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Forebrain-Specific Calcineurin Knockout Selectively Impairs Bidirectional Synaptic Plasticity and Working/Episodic-like Memory

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Summary

Calcineurin is a calcium-dependent protein phosphatase that has been implicated in various aspects of synaptic plasticity. By using conditional gene-targeting techniques, we created mice in which calcineurin activity is disrupted specifically in the adult forebrain. At hippocampal Schaffer collateral-CA1 synapses, LTD was significantly diminished, and there was a significant shift in the LTD/LTP modification threshold in mutant mice. Strikingly, although performance was normal in hippocampus-dependent reference memory tasks, including contextual fear conditioning and the Morris water maze, the mutant mice were impaired in hippocampus-dependent working and episodic-like memory tasks, including the delayed matching-to-place task and the radial maze task. Our results define a critical role for calcineurin in bidirectional synaptic plasticity and suggest a novel mechanistic distinction between working/episodic-like memory and reference memory.

Introduction

Synaptic plasticity is generally believed to be a major candidate neural mechanism for learning and memory. Neural network models propose that bidirectional modifications of synaptic efficacy such as long-term potentiation (LTP) and long-term depression (LTD) are used for memory encoding (Willshaw and Dayan, 1990; Bear and Abraham, 1996; Paulsen and Sejnowski, 2000). A large body of data supports the importance of LTP or synaptic strengthening in learning and memory (reviewed in Martin et al., 2000). However, the role of LTD or synaptic weakening in learning and memory is less understood. One view is that synaptic weakening has a negative effect on memory storage (Malleret et al., 2001). Another is that both synaptic strengthening and weakening are necessary for optimal memory storage (Willshaw and Dayan, 1990; Migaud et al., 1998).

In studying synaptic plasticity as the candidate mechanism for learning and memory, the variables one must consider include the types of learning and memory and the brain structures that support them. For instance, learning and memory supported by the hippocampus include one-trial learning leading to the formation of trial-specific, short-term memory referred to as "working memory" (Olton, 1983), as well as incremental learning that occurs over multiple trials and leads to the formation of long-lasting reference memory of information constant across trials (reviewed in Squire, 1994). The demands on a memory system imposed by one-trial learning in working memory tasks are not generally present for incremental learning, requiring both within-trial acquisition and suppression of between-trial interference. Different types and/or parameters of synaptic plasticity may very well subserve these differential requirements of a memory system.

The rodents' working memory measured, for example, by the working memory version of the 8 arm radial maze (Olton and Papas, 1979) or the delayed matching-to-place (DMP) version of the Morris water maze (Morris and Frey, 1997; Steele and Morris, 1999) probably differs from the primates' working memory characterized by sustained firing of "working memory neurons" or "delay neurons" (Goldman-Rakic, 1996). This type of rodent memory is more akin to human episodic memory (Tulving, 1983; Tulving and Markowitsch, 1998), which requires rapid formation of memory traces of unique events in time (single trial or one-time-experience learning) and the ability to distinguish those events from other related events (suppression of interference). However, it is difficult to know whether animals performing these tasks undergo the conscious recall of past unique experience and context, another integral component of human episodic memory. In this study, therefore, we refer to rodents' memory measured in Olton's radial maze task and Morris' DMP task as "working/episodic-like memory." The fact that both working and episodic-like memories rely on the hippocampus implicates the unique ability of this structure in recording on-going life events rapidly and unambiguously (Morris and Frey, 1997).

Pharmacological studies have indicated that NMDA receptor-dependent LTD is mediated by the activation of a protein phosphatase cascade (Mulkey et al., 1993,1994; Kirkwood and Bear, 1994). Following a modest activation of the NMDA receptor during LTD induction, calcineurin may be activated first and in turn activate PP1 by inactivating I-1, a PP1 inhibitor (Lisman, 1989). Calcineurin could also affect synaptic plasticity in other ways. For instance, it could reduce the NMDA receptor channel open time (Lieberman and Mody, 1994; Tong et al., 1995; Shi et al., 2000), which would lead to reduced Ca^{2+} influx through the NMDA receptor and thereby promote LTD induction. Ca^{2+}-activated calcineurin could also associate with dynamin I and dephos-

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phorylate several endocytic proteins (Liu et al., 1994; Marks and McMahon, 1998; Lai et al., 1999) and thereby enhance internalization of AMPA receptors at the postsynaptic spines and promote the expression of LTD (Beattie et al., 2000; Lin et al., 2000).

Calcineurin is a heterodimer consisting of a catalytic subunit and a regulatory subunit (Klee et al., 1998). In the brain, only one regulatory subunit, CNB1, is expressed while two isoforms of the catalytic subunit (CNAα and CNAβ) are expressed with distinct yet overlapping patterns (Takaishi et al., 1991; Kuno et al., 1992). Previously it was shown that CNAα global knockout mice, which retained partial calcineurin activity due to CNAβ, exhibited impairments in water maze learning and fear conditioning. However, the interpretation of learning deficits was complicated by the presence of a variety of other abnormalities (Zhang, 1995). A transgenic mouse line expressing a constitutively active form of CNAα exhibited reduced intermediate-phase LTP and impaired long-term memory (Winder et al., 1998; Mansuy et al., 1998a, 1998b). The same group subsequently reported that in a dominant-negative transgenic line in which calcineurin activity was partially inhibited, LTP was enhanced and so was the learning ability (Malleret et al., 2001). These studies indicated a constraining role for calcineurin in learning; the lower the level of calcineurin activity, the greater the LTP and learning ability. However, LTD was not affected in either of the two mouse lines, presumably because of the incomplete blockade of the endogenous calcineurin activity, and hence a potential role for LTD in learning and memory could not be assessed.

