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Inducible and Reversible Enhancement of Learning, Memory, and Long-Term Potentiation by Genetic Inhibition of Calcineurin

Gaël Malleret,[1,2,7] Ursula Haditsch,[3,7] David Genoux,[3] Matthew W. Jones,[4,8] Tim V. P. Bliss,[4] Amanda M. Vanhoose,[5] Carl Weitlauf,[5] Eric R. Kandel,[1] Danny G. Winder,[5] and Isabelle M. Mansuy[3,6]

[1] Howard Hughes Medical Institute
Center for Neurobiology and Behavior
Columbia University
722 West 168th Street
New York, New York 10032

[2] Laboratoire de Neurosciences Cognitives
CNRS Unité Mixte de Recherche 5807
Université Bordeaux I
Avenue des facultés
33405 Talence Cedex
France

[3] Institute of Cell Biology
Swiss Federal Institute of Technology
ETH Hönggerberg
CH-8093 Zürich
Switzerland

[4] Division of Neurophysiology
National Institute for Medical Research
Mill Hill
London NW7 1AA
United Kingdom

[5] Department of Molecular Physiology and Biophysics and Center for Molecular Neuroscience
Vanderbilt University School of Medicine
Nashville, Tennessee 37232

Summary

The threshold for hippocampal-dependent synaptic plasticity and memory storage is thought to be determined by the balance between protein phosphorylation and dephosphorylation mediated by the kinase PKA and the phosphatase calcineurin. To establish whether endogenous calcineurin acts as an inhibitory constraint in this balance, we examined the effect of genetically inhibiting calcineurin on plasticity and memory. Using the doxycycline-dependent rtTA system to express a calcineurin inhibitor reversibly in the mouse brain, we find that the transient reduction of calcineurin activity facilitates LTP in vitro and in vivo. This facilitation is PKA dependent and persists over several days in vivo. It is accompanied by enhanced learning and strengthened short- and long-term memory in several hippocampal-dependent spatial and nonspatial tasks. The LTP and memory improvements are reversed fully by suppression of transgene expression. These results demonstrate that endogenous calcineurin constrains LTP and memory.

[6] To whom correspondence should be addressed (e-mail: isabelle.mansuy@cell.biol.ethz.ch).
[7] These authors contributed equally to the work.
[8] Present address: Center for Learning and Memory, Massachusetts Institute of Technology, 400 Ames Street, Cambridge, Massachusetts 02139.

Introduction

Studies of the molecular mechanisms of learning and memory in vertebrates and invertebrates have illustrated the existence of both positive and negative regulators of synaptic plasticity and memory storage (Abel et al., 1998). For example, long-term forms of plasticity and memory in invertebrates can either be enhanced or suppressed by changing the ratio between activator and repressor isoforms of the transcription factor cAMP response element binding (CREB) protein (Bartsch et al., 1995; Yin et al., 1995). Another potential site for the control of synaptic plasticity and memory, which operates upstream of the transcriptional machinery, resides in the balance between the phosphorylation and dephosphorylation of specific substrates (Soderling and Derkach, 2000). On the one hand, kinases such as the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), the cAMP-dependent protein kinase A (PKA), and the mitogen-activated protein kinase (MAPK) are positive regulators, critical for the initiation of many forms of synaptic plasticity and for learning and memory (Lisman, 1994; Kornhauser and Greenberg, 1997; Abel et al., 1998; Lee et al., 2000). On the other hand, phosphatases such as the Ca^{2+}/calmodulin-dependent calcineurin (CN) and protein phosphatase 1 (PP1) are thought to be negative regulators inhibiting both synaptic plasticity and memory storage (Ikegami et al., 1996; Mansuy et al., 1998a; Winder et al., 1998; Ikegami and Inokuchi, 2000).

Initial clues to the antagonistic role of kinases and phosphatases came from pharmacological and genetic studies that indicated that PKA is required for the expression of persistent LTP because of its role in suppressing an endogenous phosphatase cascade (Blitzer et al., 1995; Abel et al., 1997). Consistent with this idea, overexpression of CN in forebrain of transgenic mice was found to impair an intermediate and PKA-dependent phase of LTP, as well as the transition from short- to long-term memory and memory retrieval (Mansuy et al., 1998a, 1998b; Winder et al., 1998). These results suggested that CN can act as an inhibitory constraint on PKA-dependent processes and downregulate pathways supporting synaptic plasticity and memory.

A more direct test of the idea that PKA and endogenous CN serve to balance one another would require demonstrating that relief of the inhibitory constraint applied by CN enhances both LTP and memory. We therefore generated transgenic mice in which CN activity is decreased by the expression of a specific inhibitor. To restrict the inhibitor expression both temporally and regionally in the brain, we used the reverse tetracycline-controlled transactivator (rtTA) system in combination with the promoter for CaMKIIα (Gossen et al., 1995; Mansuy et al., 1998b). With this system, we found that the regulated inhibition of CN leads to a reversible facili-

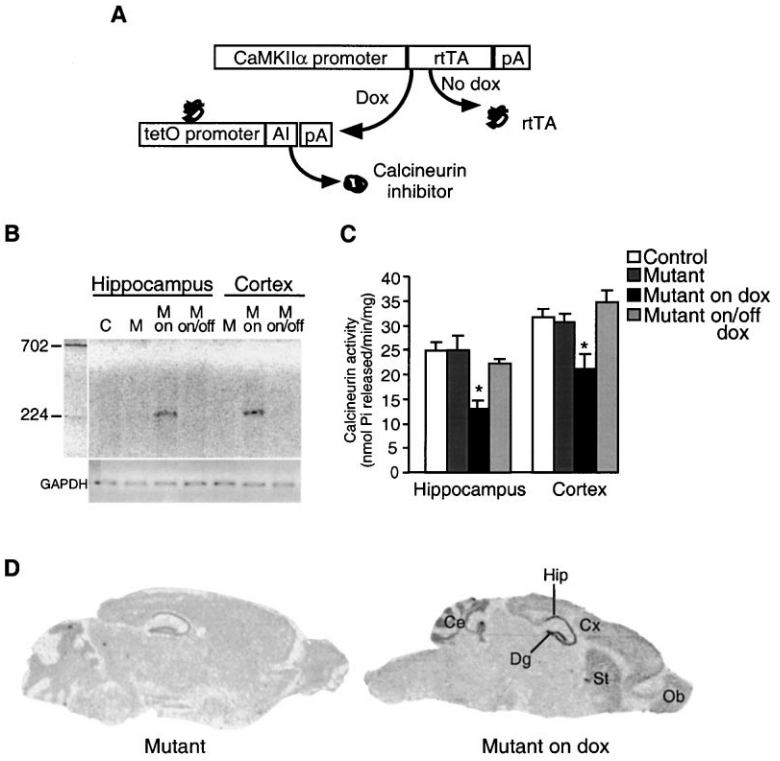

Figure 1. Generation and Characterization of the Transgenic Mice

(A) Strategy to obtain dox-dependent expression of the CN inhibitor in brain. Transgenic mice carrying the rtTA gene driven by the CaMKIIα promoter were crossed with mice carrying the tetO promoter linked to the CN autoinhibitory (AI) gene. Double transgenic (mutant) mice from this crossing express the CN inhibitor when fed dox. Without dox, rtTA does not activate gene transcription. pA: polyadenylation signal sequence.

