F89G. Among them, five of these PP1 analogs (for chemical structures, see Fig. 1*c*) were highly effective at 0.5 μM concentration (Fig. 1*d*). We have identified 1-naphthylmethyl-PP1 (NM-PP1, no. 1 in Fig. 1*c*) as the most specific inhibitor for αCaMKII-F89G. This compound provides potent inhibition at nanomolar concentration, with an IC_{50} against αCaMKII-F89G at 32 nM. Furthermore, our series of biochemical studies showed that this compound was ineffective against recombinant WT kinases such as αCaMKII ($IC_{50} > 800$ μM), CaMKIV ($IC_{50} > 800$ μM), PKCγ ($IC_{50} > 800$ μM), and CDK5 ($IC_{50} > 300$ μM).

To determine pharmacokinectics and the distribution of NM-PP1 in the brain, we used an iridium-containing catalyst to perform a hydrogen-tritium exchange reaction on NM-PP1 (29, 30) to synthesize ^{3}H-labeled NM-PP1. We have found that ^{3}H-labeled NM-PP1, via acute i.p. injection, can enter the brain quickly within 3–5 min. It reached peak levels in the brain at 20 min and decreased to basal level in 45 min (Fig. 1*e*).

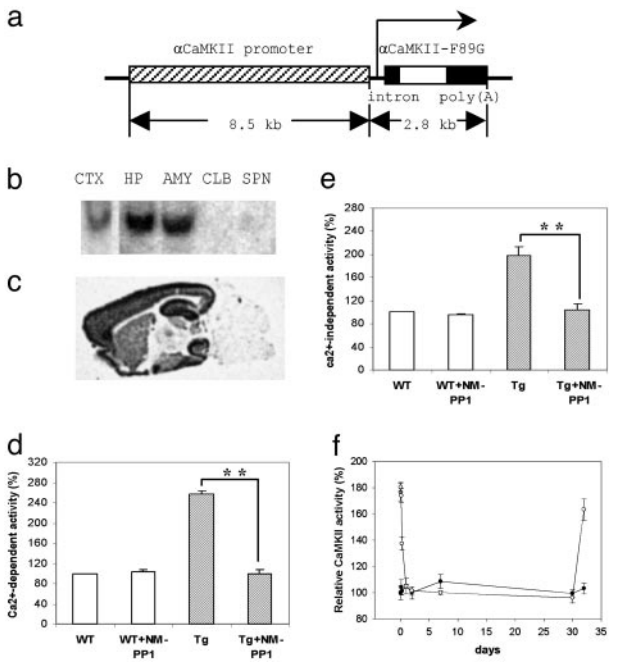

Fig. 2. Production and characterization of αCaMKII-F89G Tg mice. (*a*) The construct used to generate Tg mice overexpressing silently mutated αCaMKII-F89G in the forebrain. (*b*) Regional expression of αCaMKII-F89G transgene mRNA by Northern blot analysis. CTX, cortex; HP, hippocampus; AMY, amygdala; CLB, cerebellum; SPN, spinal cord. (*c*) Forebrain expression of αCaMKII-F89G transgene shown by *in situ* hybridization. (*d*) Ca^{2+}-dependent αCaMKII activities in WT and Tg mice. (*e*) Ca^{2+}-independent αCaMKII activities in WT and Tg mice. Enzyme activity was determined with hippocampus homogenates (0.5 μg) from adult mice and expressed as percentages of the WT control. All values are mean ± SEM. (*f*) Inhibition and reversal of αCaMKII-F89G activity *in vivo* by the oral treatment of NM-PP1 (5 μM in drinking water). NM-PP1 was administered to both αCaMKII-F89G Tg and WT mice via drinking water from day 1 to day 30. Enzyme activity was determined at day 0, 1 h, 3 h, 6 h, and days 1, 2, 7, 10, 30, and 32 ($n = 4$ mice per time point). All values are mean ± SEM.