In the present study, we knocked out CNB1, the only known regulatory subunit of brain calcineurin, in the excitatory neurons of the adult mouse forebrain. In the hippocampal Schaffer collateral-CA1 synapses of these conditional knockout mice, there was a significant leftward shift in the LTD/LTP modification threshold, leading to diminished LTD and modestly enhanced LTP across a range of stimulation frequencies, whereas 100 Hz LTP and depotentiation were normal. These physiological phenotypes correlated with a specific deficit in rapid one-trial learning ability required for the formation of working/episodic-like memory in the presence of apparently normal ability in the formation of reference memory. These results revealed a novel distinction in the underlying cellular mechanisms for these two types of learning and suggest that working/episodic-like memory that encodes one time experience is vulnerable to memory interference and is more dependent on a synaptic network with optimal bidirectional plasticity. These results support the view that LTD has a positive rather than a negative role in memory storage.

Results

Generation of the Forebrain-Specific CNB1 Knockout Mice

Two lines of mice, both in C57BL/6 genetic background, were generated—a "floxed" CNB1 line referred to as fCNB1 by ES cell-mediated gene targeting (Figure 1A) and the forebrain-specific, αCaMKII promoter-driven Cre line referred to as CW2 by transgenic approach. When tested with an LSL-lacZ reporter line, CW2 mice gave very similar patterns of Cre/loxP recombination as the previously published αCaMKII promoter-Cre line of a mixed genetic background (i.e., T29-1) (Tsien et al., 1996). That is, the X-gal staining was restricted to CA1 pyramidal neurons of the hippocampus (data not shown). We chose to use CW2 instead of T29-1 in order to avoid confounding effects of a mixed genetic background on behavior (Gerlai, 1996). With crosses between fCNB1 and CW2 mice, we obtained the mutant mice referred to as CN-KO (*fCNB1/fCNB1*, *Cre*/+) and three types of control mice as their littermates: CW2 (+/+, *Cre*/+), wild-type (+/+, +/+), and homozygous fCNB1 (*fCNB1/fCNB1*, +/+). In a series of pilot experiments, we found no significant difference among these three types of control mice, and therefore the data from them were pooled, unless indicated otherwise.

The CN-KO mice appeared grossly normal with no obvious sign of sensory or motor impairments. Staining of brain sections by hematoxylin did not reveal any gross structural abnormalities (see Figure 1B).

Absence of the CNB1 mRNA in Selected Cell Types of the Adult Forebrain

The spatiotemporal pattern of *fCNB1* gene deletion in CN-KO mice was assessed by in situ hybridization (Figure 1B and 1C). The *fCNB1* gene was deleted only in selected cell types in the hippocampus, neocortex, and amygdala (Figure 1B). In the hippocampus, it was deleted in nearly all pyramidal neurons of CA1 and in most granule cells of dentate gyrus, but not in CA3 pyramidal cells (Figure 1C). In the cortex, the *fCNB1* gene was deleted in the neurons of layers 2 and 3, and partially in layers 5 and 6; however, in most neurons of layer 4, its was unaffected (Figure 1B). The gene was also deleted in subiculum (Figure 1C), piriform cortex, and the lateral, basolateral, and basomedial nuclei of amygdala (Figure 1B, coronal sections). The reduction of *fCNB1*-expressing cells could be first detected in hippocampal CA1 (Figure 1C) and in cortex (not shown) at 5 weeks of age, and the above-described deletion pattern emerged at around 2.5 months of age and remained unchanged at least until 5 months of age, the latest time point examined (Figure 1C). The *fCNB1* expression in striatum, cerebellum, and other subcortical regions appeared normal at all ages in mutant mice (Figure 1B). These results showed that the *fCNB1* deletion in CN-KO mice was more widespread than the *NR1* deletion in the previously published CA1-NR1 KO mice (Tsien et al., 1996), even though the Cre/loxP recombination patterns determined by the LSL-lacZ reporter were similar between CW2 and T29-1 (data not shown). This probably reflects the target locus effect on the frequency of Cre/loxP recombination.

Absence of the CNA Proteins in Selected Cell Types

Previous biochemical studies showed that for the CNA subunits to express their catalytic activity, they first have to bind to the CNB regulatory subunit (Merat et al., 1985; Perrino et al., 1992; Sikkink et al., 1995). CNB not only plays a structural role in stabilizing the complex, but also is required for the maximal activation of the phosphatase activity by Ca^{2+}-calmodulin (Stemmer and Klee, 1994).