(B) RT-PCR showing transgene expression (263 bp band) in extracts from hippocampus and cortex from adult mutants on dox (M on). No expression was detected in controls treated or not treated with dox (C), in untreated mutants (M), or in mutants treated with dox then withdrawn from the drug (M on/off). GAPDH indicates equivalent DNA concentrations in the reactions.

(C) Reduced CN activity in extracts from hippocampus (Hip) and cortex from adult mutants on dox (Hip: $n = 7$ versus control ($n = 3$) or mutant ($n = 5$), $p < 0.05$, asterisk; cortex: $n = 6$ versus control ($n = 2$) or mutant ($n = 3$), $p < 0.05$, asterisk). CN activity returned to basal levels in mutants on/off dox (Hip: $n = 3$ versus mutant on dox or control, $p < 0.05$; cortex: $n = 3$ versus mutant on dox or control, $p < 0.05$).

(D) In situ hybridization on a sagittal brain section from an adult mutant on dox showing mRNA in the Hip, dentate gyrus (Dg), cortex (Cx), olfactory bulb (Ob), striatum (St), and cerebellum (Ce). No expression was detected in the absence of dox (mutant).

tation of LTP both in vitro and in vivo in the Schaffer collateral pathway to the pyramidal cells of the CA1 region in hippocampus and in the perforant pathway to the dentate gyrus, and that the enhanced LTP is PKA dependent. The facilitated LTP was accompanied by a reversible enhancement of several phases of spatial and nonspatial learning and memory. These experiments demonstrate that transient reduction of CN activity in the adult brain is sufficient to enhance synaptic efficacy and memory storage reversibly, suggesting that CN plays a determinant role in signaling pathways recruited in brain plasticity and memory. Further, our results provide direct evidence that CN acts as a negative regulator of synaptic plasticity, and of learning and memory.

Results

Doxycycline-Dependent Inhibition of CN

We achieved temporally restricted inhibition of CN in adult mouse brain by expressing the autoinhibitory domain in C terminus of CNAα (Perrino et al., 1995) with the rtTA system. Transgenic mice carrying the rtTA-responsive tetO promoter fused to the CN inhibitor gene were crossed with mice expressing rtTA in neurons under the control of the CaMKIIα promoter (Mansuy et al., 1998b) (Figure 1A). In double transgenic animals (mutants), doxycycline (dox) induced the expression of the CN inhibitor. CN inhibitor mRNA was detected in extracts from hippocampus and cerebral cortex from 3-week-old (data not shown) and adult (Figure 1B) mutants on dox while it was absent in tissue from control mice. In situ hybridization confirmed the presence of transgene mRNA in hippocampus and cortex, and revealed it also in striatum, olfactory bulb, and cerebellum (Figure 1D). In both hippocampus and cortex, transgene expression resulted in a 35%–45% decrease in CN activity (Figure 1C, $p < 0.05$). This decrease was reversed when transgene expression was suppressed by withdrawal of the mutant mice from dox. Thus, 12 days after dox removal following a 1-week treatment, transgene mRNA was no longer detected in hippocampus and cortex (Figure 1B) and CN activity returned to baseline (Figure 1C). Finally, no gross anatomical or structural changes were observed after transient expression of the CN inhibitor in adult brain (data not shown).

Inhibiting CN Facilitates LTP In Vitro

CN is thought to be critical for several forms of synaptic plasticity (Lisman, 1994; Mulkey et al., 1994; Winder et al., 1998; Zhuo et al., 1999; Lu et al., 2000). In particular, we have found that PKA-dependent forms of LTP are suppressed by CN overexpression, suggesting that CN and PKA act antagonistically to regulate a common component of LTP (Winder et al., 1998). If this were the case, one would predict that inhibiting CN would facilitate LTP in a PKA-dependent manner. To test this idea, we examined LTP elicited with a single train of 100 Hz in the Schaffer collateral pathway to area CA1 of hippocampal slices. We observed that in slices from mutants expressing the CN inhibitor, LTP was increased compared to controls (155 ± 10 versus 121 ± 6% baseline 30 min after tetanus, Figure 2A, $p < 0.05$). We next assessed whether this enhanced LTP was PKA dependent using the PKA inhibitor KT5720. Consistent with previous studies (Huang and Kandel, 1994; Abel et al., 1997), 30 min pretreatment with KT5720 had no effect

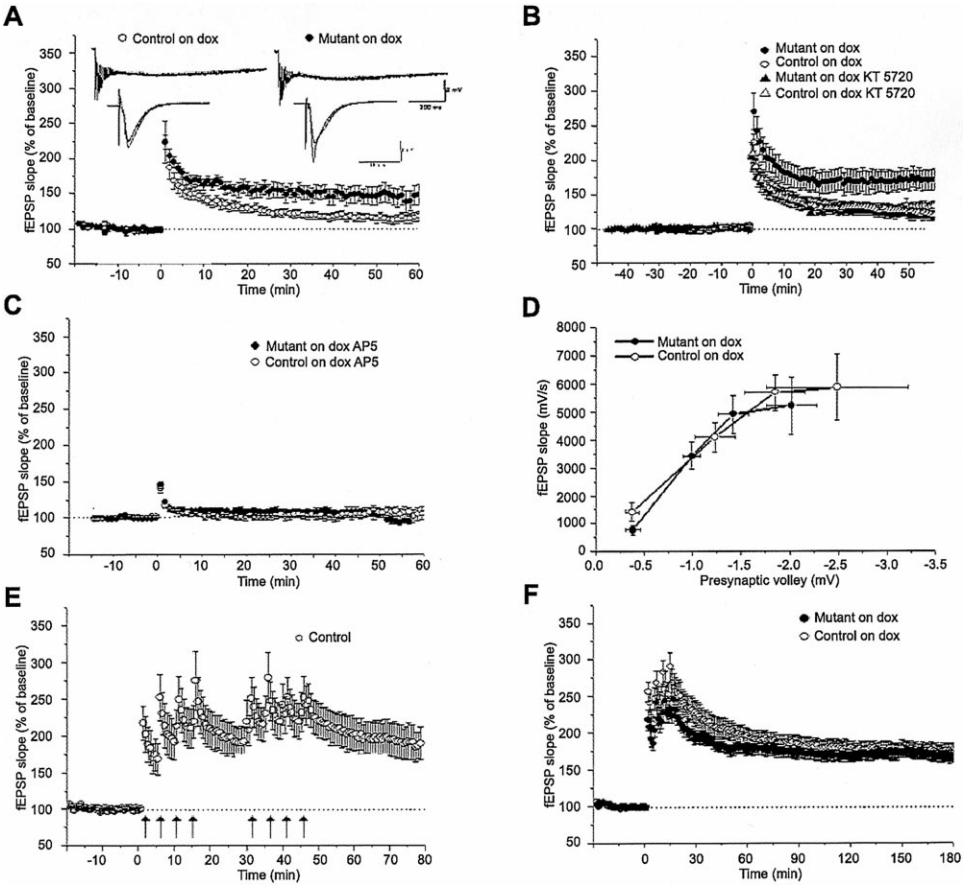

Figure 2. Enhanced 1-train but Not 4-train LTP with the CN Inhibitor

(A) LTP elicited by a single, 1 s 100-Hz train in mutant and control slices (12 slices, 7 mice in each group). Inset: top traces are responses during tetanization in controls (left) and mutants (right). For clarity of presentation, stimulus artifacts were removed. Lower traces are baseline fEPSPs just before tetanus superimposed with responses 40 min after tetanus.