Generation of Tg Mice Overexpressing αCaMKII-F89G in the Forebrain. To test the feasibility of our strategy *in vivo*, we produced Tg mice overexpressing αCaMKII-F89G in the forebrain regions using the αCaMKII promoter-driven construct (Fig. 2*a*). We produced a total of five independent CaMKII-F89G Tg founders using the pronuclear injection method. We performed Northern blot analysis and measured the expression of the αCaMKII-F89G transgene in these mice. We found that all five Tg lines expressed the expected 2.5-kb transgene mRNA in the forebrain (cortex, hippocampus, and amygdala) but not in the hindbrain or spinal cord. The αCaMKII-F89G mRNA in Tg-1 line was shown by Northern blot (Fig. 2*b*) and *in situ* hybridization (Fig. 2*c*). Because the line Tg-1 has highest transgene mRNA expression, it is used for detailed analysis in this study.

Biochemical Characterization of the Bulky Inhibitor, NM-PP1, in the Tg Mice. We measured CaMKII activity in hippocampal homogenates from Tg and control littermates. In comparison with WT littermates, the Tg mice showed a significant elevation in both Ca^{2+}-dependent and Ca^{2+}-independent CaMKII kinase activity. We observed a 2.6-fold increase of Ca^{2+}-dependent CaMKII activity in the Tg mice over the WT activity (Fig. 2*d*). Likewise, the Ca^{2+}-independent activity is nearly 2-fold higher in the Tg mice over WT controls (Fig. 2*e*). However, no changes on the levels of either βCaMKII or CaMK-IV were observed by Western blot analysis (data not shown). As expected, addition of 0.5 μM NM-PP1 to the reaction mixtures reduced both Ca^{2+}-dependent and -independent CaMKII activities back to the WT level in the Tg mice, whereas the same concentration of NM-PP1 had no effects on the total CaMKII activity of the WT littermates (Fig. 2 *d* and *e*). We further determined the IC_{50} of NM-PP1 against αCaMKII-F89G activity in Tg mice, and found it to be at 30–40 nM, identical to the *in vitro* IC_{50} curve obtained with the recombinant αCaMKII-F89G.

A single i.p. injection of NM-PP1 (16.6 ng per gram of mouse body weight) into freely behaving Tg mice completely suppressed αCaMKII-F89G activity and consistently maintained total CaMKII activity at the WT level for 8–35 min after injection (Fig. 1*d*, shaded bar). This time course fits nicely with the measured pharmacokinetics of ^{3}H-labeled NM-PP1.

We also measured *in vivo* inhibition of αCaMKII-F89G activity via noninvasive chronic oral intake (5 μM NM-PP1 in drinking water). For animals without water deprivation, this natural feeding method can result in partial inhibition of αCaMKII-F89G activity in the Tg mice by 6 h (no inhibition for the initial 3 h) and complete inhibition by 24 h after the NM-PP1-containing bottle was made available (Fig. 2*f*). The complete inhibition could be maintained as long as the NM-PP1-containing water was provided (1 mo measurement is shown in Fig. 2*f*). No observable side effects were detected. Moreover, withdrawal of NM-PP1 after 1-mo oral treatment can readily restore the higher αCaMKII activity in the Tg mice back to pretreatment level within 2 days (Fig. 2*f*). These data were used to guide our subsequent behavioral experiments.

Synaptic Electrophysiological Characterization of Tg Mice. We conducted a series of electrophysiological analyses of αCaMKII-F89G on line Tg-1. We found that the hippocampal slices from Tg mice