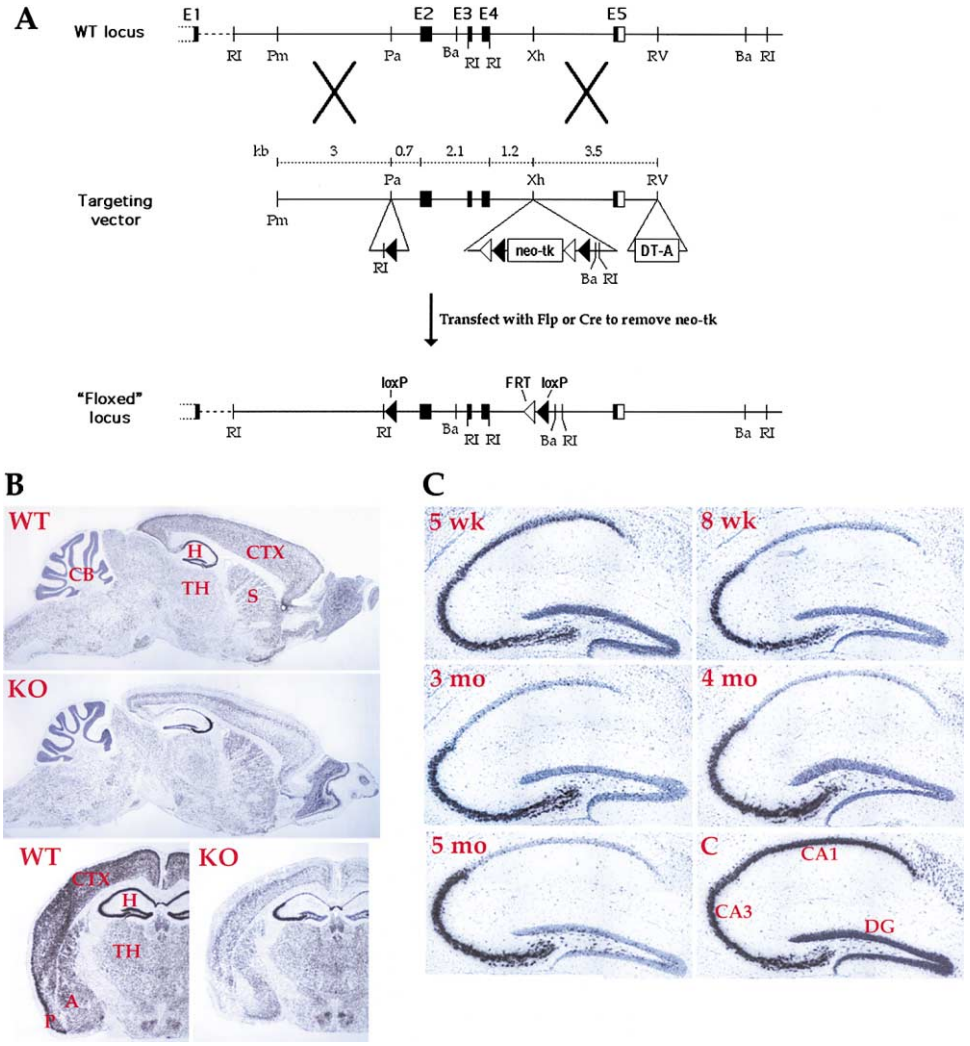

Figure 1. The Generation of Forebrain-Specific Calcineurin Knockout Mice

(A) Schematic diagram of the procedure for generating the fCNB1 ES clones. (Top) Wild-type CNB1 locus. Exons are labeled as E1–E5 in which coding regions are indicated as filled boxes. (Middle) Targeting vector in which a loxP site was inserted in intron 1, a LFNT cassette was inserted in intron 4, and a diphtheria toxin (DT-A) marker was inserted at the 3′ end. (Bottom) Floxed CNB1 locus. Restriction enzymes abbreviations are as follows: RI, EcoRI; Pm, PmeI; Pa, PacI; Ba, BamHI; Xh, XhoI; and RV, EcoRV.

(B) In situ hybridization using a CNB1 probe to demonstrate the Cre-mediated CNB1 gene deletion pattern. Whole brain sections from mice of 4 months of age include sagital sections (top and middle) and coronal sections (bottom). Bright-field images are shown in which silver grains representing mRNA signals are in black and hematoxylin counterstaining is in blue. Abbreviations are as follows: CTX, cortex; H, hippocampus; S, striatum; TH, thalamus; CB, cerebellum; A, amygdala; and P, piriform cortex.

(C) Ontogeny of Cre-mediated CNB1 deletion in the hippocampus. Sections from mutant mice with various ages are shown along with a section from a control mouse of 4 months of age.

Since CNB1 is the sole regulatory subunit in the brain, its deletion would lead to a loss of calcineurin activity. To further examine the expression of CNA and CNB proteins, we performed immunohistochemistry with anti-CNA and anti-CNB antibodies. Consistent with the in situ hybridization data, the CNB protein was undetectable beyond the background level in the cell body layer of CA1 and dentate gyrus and was substantially reduced in cortex, amygdala, and piriform cortex (Figure 2A). In the cortex, CNB was missing in most neurons except for those located in layer 4. This pattern persisted throughout all cortical regions including entorhinal cortex, subiculum, and frontal cortex, which have direct interconnections with the hippocampus. In the amygdala, CNB was missing in almost all neurons in the lateral and basolateral nuclei and partially missing in the basomedial nuclei, but it was present in the other amygdaloid nuclei. Interestingly, the distribution of the CNA protein followed closely that of the CNB protein, as demonstrated in the double staining with both types of antibodies (Figure 2A).