(B) 1-train LTP with KT5720 (1 μM) in mutants on dox (7 slices, 4 mice) or untreated mutants (5 slices, 4 mice), and in control on dox (4 slices, 3 mice) or untreated controls (15 slices, 8 mice).

(C) Similar to (A) except performed with DL-AP5 (100 μM, at least 20 min pretreatment, control, 10 slices, 3 mice; mutant, 9 slices, 3 mice).

(D) Input-output curve of synaptic transmission in slices from mutants and controls on dox (16 slices, 6 mice each).

(E) Saturation of LTP with four 1 s 100 Hz trains with 5 min interval delivered twice 20 min apart in slices from controls ($n = 6$).

(F) LTP elicited by four, 1 s 100-Hz trains with 5 min interval in controls (9 slices, 7 mice) and mutants (13 slices, 7 mice).

on 1-train LTP in control slices (134 ± 6% baseline 30 min after tetanus, Figure 2B). However, in mutant slices, the increased potentiation was suppressed by KT5720 (168 ± 17 versus 126 ± 7% baseline 30 min after tetanus, Figure 2B), suggesting it required PKA activity.

Four independent experiments suggest that the LTP enhancement does not represent a nonspecific alteration of synaptic transmission. First, the enhanced LTP was dependent on N-methyl-D-aspartate receptor (NMDA-R), as it was abolished by the NMDA-R antagonist DL-AP5 (100 μM, Figure 2C). Second, basal synaptic transmission was normal in mutants on dox as assessed by stimulus-response curves of baseline fEPSPs versus presynaptic fiber volley sizes across a range of stimulus intensities (Figure 2D). Third, the total depolarization elicited by the tetanus (estimated by the area under the fEPSPs during tetanus) was indistinguishable between mutant and control slices (inset Figure 2A; control, 2876 ± 391 mV; mutant, 2768 ± 340 mV). Fourth, the LTP enhancement could not be explained by an increased basal probability of glutamate release resulting in greater depolarization during the tetanus since paired-pulse facilitation (PPF), an index of presynaptic activity and release probability, was markedly facilitated in mutants on dox compared to controls, suggesting rather a reduced release probability in mutant mice (Figure 3E).

The enhancement of LTP observed with one 100-Hz train may be reflecting a decrease in the threshold for the induction of LTP or an enhanced maintenance of LTP. To distinguish between these possibilities, we examined LTP induced with four 100-Hz trains. 4-train LTP was nondecremental and similar in slices from mutant and control mice on dox (Figure 2F). However, 4-train LTP was not further enhanced by another 4-train tetanization in slices from controls (Figure 2E) or from mutants on dox (data not shown), indicating that the evoked LTP was saturated. These data, therefore, are consistent with a role for CN in regulating the induction of LTP.

Since pharmacological studies have suggested a role for phosphatases in synaptic changes elicited by low frequency stimulation (Mulkey et al., 1994; Thomas et

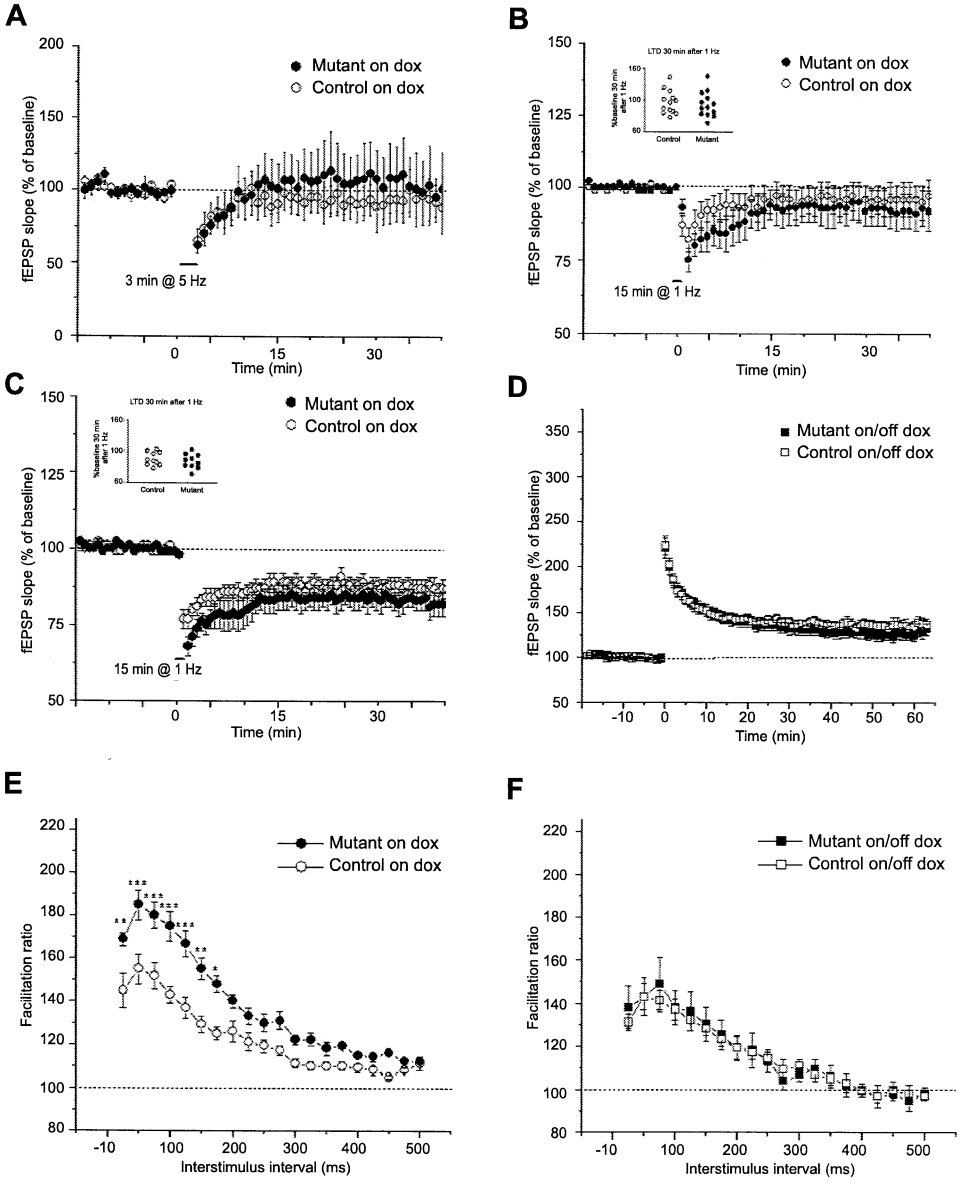

Figure 3. Normal Responses to Low Frequency Stimulation but Enhanced PPF in Mutants

(A) Responses to 3 min of 5-Hz stimulation (control, 5 slices, 2 mice; mutant, 6 slices, 2 mice).

(B and C) LTD elicited by 15 min of 1-Hz stimulation. In (B), overall responses in 13 slices from 5 controls and 5 mutants and (C), responses in 10 slices from 5 controls and 5 mutants, excluding 3 slices in each case that gave responses over 100% baseline. Insets: scatterplots of responses 30 min after stimulation.