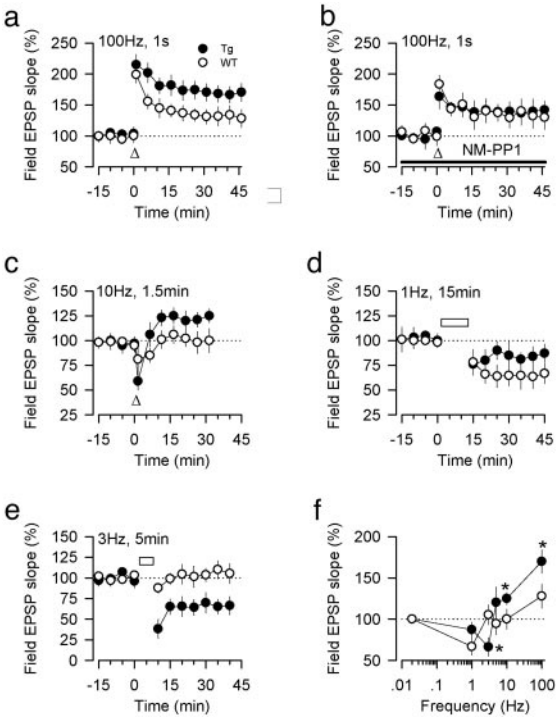

Fig. 3. CaMKII overexpression alters frequency–plasticity responses in the hippocampal Schaffer-collateral pathway. (*a*) LTP induced by a single tetanus (100 Hz, 1 s) in Tg slices (170 ± 14.3%; $n = 10$ slices/7 mice) were significantly larger than that of WT slices (128 ± 14.7%; $n = 10$ slices/7 mice). (*b*) NM-PP1 (0.5 μM) reduced LTP in the Tg slices to normal level (Tg, 11 slices/8 mice; WT, 11 slices/8 mice). (*c*) Larger LTP was also produced by 10 Hz (1.5 min) stimulation in Tg slices (125 ± 7.1%; $n = 8$ slices/8 mice) in comparison with WT (100 ± 12.2%; $n = 7$ slices/7 mice). (*d*) No statistical difference in 1-Hz-induced LTD was produced between Tg and WT mice. We noted that LTD in Tg slices seemed to show a smaller trend, but it did not reach a statistically significant level. (*e*) Pronounced LTD was produced in Tg slices by 3-Hz (5-min) stimulation. Representative traces before and after 3-Hz stimulation from Tg slices are shown. Tg/artificial cerebrospinal fluid (ACSF), $n = 14$ slices/9 mice; WT/ACSF, $n = 7$ slices/5 mice. (*f*) A plot for the summarized bidirectional plasticity–frequency response curves between the 1- to 100-Hz frequency ranges in Tg and WT slices. Note that the LTD peak is moved to 3 Hz in Tg mice. All values are mean ± SEM. Statistical differences were evaluated by using one-way ANOVA and two-tailed *t* test (*, $P < 0.05$).