Figure 2B shows the hippocampal distribution of CNA in finer detail, which is identical to that of CNB (data not shown). Since it is known that calcineurin is present in dendrites, axons, and cytosolic parts of soma but not in nuclei (Kuno et al., 1992), the expression pattern in

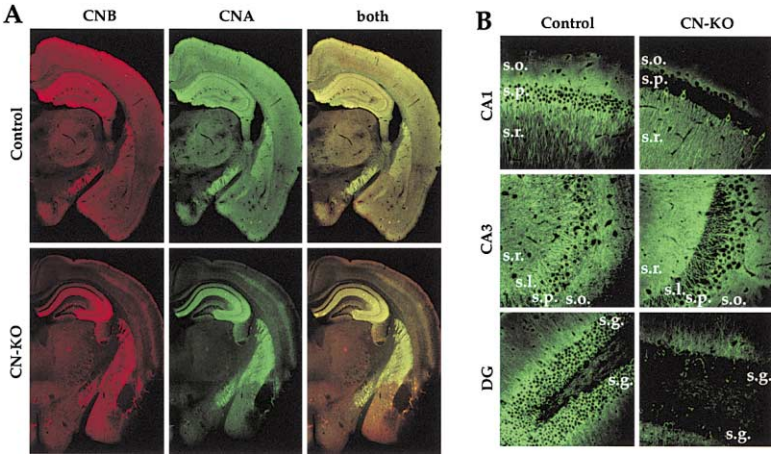

Figure 2. Immunohistochemical Staining with Anti-CNB and Anti-CNA Antibodies

(A) Coronal sections from mice of 5 months of age showing the general distributions of CNB and CNA proteins double-stained with anti-CNB and anti-CNA simultaneously. CNB and CNA proteins exhibit identical patterns in either control (top) or mutant (bottom) sections. In the mutant sections, both proteins are missing in selected cell types of the hippocampus, cortex, and amygdala. Immunostaining of another group of mice of 12 weeks of age exhibited similar patterns (data not shown).

(B) Higher magnification images of the hippocampal subfields showing the loss of CNA protein in CA1 pyramidal cells and dentate gyrus (DG) granule cells but not CA3 pyramidal cells. Abbreviations are as follows: s.p., stratum pyramidale; s.r., stratum radiatum; s.o., stratum oriens; s.l., stratum lucidum; and s.g., stratum granulosum.

the hippocampus could be delineated as follows in light of the known anatomy of the hippocampus. In stratum pyramidale of CA1, CNA was undetectable in the soma of all but a few pyramidal neurons. Consistent with this, there were few stained dendrites in stratum radiatum of CA1. The staining remaining in stratum radiatum and stratum oriens seems to come from the Schaffer collateral axons. In CA3, CNA was present everywhere except stratum lucidum where mossy fibers terminate. In stratum lucidum the dendrites were still stained, indicating that in this area CNA is missing only in the mossy fiber axons. In dentate gyrus, the staining was missing in most granule cells. Thus, our data indicate that deletion of the regulatory subunit, CNB1, leads to a loss of the CNA subunits and hence the calcineurin activity from CA1 and dentate but not from CA3 neurons. The loss of CNA may be due to degradation which may occur in the absence of the CNB subunit.

Impaired LTD But Normal LTP and Depotentiation at the Schaffer Collateral-CA1 Synapse

We carried out a series of electrophysiological recordings with a focus on the Schaffer collateral-CA1 synapse in hippocampal slices. LTD induced by low-frequency stimulation (LFS, 900 pulses at 1 Hz) was significantly reduced in mutant slices (CN-KO: 92% $\pm$ 4%, n = 16 slices from four mice; control: 81% $\pm$ 5%, n = 12 slices from four mice; p = 0.03, Student's t test; Figure 3A). In contrast, no difference between genotypes was observed when LTP was elicited with strong conditioning stimulation (100 Hz, 1 s; CN-KO: 149% $\pm$ 6%, n = 11 slices from six mice; control: 152% $\pm$ 6%, n = 13 slices from six mice; p = 0.73; Figure 3D).

To study plasticity under conditions where the processes of LTP and LTD compete (near the LTD/P modification threshold), we used a series of intermediate stimulation frequencies. A train of 900 pulses at 10 Hz revealed a slight potentiation in CN-KO (109% $\pm$ 9%, n = 10 slices from four mice) but not in control slices (96% $\pm$ 5%, n = 12 slices from four mice; Figure 3B), and a train of 100 pulses at 40 Hz generated LTP that was greater in CN-KO (146% $\pm$ 8%, n = 11 slices from six mice) than in controls (128% $\pm$ 6%, n = 13 slices from six mice; Figure 3C). A two-way ANOVA on these data confirmed that there is a significant main effect of genotype at these frequencies ($F[1,42] = 5.167$, $p < 0.03$).

The consequences of different types of conditioning stimulation are summarized in the frequency-response functions of Figure 3E. The modification function is shifted in the mutant mice, favoring LTP over LTD across a range of stimulation frequencies [highly significant at $p < 0.005$ for stimulation frequencies of 1, 10, and 40 Hz ($F[1,68] = 9.3$, two-way ANOVA)]. Thus, deletion of calcineurin significantly altered the properties of bidirectional synaptic plasticity. Because calcineurin is depleted specifically on the postsynaptic and not on the presynaptic side of the Schaffer collateral-CA1 synapse, these data support an essential role for postsynaptic calcineurin in bidirectional synaptic plasticity.

Previous studies have demonstrated that depotentiation and homosynaptic LTD are both NMDA receptor dependent and have many common properties (Bear and Abraham, 1996). To examine depotentiation, we induced maximal LTP by theta-burst stimulation (TBS), and LFS was delivered to induce depotentiation 30 min after TBS (Figure $3F_1$). To better illustrate the extent of depotentiation, the potentiated fEPSPs from the final 10 min of LTP prior to delivery of LFS were renormalized to baseline and presented in Figure $3F_2$. Synaptic response was depressed to the same extent in both mutant and control slices (CN-KO: 68% $\pm$ 5% of potentiated baseline, n = 13 slices from four mice; control: 71% $\pm$ 3%, n = 12 slices from four mice; p = 0.78, Student's t test; Figure $3F_2$). Thus in mutant slices, the same LFS that was unable to induce normal LTD in naive synapses (Figure 3A) fully reversed LTP when delivered 0.5 hr after TBS (Figures $3F_1$ and $3F_2$). Hence, calcineurin appears to be involved preferentially in synaptic depression de novo, but not in the processes that render LTP vulnerable to disruption for a few hours after induction.