(D) Reversal of 1-train LTP enhancement in mutants on/off dox (22 slices, 11 mice). Control on/off, 22 slices, 11 mice.

(E) PPF at various interstimulus intervals is increased in slices from mutants on dox versus controls on dox (10 slices, 3 mice in each case). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one way ANOVA ($p < 0.0001$).

(F) Reversal of PPF increase in slices from mutants on/off dox (6 slices, 3 mice). Control on/off, 6 slices, 3 mice.

al., 1996), we next determined the effect of the CN inhibitor on responses induced by low frequency stimulation. As shown in Figure 3A, three minutes of 5-Hz stimulation, a crossover frequency between those producing LTP and those producing long-term depression (LTD) (Bienenstock et al., 1982), resulted in responses that were not different from baseline in both control and mutant slices. Further, consistent with previous observations (Winder et al., 1998), 15 min of 1-Hz stimulation did not produce any response in slices from adult mice. By contrast, 15 min of 1-Hz stimulation induced very modest LTD in area CA1 that was similar in mutant and control slices from young animals (3.5–4.5 weeks, Figure 3B). The modest averaged population LTD was in part due to the fact that this protocol elicited a lasting synaptic enhancement on occasion (inset Figure 3B). The occasional enhancement of synaptic transmission that we observed (to a similar degree in both mutant and control populations) obscures the fact that in most individual experiments, LTD was observed. Indeed, when these

individual experiments were excluded from re-analysis (three from both populations, inset Figure 3C), robust LTD was observed, which again was indistinguishable between mutant and control slices (Figure 3C). Thus, although we cannot rule out subtle differences in LTD induction between mutant and control slices, these data demonstrate that LTD can be induced in slices from mutant mice on dox. These results corroborate recent genetic studies revealing normal LTD in slices from mice lacking CNAα (Zhuo et al., 1999), but they contrast with previous pharmacological studies indicating that LTD is blocked by CN inhibition (Mulkey et al., 1994).

Finally, to determine whether the enhancement of 1-train LTP and PPF was a direct effect of the transgene, we assessed whether suppression of transgene expression in adult could reverse this enhancement. Slices from mutants in which transgene expression was induced for 1 week then blocked by dox removal for 2 weeks prior to experimentation (on/off dox) displayed similar 1-train LTP (131 $\pm$ 6% baseline 30 min after potentiation, Figure 3D) and PPF (Figure 3F) to control slices, indicating that the enhancement of LTP and PPF is a direct effect of the CN inhibitor.

LTP Is Also Enhanced In Vivo in Both CA1 and Dentate Gyrus

Previous studies of LTP in genetically modified mice have revealed discrepancies between results obtained in vitro or in vivo (Errington et al., 1997). We therefore examined LTP in vivo in both anesthetized and awake mice. In area CA1, weak tetanic stimulation (50 pulses at 100 Hz) of Schaffer collateral afferents induced a transient potentiation of the fEPSP slope in anesthetized mutants on dox. In contrast, this tetanus produced only slight post-tetanic potentiation in control mice (Figure 4A). A subsequent, stronger tetanus (two trains at 100 Hz) induced significant potentiation during the first 10 min after the tetanus in control mice ($p < 0.05$, Figure 4A). Consistent with the in vitro data, this initial potentiation was significantly greater in mutant versus control mice ($p < 0.05$) so that 50–60 min after the tetanus, only mutant mice maintained significantly enhanced responses ($p < 0.05$). Basal synaptic efficacy appeared normal in mutant mice since stimulus-response curves were indistinguishable from those in control mice (data not shown). These results are consistent with the facilitatory effect on LTP observed in the rat after chronic injection of antisense oligodeoxynucleotides against CN (Ikegami et al., 1996).

In contrast to in vitro, in vivo PPF was similar in mutant and control animals, with both groups showing maximal facilitation at an interstimulus interval of 30 ms (facilitation ratio: mutant, 183 $\pm$ 9.9%; control, 191 $\pm$ 14%, data not shown). At present, the cause of this difference between preparations is unclear. One possibility is that while in vivo recordings were performed in the dorsal 1/3 of the hippocampus, in vitro recordings were from slices of the middle 2/3 of the hippocampus. PPF was reported to be greater in more dorsal than ventral portions of the hippocampus (Papatheodoropoulos and Kostopoulos, 2000). Regardless, the fact that similar modulation of LTP is seen in both preparations suggests that altered PPF does not play a critical role in the enhancement of LTP.

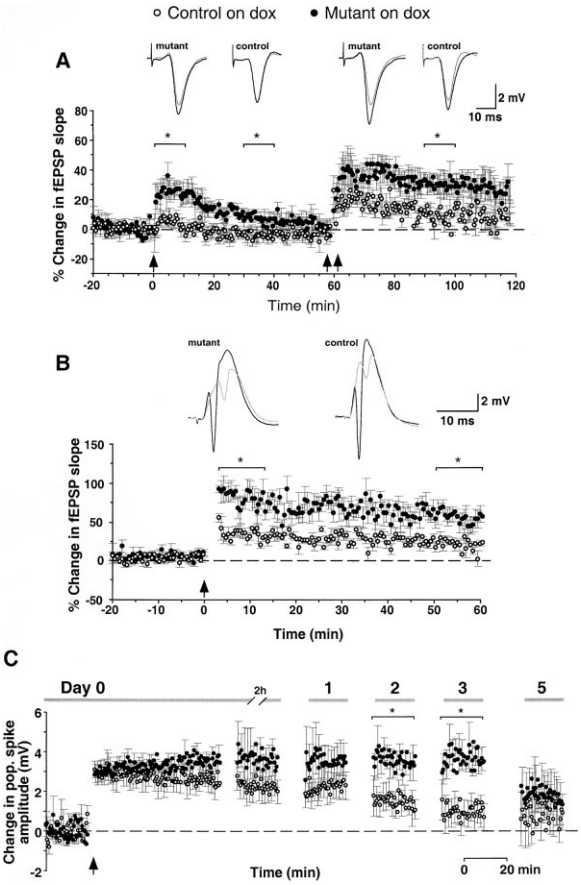

Figure 4. Enhanced In Vivo LTP in Both Anesthetized and Awake Mice

(A) CA1 LTP in anesthetized mice. Test stimuli were delivered at 0.033 Hz at an intensity that evoked 50% maximal fEPSP slope. 50 stimuli at 100 Hz (arrow) induced a larger transient increase in fEPSP slope in mutant ($n = 4$) than in control ($n = 5$) mice on dox. 1 hr after the initial tetanus, 2 trains of 50 stimuli at 100 Hz (arrows) induced enhanced LTP in mutant ($n = 4$) versus control ($n = 3$) mice on dox. Insets: representative field potentials 10 min after the first (left) or the second (right) tetanus. Potentiated responses (black) are superimposed on control responses (gray).

(B) Dentate gyrus LTP in anesthetized mice. Test stimuli were delivered at 0.033 Hz at an intensity that evoked a population spike amplitude of 1–3 mV. Tetanic stimulation (arrow, 6 series of 6 trains of 6 stimuli at 400 Hz, 200 ms between trains, 20 s between series) induced larger LTP in mutant ($n = 3$) versus control ($n = 3$) mice on dox.