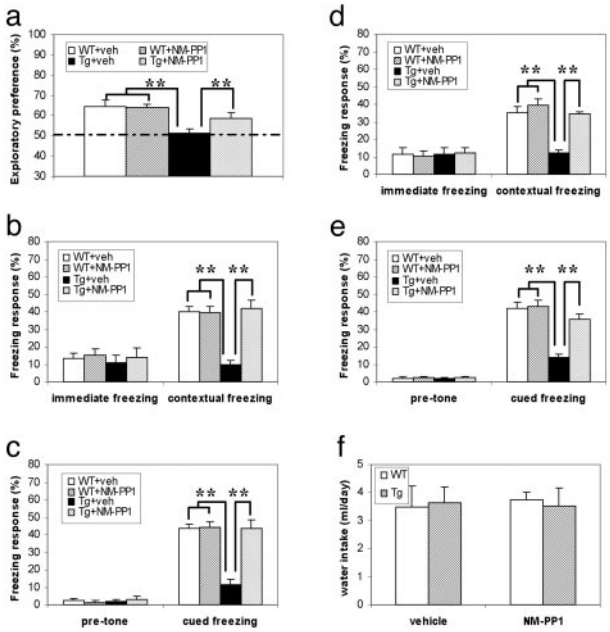

Fig. 4. NM-PP1 readily reversed performance deficits of αCaMKII-F89G Tg mice in several memory tests. (*a*) Reversible deficits in novel object recognition test in Tg mice. Significant deficits were observed in Tg mice with vehicle ($n = 12$), compared with WT mice with vehicle ($n = 10$), with NM-PP1 ($n = 11$), and Tg mice with NM-PP1 ($n = 12$; $F_{3,41} = 12.8$, $P < 0.01$). (*b* and *c*) NM-PP1 treatment reversed contextual (*b*) and cued (*c*) conditioning memory deficits (1-day retention) in Tg mice. Significant deficits in contextual ($F_{3,60} = 13.86$, $P < 0.001$) and cued ($F_{3,60} = 14.35$, $P < 0.001$) freezing were observed in Tg mice treated with vehicle ($n = 13$), compared with WT mice with vehicle ($n = 17$) or with NM-PP1 ($n = 21$) and Tg mice with NM-PP1 ($n = 13$). (*d* and *e*) Performance deficits in contextual and cued conditioning memory deficits (1-mo retention) in Tg mice can be reversed by NM-PP1. Significant deficits in contextual ($F_{3,57} = 10.55$, $P < 0.001$) and cued ($F_{3,57} = 9.57$, $P < 0.01$) freezing were observed in Tg mice treated with vehicle ($n = 12$), but not in the WT mice with vehicle ($n = 18$), WT mice with NM-PP1 ($n = 19$), and Tg mice with NM-PP1 ($n = 12$). All values are mean ± SEM. **, $P < 0.01$, post hoc analysis between Tg and WT mice. (*f*) Daily intake of either vehicle or NM-PP1 containing water showed no significant difference between WT ($n = 2$ 0) and Tg ($n = 24$) mice. All values are mean ± SD.

exhibited normal input/output function, indistinguishable AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor-mediated field excitatory postsynaptic potentials, and normal paired-pulse facilitation (see Fig. 7, which is published as supporting information on the PNAS web site), suggesting that both presynaptic function and postsynaptic AMPA receptors are normal in Tg animals.

We then investigated bidirectional modifications of long-lasting synaptic changes, evoked by various frequency stimulations (1–100 Hz). First, we applied a single tetanic stimulation (100 Hz, 1 s) and found that it evoked larger long-term potentiation (LTP) in Tg slices in comparison with that in WT slices (Fig. 3*a*). 100 Hz-induced LTP could be reversed to normal levels in Tg slices by 0.5 μM NM-PP1 (Fig. 3*b*). Moreover, a modestly larger LTP in response to 10-Hz stimulation for 1.5 min (900 pulses) in the Tg slices was further observed in comparison with that of control slices (Fig. 3*c*). However, 5-Hz stimulation for 3 min (900 pulses) did not produce synaptic potentiation in both groups of mice (Tg, $n = 6$ slices per six mice; mean 120.5 ± 18.8%; WT, $n = 8$ slices per eight mice, mean 94.6 ± 13.3%; data not shown).

We then measured long-term depression (LTD) in CA3-CA1 synapses. We found that a 1-Hz stimulation produced statistically similar LTD in control animals and Tg mice (Fig. 3*d*). Surprisingly, we found that 3-Hz, 5-min stimulation evoked significant LTD in slices of Tg mice but not in WT mice (Fig. 3*e*). In addition, 3-Hz-induced LTD could be completely prevented by 0.5 μM NM-PP1 (data not shown). Thus, our experiments revealed that the expression of CaMKII-F89G in Tg mice altered the bidirectional plasticity response curve, most strikingly with the shifted peak of LTD response to 3 Hz (Fig. 3*f*).

Reversal of Learning and Memory Deficits by NM-PP1 Treatment in Tg Mice. Visual inspection of CaMKII-F89G Tg mice did not reveal any gross abnormalities in their body weight, feeding, mating, or open-field activity. To test whether Tg mice have altered learning and memory, we conducted learning tasks relevant to the forebrain regions. All behavioral experiments were performed on the littermate WT and Tg mice and coconducted by two or three experimenters blind to the genotype of each mouse.

First, we evaluated visual recognition memory in αCaMKII-F89G Tg mice using the novel object recognition paradigm and found that these Tg mice were impaired on the 1-day retention test (Fig. 4*a*). The memory deficit of the Tg mice could be reversed completely by the NM-PP1 treatment (5 μM) in the drinking water starting from 2 days before training (Fig. 4*a*).