The average stimulating intensity needed to obtain half-maximal fEPSP response was indistinguishable between the mutants and controls (CN-KO: 19.9 $\pm$ 1.4 μA, n = 22; control: 21.7 $\pm$ 1.4 μA, n = 26; p = 0.19, Student's t test), as was the fEPSP slope at half-max (CN-KO: 0.32 $\pm$ 0.02 mV/msec, n = 22; control: 0.36 $\pm$ 0.03 mV/

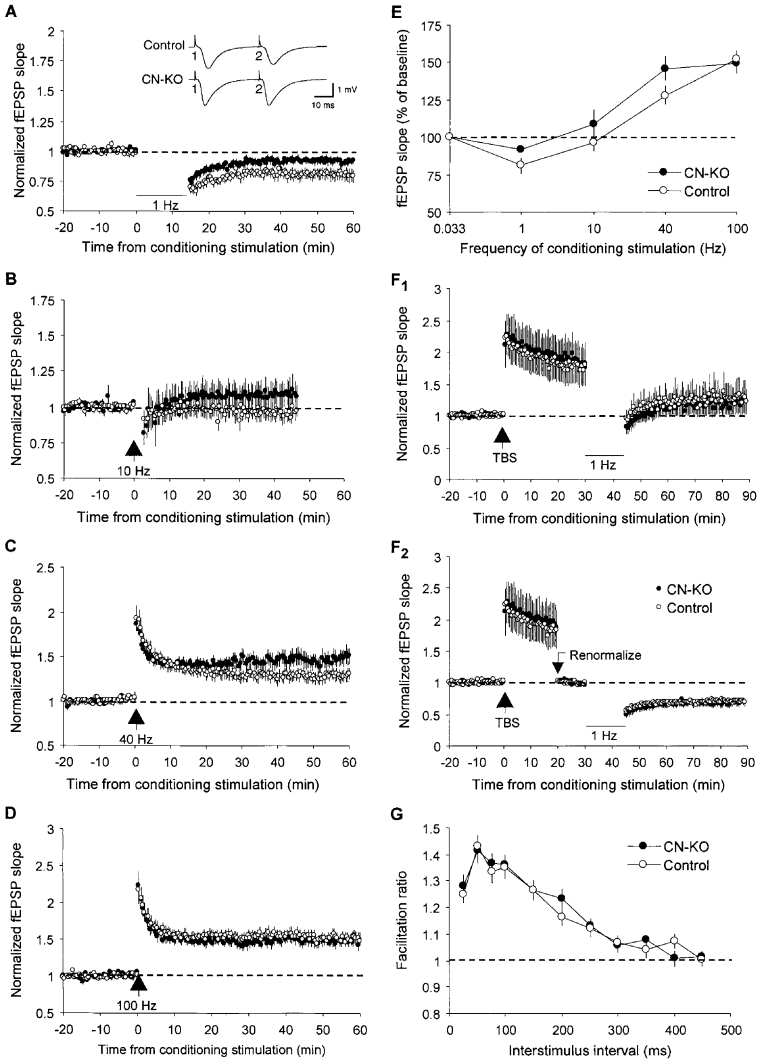

Figure 3. Impaired LTD and Frequency-Dependent Bidirectional Synaptic Plasticity, But Normal 100 Hz LTP and Depotentiation in Schaffer Collateral-CA1 Synapse

Filled circles indicate CN-KO data, and open circles indicate control data.

(A) Stimulation (1 Hz) induced normal LTD in control slices but significantly diminished LTD in mutant slices.

(B) Stimulation (10 Hz) induced modest potentiation in the mutant slices.

(C) Stimulation (40 Hz) induced enhanced LTP in the mutant slices.

(D) Control and mutant slices showed no difference in LTP induced by 1 tetanus of 100 Hz.

(E) Summary of the fEPSP changes with different stimulation frequencies.

(F_1) Following TBS-induced LTP, LFS given at 30 min post-TBS elicits depotentiation of comparable magnitude in both mutant and control slices.

(F_2) Data presented in (F_1) were replotted by renormalizing to baseline the potentiated fEPSPs from the final 10 min of LTP prior to delivery of LFS.

(G) Paired-pulse facilitation was not altered in the mutant slices across all interstimulus intervals.

msec, n = 26; p = 0.12). There was no difference in paired-pulse facilitation at all interstimulus intervals tested (Figure 3G), and PTP also appeared normal in the mutant slices as seen in the 100 Hz (Figure 3D) and 40 Hz (Figure 3C) stimulations. These results suggest that basal synaptic transmission and short-term plasticity are normal in the mutants.

Normal NMDA Receptor Properties

To test whether calcineurin alters the functional properties of the NMDA receptor, we pharmacologically isolated NMDA EPSCs in CA1 pyramidal cells. Hippocampal slices were bathed in ACSF containing CNQX, picrotoxin, glycine, and high concentrations of divalent cation. Cells were voltage-clamped at +40 mV to remove the Mg^{2+} block. Stimulation was adjusted so that evoked currents were near 100 pA. Under these conditions, isolated NMDA currents from mutants (n = 13 cells) demonstrated a typical J-shaped I-V relation (Figure 4A), which did not differ from controls (n = 9 cells) and had a reversal potential near 0 mV. The stimulation intensity used to achieve ~100 pA response also did not differ (data not shown).