(C) Dentate gyrus LTP in awake mice. Test stimuli and tetanic stimulation as in (B), with 20 min recording sessions apart from the first hour after LTP induction. More persistent LTP in mutant ($n = 4$) than in control ($n = 4$) mice on dox 2 and 3 days after induction. Asterisks indicate significant difference in mean level of potentiation over the time period indicated by brackets, $p < 0.05$ by Student's t test.

In dentate gyrus of anesthetized mice, 400 Hz tetanic stimulation induced a stable and significant LTP of fEPSP in all mice tested (Figure 4B). As in CA1, LTP was significantly greater in mutant compared to control mice ($p < 0.05$). Associated increases in population spike amplitude were also larger in mutants (by 4.9 $\pm$ 1.5 mV, 50–60 min post-tetanus) than in controls (by 3.1 $\pm$ 0.7 mV), although this enhancement of spike potentiation was not significant (data not shown). Stimulus-response

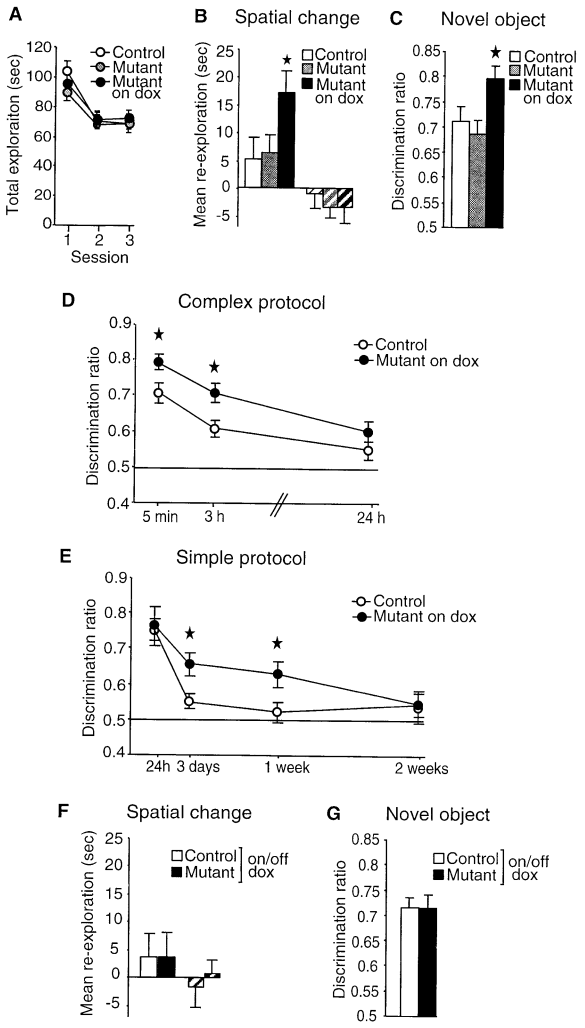

Figure 5. Reversible Improvement of Short- and Long-Term Memory on the Object Recognition Task

(A) Mean exploration during training. Control (pooled), n = 12; mutant, n = 16; mutant on dox, n = 16.

(B) Reaction to the spatial change. Increased re-exploration of the displaced object (plain, left three bars) in mutants on dox (n = 16, versus control (pooled), n = 11 or mutant, n = 16, all $p < 0.05$, asterisk). No re-exploration of the undisplaced objects (hatched, right three bars) was observed. Re-exploration was calculated by subtracting the exploration time before and after the spatial change.

(C) Reaction to a novel object. Discrimination ratio is the time spent exploring the novel object over total exploration time (for 3 objects, total time is the mean time for familiar plus time for novel). Ratio of 0.5 reflects equal exploration of all objects. Higher discrimination ratio indicating increased exploration of the novel object in mutants on dox (n = 16, versus control (pooled), n = 10, $p < 0.05$, asterisk, or mutant, n = 14, $p < 0.01$).

(D and E) Increased discrimination ratio in mutant mice on dox (D) 5 min and 3 hr after introduction of the novel object with the complex protocol (mutant on dox, n = 8 versus control (pooled), n = 8, $p < 0.05$, asterisk) or (E) 3 days (mutant on dox, n = 10 versus control (pooled), n = 9, $p < 0.02$, asterisk) and 1 week (mutant on dox, n = 10 versus control (pooled), n = 14, $p < 0.05$, asterisk) after introduction of the novel object with the simple protocol. No difference in exploration was observed between groups (D) at 24 hr with the complex protocol (control (pooled), n = 6; mutant on dox, n = 6) or (E) both at 24 hr and 2 weeks (control (pooled), n = 8; mutant on dox, n = 8) with the simple protocol.

(F and G) Reversibility of the improvement. No difference after 5 min in (F) re-exploration of the displaced object (plain, left two bars) or the undisplaced objects (hatched, right two bars) and (G) discrimination between the novel and the familiar objects in mutant and control mice on/off dox (control, n = 7; mutant, n = 11).

curves and PPF again appeared normal in mutants (data not shown), once more indicating normal basal transmission and excitability.

We next examined the time course of LTP over several days in the dentate gyrus of awake animals with chronically implanted electrodes. LTP was induced using a pattern of tetanic stimulation identical to that in anesthetized mice. Population spike amplitudes were potentiated to similar extents one and three hours after LTP induction (Figure 4C). However, spike amplitudes in mutant mice remained robustly elevated for at least 3 days after tetanic stimulation ($p < 0.05$ versus control group) while in control mice, potentiation was no longer significantly different from baseline after 3 days. By 5 days, although spike amplitudes remained potentiated in mutant animals, there was no significant difference between genotypes. Potentiation is expressed as change in population spike amplitude since the initial fEPSP slope was often obscured by the spike in awake animals.

CN Inhibition Enhances Short-Term Memory for Object and Spatial Configurations

To examine whether a reduction in CN activity leading to increased LTP might improve memory, we first tested the animals on a spontaneous object recognition task. This task is based on the discrimination between a familiar and a novel situation and requires the hippocampus (Vnek and Rothblat, 1996). We assessed the ability of the animals to discriminate either between a novel and a familiar spatial location of an object, or between a novel and a familiar object. Mice were first trained with a complex protocol for which they were placed in an arena containing three novel objects that they were allowed to explore for three sessions of 5 min each. In the first session, all groups spent a similar amount of time exploring the objects (25%–35% total time), then exploration decreased, indicating comparable habituation to the objects and attention in all groups (Figure 5A). No difference in basic locomotor activity or in thigmotaxis, an index of anxiety characterized by motion along the walls, was observed between groups (data not shown).

Five min after these initial sessions of exploration, one object was moved to a novel location in the arena. In response to the change in objects configuration, all groups tended to re-explore the displaced object more than the nondisplaced objects, indicating they perceived the change in spatial arrangement (Figure 5B). However, mutant mice on dox spent significantly more time re-exploring the displaced object than did control mice [$F(1,25) = 4.272$, $p < 0.05$] or mutant mice off dox [$F(1,30) = 4.495$, $p < 0.05$], suggesting enhanced memory for the initial configuration. No significant difference in re-exploration of the nondisplaced objects was observed between the groups.