We then assessed two forms of associative memory in these mice: contextual- and cued-fear conditioning. The former is hippocampal-dependent, whereas the latter is amygdala-dependent (36–38).

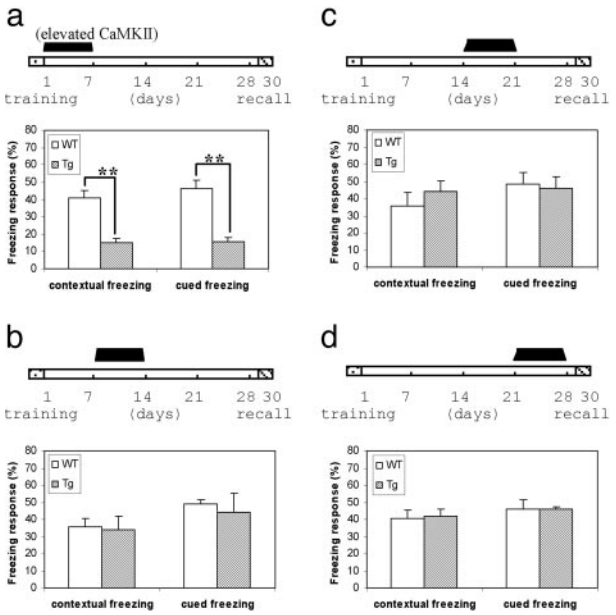

Fig. 5. Effects of inducible manipulation of elevated CaMKII levels between the first to fourth week on the formation of 1-mo long-term contextual- and cued-fear memories. (*a*) Significant deficits were observed in contextual ($F_{1,21} = 17.6$, $P < 0.01$) and cued ($F_{1,16} = 30.0$, $P < 0.001$) freezing in Tg mice ($n = 11$) in which CaMKII-F89G activity was made available only in the first posttraining week (days 1–7) compared with WT mice ($n = 12$). CaMKII activity measurement showed that NM-PP1 withdrawal allowed the partial expression of the CaMKII-F89G activity at 6 h and full expression by 12 h on day 1. (*b–d*) No effects were observed on contextual and cued freezing when elevated CaMKII were temporally introduced during days 8–14 (*b*; Tg, $n = 9$; WT, $n = 9$; $P > 0.05$); during days 15–21 (*c*; Tg, $n = 10$; WT, $n = 10$; $P > 0.05$), or during day 22–28 (*d*; Tg, $n = 9$; WT, $n = 9$; $P > 0.05$). All values are mean ± SEM. **, $P < 0.01$, post hoc analysis between Tg and WT mice. The black bar above the time line indicates the duration for the presence of elevated total CaMKII activity (when αCaMKII-F89G activity in Tg mice was not inhibited by NM-PP1).

Both types of fear conditioning require the activation of NMDA receptors. We have found that CaMKII-F89G Tg mice were also impaired in both contextual- and cued-fear paradigms, as indicated by fewer freezing responses during the 1-day retention test (Fig. 4 *b* and *c*) and 1-mo retention tests (Fig. 4 *d* and *e*), whereas Tg mice with NM-PP1 treatment exhibited normal contextual- and cued-fear freezing responses in either 1-day retention (Fig. 4 *b* and *c*) or 1-mo retention (Fig. 4 *d* and *e*) tests. Therefore, these results suggest that overexpression of αCaMK-F89G in the forebrain of Tg mice is sufficient to produce memory phenotypes, and those changes can be readily reversed by NM-PP1.

We excluded the possibility that the rescuing effects by NM-PP1 were due to "memory enhancing" effects in those tests by measuring the performance of WT mice. We found that NM-PP1 had no effect in all three tests (Fig. 4 *a–e*). Furthermore, we excluded the difference in drinking NM-PP1-containing water between WT and Tg mice as a potential factor because they drank the same amount of water (vehicle) or drug-containing water (Fig. 4*f*).