To determine the deactivation kinetics of NMDAR-mediated EPSCs, 30–60 evoked EPSCs were averaged, and the weighted time constant τ_w was calculated for each neuron. We found that NMDA current decay did not differ in cells from CN-KO and control mice of 3 months of age (Figure 4B; control n = 13 cells from four mice, τ_w = 119.4 ± 9.8 ms; CN-KO n = 19 cells from four mice, τ_w = 102.5 ± 12.1 ms; p = 0.32, t test).

It has been shown recently that temporal summation of NMDAR-EPSCs is extremely sensitive to even modest changes in NMDA current duration (Philpot et al., 2001). Thus, we used 40 Hz trains of 11 stimulation pulses, given every 6 s for 3 min (for a total of 30 trains), to determine whether the summation of NMDA currents differed in cells from CN-KO and control mice (Figure 4C). Current responses were averaged for all 30 trains. Quantification was performed by comparing the ratios of the average peak EPSC amplitude of each cell's response to the eleventh pulse to the response to the first pulse. At 40 Hz stimulation, cells from both CN-KO and control mice exhibited similar temporal summation (control n = 12 cells from four mice, 11^{th} pulse/1^{st} pulse = 1.84 ± 0.13; CN-KO n = 17 cells from four mice, 11^{th}

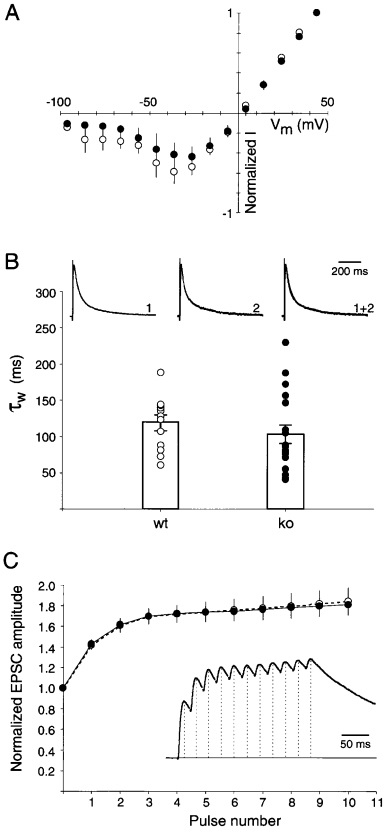

Figure 4. Deletion of Calcineurin Does Not Alter NMDA Receptor Current Properties

(A) I-V curve of normalized NMDA currents from control (open circles) and mutant (closed circles) cells. Voltage was adjusted by the measured junction potential, and currents were normalized to the response at +44 mV.

(B) Scatter plot with mean and SEM of the weighted time constant (τ_w) of the NMDAR EPSC decay. Traces depict normalized NMDAR EPSC from control (1) and mutant (2) mice, as well as an overlay of the two (1 + 2).

(C) Average temporal summation of NMDAR EPSCs to 40 Hz stimulation. Currents were normalized to the peak response evoked by the first pulse. Inset depicts a sample trace, and dashed lines demonstrate how total amplitude was measured for each pulse.

pulse/1st pulse = 1.81 ± 0.10; p = 0.84, Student's t test). Taken together, our data demonstrated that NMDAR channel properties were not altered in the CN-KO mice, in contrast to previous studies using calcineurin inhibitors (Lieberman and Mody, 1994; Tong et al., 1995; Shi et al., 2000). This could reflect the difference between developing and adult animals or between acute and chronic perturbations.

Normal Contextual and Cued Fear Conditioning

We subjected the CN-KO and control mice to a fear-conditioning paradigm. The CN-KO (n = 21) and control mice (n = 21) displayed similar levels of freezing to the context ($F[1,40] = 1.509$, $p = 0.23$) as well as to the tone ($F[1,40] = 0.053$, $p = 0.82$), indicating that the CN-KO mice are not impaired in either contextual or cued fear conditioning (Figure 5A). We also examined whether the knockout would affect memory extinction, a form of

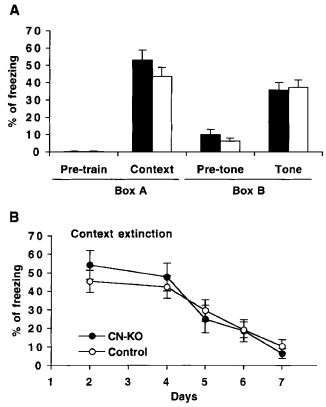

Figure 5. Normal Contextual and Cued Fear Conditioning

(A) Freezing responses after training with three tone-shock pairs. Pretrain indicates the freezing levels in Box A (the shocking chamber) before the onset of training. Context indicates the freezing levels in Box A after training. Pretone indicates the freezing levels in Box B after training and before the tone testing. Tone indicates the freezing levels in Box B during the presentation of tone.

(B) Freezing responses in Box A during the extinction test following the tests for contextual and cued fear shown in (A). Training was given on day 1. Context test was done on day 2. Tone test was done on day 3. Context extinction test was subsequently done from day 4 to day 7.

relearning. For this purpose the mice that had been trained were repeatedly exposed to the training chamber for several days. Mutant (n = 16) and control (n = 17) mice gradually reduced their freezing responses to the context at a similar rate ($F[1,31] = 0.083$, $p = 0.78$, repeated measures ANOVA; Figure 5B). Thus, the mutants do not seem to be impaired in memory extinction.