Following a session of habituation to the novel configuration, one of the familiar objects was replaced with a

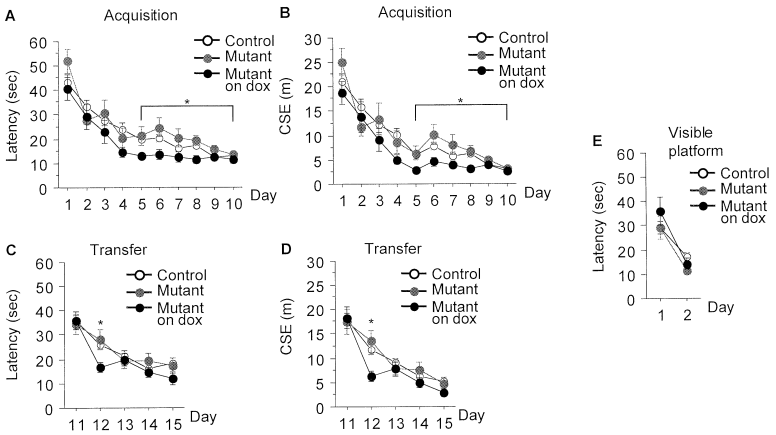

Figure 6. Enhanced Spatial Learning and Memory

(A–D) Mean escape latency and CSE per day across days on the Morris water maze. Mutants on dox (n = 12) have significant lower escape latency (asterisks) (A) from day 5 to day 10 during acquisition (mutant on dox versus control (pooled), n = 60, $p < 0.05$ or mutant (n = 16), $p < 0.01$) and (C) on day 12 during transfer (mutant on dox versus control, $p < 0.04$ or mutant, $p < 0.03$), and significant lower CSE (asterisks) (B) from day 5 to day 10 during acquisition (mutant on dox versus control, $p < 0.04$ or mutant, $p < 0.01$) and (D) on day 12 during transfer (mutant on dox versus control, $p < 0.03$ or mutant, $p < 0.03$). (E) Normal learning on a cued version of the Morris water maze.

novel object and a discrimination ratio was determined. After a 5 min delay, mutant mice on dox showed increased discrimination between novel and familiar objects with a significantly stronger preference for the novel object compared to other groups [control, $F(1,24) = 5.287$, $p < 0.05$; mutant, $F(1,28) = 9.414$, $p < 0.01$]. However, all groups explored the novel object more than the familiar ones (Figure 5C). These results suggest that memory for spatial location of object and for object is improved by the CN inhibitor at short retention intervals.

Intermediate to Long-Term Memory Is Also Improved by CN Inhibition

To assess whether enhanced memory could also be observed after longer retention intervals, the discrimination ratio was examined 3 hr and 24 hr after acquisition. Again, mutant mice on dox displayed greater preference for the novel object than control mice after 3 hr [$F(1,14) = 8.18$, $p < 0.05$], although both groups explored the novel object preferentially (Figure 5D). However, by 24 hr, the discrimination ratio was low in both groups (Figure 5D). These results suggested that the CN inhibitor improves memory not only at short but also at intermediate retention intervals.

Next, to examine whether long-term memory could also be improved, we trained the animals on a simple and more intense protocol. This protocol consisted of two training sessions with two objects, and after a retention interval of 24 hr, 3 days, 1 week, or 2 weeks, one familiar object was replaced with a novel object. We found that after 24 hr, both control mice and mutant mice on dox displayed a large preference for the novel object, indicating robust memory for the familiar object (Figure 5E). Strikingly after 3 days [$F(1,17) = 8.267$, $p < 0.02$] or after 1 week [$F(1, 22) = 4.375$, $p < 0.05$], mutant mice on dox still clearly preferred the novel object while control mice did not show any preference for this object (Figure 5E). Two weeks later, however, mutant mice explored both objects similarly, suggesting decayed memory for the familiar object (Figure 5E). These results clearly indicated that the CN inhibitor allowed long-term memory to persist for over a week longer in mutant mice than in control mice.

The Memory Enhancement Is Reversible

To demonstrate that the enhancement in memory was a direct consequence of CN inhibition, we took advantage of the dox dependence of transgene expression and examined whether its suppression could reverse the memory improvement. Mutant mice displaying enhanced memory with the transgene were withdrawn from dox, then trained and tested again 2 weeks later using a different set of objects. Suppression of CN inhibition in these mutants reversed the increase in exploration of both the displaced [on/off dox versus on dox, $F(1,30) = 4.495$, $p < 0.05$] (Figure 5F) and the novel object [on/off dox versus on dox, $F(1,25) = 5.275$, $p < 0.05$] (Figure 5G). Mutant mice not withdrawn from dox and tested again after 2 weeks exhibited enhanced exploration similar to that observed during the first testing session (data not shown). These results indicated that the enhancement in memory is reversible and therefore is a direct consequence of transgene expression in the adult.

Spatial Learning and Memory Is Facilitated with the CN Inhibitor

To assess whether memory was enhanced on other behavioral tasks, we used the Morris water maze, a hippocampal-dependent task that challenges spatial learning and memory (Morris et al., 1982). On this task, animals learn the position of a hidden escape platform in a circular pool using distal cues. During acquisition, mice from all groups showed a decrease in escape latency (Figure 6A) and path length (data not shown) across days, indicating learning of the platform position (all groups, $p < 0.0001$). Similarly after the platform was moved to a different location in the pool (transfer), a decrease in latency and path length was observed across time (Figure 6C), reflecting learning of the second platform position.

Strikingly however, mutant mice on dox displayed significantly lower escape latencies and shorter path length than control mice (latency, [$F(1,70) = 4.34$, $p < 0.05$]; path length, [$F(1,70) = 3.98$, $p < 0.05$]) or than untreated mutant mice (latency, [$F(1,26) = 8.97$, $p < 0.01$]; path length, [$F(1,26) = 7.44$, $p < 0.05$]). This effect was particularly pronounced between day 5 and day 10 of acquisition (Figure 6A) and on day 12 of transfer (Figure 6C). Thus, after 5 days of acquisition, mutant mice on dox showed minimal escape latencies (10–15 s) (no session effect from day 5 to 10; $p > 0.8$), while controls needed almost twice as much training, 4 additional days, to

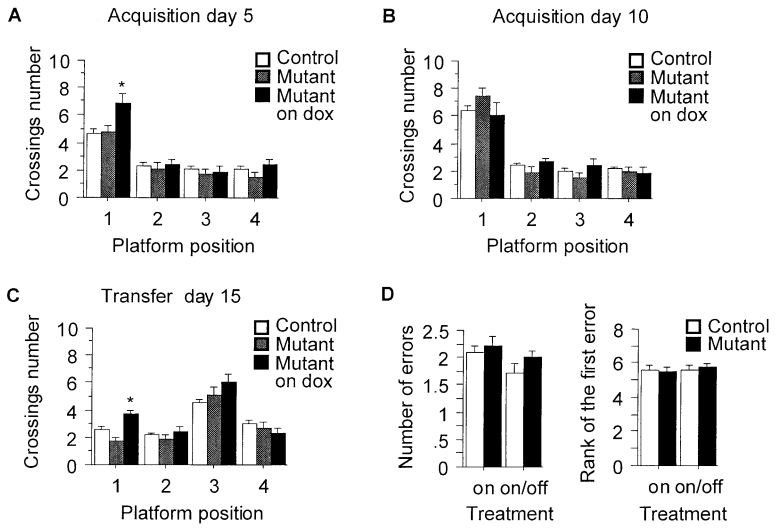

Figure 7. Enhanced Long-Term Memory and Normal Working Memory

(A–C) Probe trials in the Morris water maze on (A) day 5 or (B) day 10 of the acquisition phase and (C) day 15 of the transfer phase. Mean number of platform crossings in the training quadrant (Position 1 during acquisition, 3 during transfer) and in corresponding zones in other quadrants. Significant increase in platform crossings (asterisks) in the first training quadrant in mutant on dox (versus control, $p < 0.01$ or mutant, $p < 0.05$) on day 5 and on day 15 (versus control, $p = 0.01$ or mutant, $p = 0.0001$).