Temporally Restricted Alteration of αCaMKII Level During the First, but Not Second, Third, or Fourth, Posttraining Week Disrupts Long-Term Memory Consolidation. Recent studies suggest that the NMDA receptor is crucial for postlearning consolidation of long-term memories (1, 39). We decided to use our technique to further examine the role of CaMKII in memory consolidation. More specifically, we set to examine two major questions. Is there a critical time window for CaMKII reactivation during memory consolidation? How sensitive is memory consolidation to numerical changes in the level of CaMKII activity?

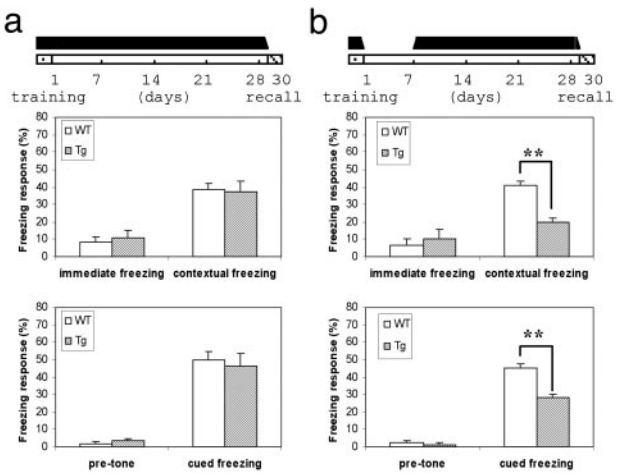

Fig. 6. (*a*) Tg mice with elevated CaMKII activity during training and consolidation exhibited normal contextual- and cued-fear memories (Tg, $n = 8$) in comparison with WT ($n = 9$; $P > 0.05$). NM-PP1 was injected 15 min before retrieval i.p. so retrieval occurred with normal CaMKII levels in Tg mice. (*b*) Temporally controlled switching of CaMKII activity from the elevated level to the normal level during the first postlearning week also disrupted long-term memory consolidation in the Tg mice. Mice received NM-PP1 during days 1–7 by oral treatment and i.p. 15 min before retrieval. Significant deficits in contextual ($F_{1,16} = 33.5$, $P < 0.01$) and cued ($F_{1,16} = 35.5$, $P < 0.01$) freezing were observed in αCaMKII-F89G Tg mice ($n = 8$) compared with WT ($n = 9$). All values are mean ± SEM. **, $P < 0.01$, post hoc analysis between Tg and WT mice. The black bar above the time line indicates the duration for the presence of elevated total CaMKII activity.

To address these issues, we pretreated both Tg and their littermate control mice with NM-PP1 2 days before training and then subjected these animals to fear conditioning so that memory acquisition occurred with the normal amount of CaMKII in the brain of Tg mice. By varying the time of NM-PP1 withdrawal from the posttraining week 1 to week 4, we determined the effect of alteration of total CaMKII activity on the consolidation of 1-mo contextual and cued long-term memory. We found that temporally controlled expression of αCaMKII-F89G (by removing NM-PP1 from drinking water) during the first postlearning week resulted in significant impairment in the formation of both 1-mo contextual- and cued-fear memory in Tg mice (Fig. 5*a*). In contrast, the temporal expression of αCaMKII-F89G in the second (Fig. 5*b*), third (Fig. 5*c*), or fourth (Fig. 5*d*) postlearning week had no effects on the formation of both long-term contextual- and cued-fear memories.

In addition, the constitutive presence of αCaMKII-F89G during training and the following 4 wk (but not during retrieval) also had no effects on the formation of both long-term contextual- and cued-fear memories (Fig. 6*a*), suggesting that learning and consolidation could occur normally in the brain with elevated CaMKII levels. To further assess the sensitivity of changing CaMKII in the first posttraining week on memory consolidation, we allowed a group of Tg mice to acquire fear memories with elevated CaMKII and then to consolidate with a normal amount of CaMKII during the first week before switching back to the elevated level in the second to fourth posttraining week. Interestingly, such a manipulation also disrupted the formation of 1-mo long-term memories (Fig. 6*b*). Therefore, our above results show that long-term memory formation indeed requires the reactivation CaMKII, and more importantly, there is a stringent requirement during the first postlearning week for a precise CaMKII reactivation level that needs to be closely matched to the level used during initial learning.