Intact Spatial Reference Memory in the Morris Water Maze Task

We subjected the mutant and control mice to the standard hidden-platform version of the Morris water maze task (Morris et al., 1982). All groups of mice (CN-KO, n = 21; floxed, n = 15; CW2, n = 11; wt, n = 13) decreased their escape latencies over ten days of training, although control groups achieved a slightly shorter latency than mutants at the end of the training session (Figure 6A). This difference could be due to the slightly slower swimming speed of the CN-KO mice (Figure 6B). Figure 6C shows that the CN-KO mice spent a similar percentage of time near the wall as did the controls, so the mutants did not exhibit a pronounced thigmotaxic or wall-hugging tendency that could have affected their performance. In a probe trial conducted on day 10 (Figure 6D), both mutant and control mice spent significantly more time in the target quadrant than in the other three quadrants ($F[3,174] = 117.84$, $p < 0.0001$, two-way ANOVA), and no genotype-associated difference was observed ($F[1,58] = 0.039$, $p = 0.85$). The average number of times mutant or control mice crossed the platform location during the probe trial was also indistinguishable (Figure 6D). Thus, the spatial reference memory required to perform the water maze task does not seem to be affected by the lack of calcineurin in the forebrain.

To assess the ability of relearning, these mice were

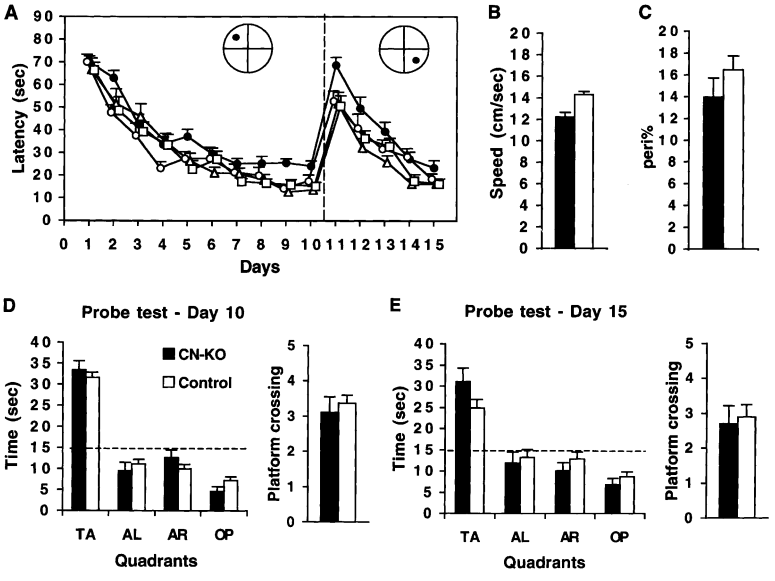

Figure 6. The Hidden Platform Morris Water Maze Task

(A) The 10 day spatial reference memory training plus 5 day reversal training. Escape latencies were averages of four trials for each day. Escape latencies of all three types of controls were shown separately to demonstrate that there was no significant difference among them. Subsequently they were pooled as one single control group. Closed circles indicate mutants, open circles indicate homozygous fCNB1 control, open triangles indicate CW2-Cre control, and open squares indicate wild-type control.

B) Swimming speeds averaged from all trials on days 10 and 11.

(C) Percentage of time spent in the peripheral area of the maze, i.e., a 15-cm-wide zonal area adjacent to the wall. The data presented are averages from all trials on days 10 and 11.

(D) Probe test on day 10, after the first-platform training. Left graph shows the quadrant occupancy. Right graph shows the number of crossings at the original platform location.

(E) Probe test on day 15, after the second-platform training. Left graph shows the quadrant occupancy. Right graph shows the number of crossings at the new platform location. Abbreviations are as follows: TA, target quadrant; AL, adjacent left quadrant; AR, adjacent right quadrant; and OP, opposite quadrant. Dotted lines in all probe tests depict chance level (15 s) in random searching.

subsequently given a 5 day reversal training in which the hidden platform was moved to the opposite quadrant (Figure 6A). CN-KO mice demonstrated a transient deficit in escape latency on the first day of the reversal training (day 11; $p < 0.01$ between CN-KO and any control groups, Student's t test). However, they were able to improve their performance to a level similar to that of the controls by day 15 ($p > 0.1$ between CN-KO and any control groups). The results of the probe trial conducted on day 15 were indistinguishable between genotypes, as scored either by quadrant occupancy or platform-crossing criterion (Figure 6E). These results demonstrate that the mutant mice were impaired in learning the new location of the hidden platform initially but that this deficit could be overcome by repeated training.

Impaired Working/Episodic-Like Memory in Delayed Matching-to-Place Task

The ability to encode on-going events rapidly and unambiguously is essential for the formation of working/episodic-like memory. The impairments observed in the initial stage of the reversal training in the water maze suggested that the CN-KO mice might be deficient in this type of learning. Therefore, we further tested the mice in the delayed matching-to-place (DMP) task, which measures rodents' working or episodic-like memory and has been shown to depend on the integrity of the hippocampus and NMDA receptor activation (Morris and Frey, 1997; Steele and Morris, 1999; Chen et al., 2000).

Mice were trained to navigate to a hidden platform at a fixed location in the water maze until reaching a criterion or finishing a maximal number of trials. This constitutes one training session. Starting on the next day, a new session began in the same manner except that the platform was moved to a new location in the same water maze. A total of six sessions were completed, i.e., six different hidden platform locations were learned sequentially. The reduction of the escape latency in the second trial compared to the first trial ("saving time") within a session reflects the animal's ability to acquire memory for the platform location rapidly based on a single exposure against the interference by the memories of other platform locations acquired in previous sessions.