(D) Mean errors and rank of the first error per day on the radial arm maze. No difference between mutant ($n = 8$) or control ($n = 10$) whether on dox (on) or withdrawn from dox after treatment (on/off).

reach comparable levels of performance (significant session effect through day 5 to 10, $p < 0.05$ for all control groups) (Figure 6A). Similarly, during transfer, mutant mice on dox reached minimal latencies after 2 days (no session effect from day 12 to 15; $p > 0.09$) while controls needed one more day to reach comparable latencies (significant session effect from day 12 to 15; $p < 0.05$ for all control groups) (Figure 6C). The difference in latency between mutants on dox and controls was seen only on day 5 of acquisition as opposed to day 2 of transfer because, presumably, initial days of acquisition are used for procedural learning that is no longer necessary on transfer (Bannerman et al., 1995). No difference in performance was found between control groups. The analyses of noncognitive parameters also revealed no difference in learning on the cued version of the task (Figure 6E), in swimming speed or in thigmotaxis (data not shown). These results suggested that the improved performance resulted from enhanced hippocampal-dependent spatial learning and memory and not from changes in motor or motivational/emotional processes.

A decrease in latency or path length, however, does not necessarily indicate place learning as mice can adopt efficient nonspatial strategies such as circular swimming (Gallagher et al., 1993; Wolfer et al., 1998). To specifically evaluate spatial performance, we measured the animal's proximity to the platform with the cumulative search error (CSE), an index of navigation precision. CSE was determined by adding together the distance between the animal and the platform every second for each trial. Consistent with the decrease in latency, all groups showed a decrease in CSE over both training phases ($p < 0.0001$). But again, mutant mice on dox displayed significantly lower CSE than controls [$F(1,70) = 4.66$, $p < 0.05$] or than mutants [$F(1,26) = 7.49$, $p < 0.05$], particularly between acquisition day 5 and 10 (Figure 6B), and on transfer day 12 (Figure 6D).

We next evaluated memory for the platform position on days 5, 10, and 15 by removing the platform from the pool and measuring the searching time and number of platform crossings in each quadrant of the pool. For each probe trial, all groups spent more time in the training quadrant than in other quadrants (data not shown), revealing a good memory for the training quadrant. However, on day 5, mutant mice on dox swam across the platform area in the training quadrant significantly more often than controls [$F(1,70) = 7.52$, $p < 0.01$] or than mutants [$F(1,26) = 7.35$, $p < 0.05$], suggesting a more precise memory for the platform position (Figure 7A). At the end of acquisition (day 10), this difference was no longer apparent as controls and mutants reached level of performance comparable to mutants on dox (Figure 7B). Similarly, by the end of transfer (day 15), all groups swam across the platform area in the second training quadrant more often than in other quadrants. Interestingly, mutants on dox also crossed the area where the platform was located during acquisition significantly more often than controls [$F(1,70) = 6.53$, $p = 0.01$] or than mutants [$F(1,26) = 20.68$, $p = 0.0001$], suggesting intact memory for the first platform location (Figure 7C).

Working Memory Is Normal

We next assessed whether memory was improved on very short delays by examining working memory. Working memory is an immediate and rapidly decaying memory thought to be anatomically sustained by a prefrontal cortex-hippocampus network (Olton and Feustle, 1981; Floresco et al., 1997). We used a classical random foraging paradigm for which animals had to retrieve food baits in eight arms of a radial arm maze (Cassel et al., 1998). The optimal strategy for the animals to be rewarded is to remember the arms already visited while performing the task. On this task, no significant difference in the number of errors or the rank of error between groups was found, suggesting no effect of the CN inhibitor on working memory (Figure 7D).

Discussion

The Inhibition of CN Enhances LTP and Several Phases of Memory

The main finding of our study is that the regulated inhibition of the phosphatase CN leads to enhanced LTP both in vitro and in vivo, and to improved learning and memory

storage. The parallel in time course of the increased persistence of LTP in awake animals and of the memory improvement strongly suggest a correlation between the duration of LTP and memory storage. Improved cognitive performance was observed both on spatial and nonspatial hippocampal-dependent tasks, consistent with the multipurpose role of the hippocampus in human declarative memory (Squire, 1992). Moreover, with different tasks, different temporal components of memory were improved. Thus, the complex object recognition task, involving brief training sessions, multiple objects, spatial transfer, and object change, elicited a weak form of memory that was strengthened at early and intermediate time points by the CN inhibitor, but did not persist longer in mutants than in controls. By contrast, a more robust form of memory elicited by a more intense training was maintained and persisted for over a week longer in mutants expressing the CN inhibitor when compared to controls.

Facilitated learning and memory was also observed on the Morris water maze and was evident not only with traditional measurements of spatial performance such as escape latency, but also with more specific aspects of performance such as the precision of navigation. These measures suggested that mutant mice expressing the CN inhibitor retained spatial information more efficiently than controls. The persistent memory for the first platform position associated with the efficient learning of a second platform position suggested an overall enhanced capacity for memory storage with the CN inhibitor. Further, the rapid adaptation to spatial changes observed in mutants expressing the CN inhibitor on both the Morris water maze and the object exploration task suggested increased cognitive flexibility, a process that depends on the hippocampus (Day et al., 1999).

It should be noted that some of our results contrast with those obtained recently in rat where antisense oligonucleotides against CN did not enhance learning and memory on the Morris water maze (Ikegami and Inokuchi, 2000). This discrepancy may be due to different levels of CN inhibition achieved with the two approaches, or to the choice of the animal model or of behavioral paradigms. Finally, on all tasks, the cognitive enhancement observed in the mutant mice was quite remarkable considering the high level of performance in all control groups.

A Molecular Gate for LTP and for Memory Storage

One of the molecular mechanisms allowing transmitted signals to persist or decay is thought to be the balance between phosphatase and kinase activity (Blitzer et al., 1995; Wang and Kelly, 1997). Much evidence suggests that PKA and CN specifically regulate this balance and thereby serve as a gate for LTP (Blitzer et al., 1995, 1998; Thomas et al., 1996; Winder et al., 1998). In the current study, we provide further evidence in support of this model by demonstrating that shifting the endogenous balance away from calcineurin activity positively modulates synaptic plasticity in a PKA-dependent manner. Further, together with our previous results (Mansuy et al., 1998a), the current data indicate that altering CN activity transiently in the adult brain is sufficient to positively or negatively control synaptic plasticity and memory storage. The effects observed suggest that CN is essential both for early events of plasticity and memory and for downstream pathways that contribute to persistent changes in plasticity and memory storage.