Discussion

We described the conditional protein knockout/manipulation technique that is based on the concept of convergent engineering

of both protein and its unique chemical inhibitor to achieve monospecific inhibition of a single kinase in one region of the mouse brain. This method combines high temporal resolution (within 3–8 min) of chemical inhibition with molecular and spatial specificity that genetic techniques can offer. The sensitized inhibitor exhibits unprecedented molecular specificity that does not inhibit unmodified CaMKII and other kinases; thus, it can be used for both chronic and acute knockout experiments.

Our technique can be easily modified for expanded applications. For example, spatial-specificity of protein knockout can be achieved through application of a subregion-specific Cre/loxP system (6–8). In addition, our technique can also be used to produce a knockin mouse in which one can use the bulky inhibitor to block the function of the sensitized kinase. Finally, the prevalence of structural modules in proteins indicates broad applications of our strategy to other classes of protein (e.g., receptors, proteases, acetylases, etc.; refs. 40–42).

With this technique, we set out to dissect and manipulate molecular and temporal processes during memory consolidation. Previous genetic studies suggest the importance of CaMKII for normal learning behaviors (24, 26, 27). Unfortunately, because those techniques either lacked temporal regulation or offered only limited temporal resolution (e.g., slow induction that takes ≈6 wk using the tTA system in ref. 27), the role of CaMKII in distinct stages of memory process was not analyzed. In addition, it was argued that the behavioral deficits in those genetically modified mice could be due to either developmental alteration or to disruption of other signal molecules independent of CaMKII activity, such as trapping calmodulin.

To examine these crucial issues, we directly manipulated the αCaMKII activity level and probed how changes of CaMKII activity affected synaptic plasticity and long-term memory formation. Our results show that higher amounts of αCaMKII can lead to larger LTP (by 100-Hz tetanus) as well as to pronounced LTD (by 3-Hz stimulation), suggesting that the level of αCaMKII plays broader roles in determining the degrees and directions of synaptic plasticity. Interestingly, we found that Tg mice with the elevated αCaMKII in the forebrain could acquire and consolidate memories indistinguishable with that of WT mice (Fig. 6*a*), thereby showing a certain degree of tolerance within the brain in using different plasticity-frequency curves for encoding the same memories. This finding is consistent with the emerging view that the simple correlation between LTP level and learning behaviors could be often misleading (43, 44). Were a similar shift in the plasticity-frequency curve to also be produced in other forebrain regions (such as the amygdala or cortex) of the Tg mice by excessive αCaMKII, altered plasticity frequency responses by overexpression CaMKII-F89G could form a physiological basis for the observed behavioral phenotypes in these mice.

Taking advantage of our Tg mice in which two distinct bidirectional plasticity-frequency curves or states can readily switch back-and-forth via NM-PP1, we studied the effects of changing plasticity states on the formation of 1-mo-old long-term memories in the brain. Our results show that switching back-and-forth between two curves (or plasticity states) in the first postlearning week severely disrupted synaptic consolidation of long-term memories (Figs. 5*a* and 6*b*). Lack of any behavioral effects during the second, third, and fourth postlearning week demonstrates the specific time window for such a molecular interference. Finally, temporal disruptions of both contextual (hippocampal–dependent) and cued (hippocampal-independent) memories by shifting CaMKII reactivation levels in the first postlearning week not only are consistent with the notion that long-term memory formation requires multiple rounds of SRR (1, 16, 17), but also further suggest that the SRR process may represent a general mechanism for the consolidation of many types of memories.

In conclusion, we developed an inducible, reversible, and region-specific protein knockout technique by using convergent protein engineering and rational design and synthesis of a genetically sensitized chemical inhibitor. By using this technique, we have specifically, reversibly, and rapidly manipulated the properties of CaMKII-mediated synaptic plasticity in the forebrain during distinct stages of memory processes. Our electrophysiological and behavioral analyses reveal the critical temporal window during which precise CaMKII reactivation is crucial for the formation of long-term memory in the brain.

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