Mechanistically, early and transient forms of plasticity and memory are known to rely on the covalent modification of pre-existing proteins while long-term forms require activation of transcription factors such as CREB, and protein synthesis (Dash et al., 1990; Bourtchuladze et al., 1994; Impey et al., 1996). One possible mechanism for the facilitatory effect of the CN inhibitor may be a decrease in the activity of PP1, a protein phosphatase positively regulated by CN through dephosphorylation of inhibitor-1 (I-1). PP1 inhibition has been shown to promote the induction of LTP (Blitzer et al., 1995; 1998), whereas increased PP1 activity, produced by genetic suppression of I-1, has been shown to affect certain forms of LTP in some hippocampal regions (Allen et al., 2000). Since PP1 is effective in modulating CaMKII, a kinase critical for the transmission of postsynaptic signals required for the induction of LTP (Lledo et al., 1995; Otmakhov et al., 1997), it is possible that increased CaMKII activity mediated by lower PP1 activity facilitates the induction of LTP (Makhinson et al., 1999). Raising the signal for the induction of LTP, through genetic upregulation of NMDA-R function, has previously been demonstrated to enhance LTP, learning, and memory (Tang et al., 1999). Our findings suggest that LTP and memory enhancements can be similarly achieved by relieving a constraint downstream of the NMDA-R and that this constraint is exercised by CN.

The effect of the CN inhibitor on long-lasting changes in plasticity and memory may be mediated by modulation of transcriptional control. Thus, the prolonged maintenance of LTP and of memory may arise from augmented CREB transcriptional activity via reduced CREB dephosphorylation by PP1 (Hagiwara et al., 1992). In this context, it is important to note that unlike the phenotypes observed in *Drosophila* mutants expressing active CREB (Yin et al., 1995), the CN inhibitor did not convert labile memory into long-lasting memory. It was, however, able to strengthen or prolong different phases of memory, suggesting that CN inhibition modulates rather than mediates memory processes.

The PKA and CN pathways may also interact antagonistically at sites other than CREB. For example, CN can inhibit specific isoforms of adenylyl cyclase required for PKA activation (Paterson et al., 1995). Similarly, PKA and CN can regulate, in opposite ways, phosphorylation sites on key proteins in synaptic transmission, such as the NMDA-R (Tong et al., 1995) or the γ-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor (see for review Yakel, 1997; Banke et al., 2000). The effect of inhibiting CN may also occur through processes additional to or independent of the cAMP pathway. For instance, CN inhibitor may modulate Ca^{2+}-dependent kinases such as CaMKII or PKC through control of intracellular Ca^{2+} mobilization by regulation of inositol 1,4,5-triphosphate receptors (Cameron et al., 1995). Finally, the PKA/CN gate most likely represents only one of several activator/suppressor mechanisms regulating plasticity and memory (Abel et al., 1998).

A Genetic System for Modulating Cognitive Functions

Several genetic approaches have been used to study the molecular mechanisms of hippocampal functions such as memory (Jerecic et al., 1999). Standard genetic techniques, however, have suffered from the limitation that the genetic modification is permanent. Here we confirmed the usefulness of the rtTA system for such studies by showing that inducible and reversible transgene expression allows temporary improvement of complex cognitive functions and of brain plasticity. The ability to achieve such reversible improvements in the adult animal demonstrates that no permanent changes in neuronal circuits are involved and that the effects result specifically from molecular and biochemical changes elicited by a reduction in CN activity. In this context, the rtTA system could be further exploited to assess the timing of the requirement of CN in such processes, for instance in various stages of memory storage such as memory consolidation or retrieval since CN was previously suggested to be involved in both processes (Mansuy et al., 1998b).

Finally, we would emphasize that transgene expression was found in several brain structures and, therefore, we cannot exclude the contribution of other structures, in addition to the hippocampus, to the enhancement of long-term memory. Restricting transgene expression further via local injection of dox should clarify this issue. Overall, however, our results may provide a clear target for potential treatment of learning and memory disorders.

Experimental Procedures

Generation of Transgenic Mice

A founder carrying the tetO-AI transgene was crossed to C57Bl/6J then to CaMKIIα promoter-rtTA animals (Mansuy et al., 1998b). RT-PCR and in situ hybridization were performed as previously described using oligonucleotides specific for tetO-AI (Mansuy et al., 1998a). Phosphatase assays used a nonradioactive CN assay kit (Biomol. Research Laboratories). ANOVAs were used to compare activity between groups.

Treatment and Genotypes

For all experiments, dox (Mutual Pharmaceutical Co., Philadelphia, PA) was administered at 6 mg/g food at least 1 week before experimentation. Control mice were either treated or not treated with dox and for all behavioral tests, results from both groups were pooled. Control groups were wild types and mice carrying either one of the two transgenes.

Electrophysiology

The experimenter was blind to genotype and drug treatment throughout. Field potential recordings from area CA1 of mouse hippocampal slices were performed as previously described (Winder et al., 1998). Dox was present in the ACSF at 6 ng/ml for recordings. For in vivo experiments, mice were anesthetized with urethane (1.8 mg/kg i.p. for nonrecovery) or sodium pentobarbitone (60 mg/kg i.p. during electrode implantation). For CA1 recordings, a bipolar stimulating electrode was positioned in one hippocampus and a micropipette recording electrode in the contralateral stratum radiatum. For dentate gyrus recordings, a stimulating electrode was placed in the medial perforant path and the recording electrode in the ipsilateral hilus. Nichrome wire electrodes were fixed with dental cement for recordings from awake mice (Errington et al., 1997). Test responses were evoked by 60 μs monophasic stimuli at 1 per 30 s. fEPSP potentiation is expressed as percentage change relative to the mean control response during the 10 min prior to tetanic stimulation. Population spike potentiation is expressed as millivolt change in spike amplitude. Data are given as mean $\pm$ SEM.

Behavior and Data Analyses

For all behavioral tasks, mutant and control littermates (males, 3–5 months old) were used. Statistical analyses used ANOVAs with genotype as the between-subject factor, and session (exploration task), day, area (quadrant or platform in the Morris water maze), or treatment (8-arm radial maze) as within-subject factors. Mean $\pm$ SEM are presented.

Object Exploration

Mice were habituated to the arena (63 $\times$ 51 $\times$ 25 cm) for 4 days then tested with either a complex or simple protocol. The complex protocol consisted of five successive 5-min training sessions with 5-min inter-trial interval (ITI) (Buhot and Naïli, 1995) and a last 5-min session either 5 min, 3 hr, or 24 hr later. The simple protocol was of two 10-min sessions, 10-min ITI, and a last 5-min session either 24 hr, 3 days, 1 week, or 2 weeks later. The time spent exploring each object was recorded with a videotracking system (Viewpoint, France) tracing a phosphorescent tag on the animals' heads.

Morris Water Maze

The task was performed as previously described (Malleret et al., 1999) with three training phases: 2 days with a visible platform followed by 10 days (acquisition phase) with a hidden platform in the training quadrant, then 5 days (transfer phase) with the hidden platform in the opposite quadrant. For each phase, four trials, 120 s maximum and 15-min ITI were given daily, probe trials were 60 s. The animals' trajectories were recorded with a videotracking system (HVS Image Analyzing VP-118).

8-Arm Radial Maze

Food-deprived males (90% ad libitum weight) on dox were habituated for a week to retrieve food pellets in cups placed at the end of each arm of an elevated 8-arm radial maze. Mice were tested for 1 week and allowed eight visits each day then dox was removed and animals were tested again 2 weeks later. Errors (runs into an already visited arm) and rank of the first error were recorded.

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