After several training sessions, control mice were able to rapidly remember the new platform location and reduced their escape latency at a much faster rate than CN-KO mice (Figure 7A). Both CN-KO and control mice showed learning across the first five trials within each session (CN-KO: $F[4,52] = 3.552$, $p = 0.0123$, $n = 14$; control: $F[4,56] = 14.409$, $p < 0.0001$, $n = 15$; two-way repeated measures ANOVA). However, there was a significant difference between genotypes ($F[1,27] = 55.26$, $p < 0.0001$, three-way repeated measures ANOVA) and a significant trial $\times$ genotype interaction ($F[4,108] = 3.444$, $p = 0.011$). Much of the latency differ-

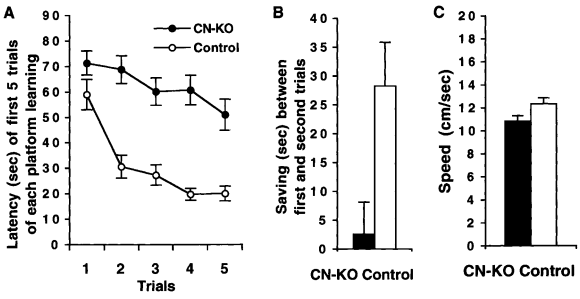

Figure 7. Delayed Matching-to-Place Task

(A) Latencies of the first five trials of new platform training, averaged from the last two training sessions (the fifth and the sixth platform locations). See supplemental data for more data on all training sessions.

(B) The reduction of latencies (saving time) between the first and second trials of each session, averaged from the last two training sessions.

(C) Swimming speeds averaged from the first two trials of the last two training sessions.

ence between genotypes was due to the difference in the saving time (Figure 7B; $p = 0.009$, Student's t test), indicating that CN-KO mice are impaired in one-trial learning. The mutant mice swam slightly slower than the control mice (Figure 7C, $p = 0.033$, t test), but this difference only slightly increased the latency and did not account for the difference in learning ($F[1,26] = 54.01$, $p < 0.0001$, two-way ANCOVA using swim speed as a covariance).

Impairment in the Spatial Working Memory Version of the 8 Arm Radial Maze

We also assessed the effects of the knockout on the spatial working memory version of the 8 arm radial maze task, known to be dependent on the hippocampus (Olton and Papas, 1979; Becker et al., 1980). In each trial the mouse must visit each of the eight arms only once to consume the food rewards most effectively, because reentry of a visited arm is not rewarded. Such a revisit is counted as a working memory error. In this task, animals must rapidly establish and maintain memory of visited arms based on single within-trial exposures and must also suppress interference by the memory obtained during previous trials.

The mice were trained with one trial per day for 16 days. Control mice improved their performance over training ($F[7,70] = 15.459$, $p < 0.0001$, $n = 11$, repeated measures ANOVA), whereas mutant mice did not improve ($F[7,70] = 1.063$, $p = 0.3966$, $n = 11$) and made significantly more revisiting errors than controls ($F[1,20] = 38.687$, $p < 0.0001$; two-way repeated measures ANOVA; Figure 8A). The number of different arm choices in the first eight entries (Figure 8B) is another measure of working memory performance. The number ranges from 5.3 for a chance performance to 8 for a perfect performance. Control mice improved significantly more than CN-KO mice with training ($F[1,20] = 48.512$, $p < 0.0001$).

Figure 8C shows that mutants and controls made similar numbers of omission errors, i.e., visiting an arm but not taking the food, at the beginning of the training but almost none subsequently, demonstrating that the mutants did not have a pronounced motivational impairment. That the mice used a spatial search strategy rather than a nonspatial serial search strategy is demonstrated in Figure 8D, which compares the percentage of each type of successive arm choice expressed by the angle shift between the two arms. For both control and CN-KO mice, the percentage of choices for 45° (adjacent) arms was not greater than that for the other arms ($p > 0.05$, one-tailed paired t test), indicating that neither group used the serial search strategy. Although mutant mice ran slightly faster than controls during training (Figure 8E), the difference in running speed could not account for the nearly complete lack of improvement in working memory ($F[1,19] = 26.50$, $p < 0.0001$, two-way repeated measures ANCOVA using speed as a covariance; also see Miyakawa et al., 2001).

Discussion

In this study, we produced a line of forebrain-specific, adult-onset calcineurin knockout mice and found that they are selectively impaired in hippocampus-dependent one-trial learning, which is essential for the acquisition of working/episodic-like memory, but that they are normal in reference memory acquisition in multitrial tasks. These behavioral impairments were correlated with an LTD deficiency at Schaffer collateral-CA1 synapses and a resulting shift in the properties of bidirectional synaptic plasticity.

Selective Impairments in Working/Episodic-like Memory

Previous studies with human patients indicated that the hippocampus is important for both episodic (event) and semantic (fact) declarative memories (Squire, 1994). In rodents, the hippocampus has long been recognized as a critical structure for encoding spatial information (O'Keefe and Nadel, 1978; Olton and Papas, 1979; Morris et al., 1982), and recent studies have indicated that the rodent hippocampus also plays an important role in carrying out certain nonspatial learning tasks that require temporal (Moyer et al., 1990) or relational (Bunsey and Eichenbaum, 1996) associations. Rodent hippocampus has also been shown to be critically involved in the formation of spatial working memory (Olton and Papas, 1979), and more recently has been suggested to underlie the formation of episodic-like memory (Morris and Frey, 1997; Steele and Morris, 1999; Wood et al., 2000).

In this study, the integrity of reference memory capability of the calcineurin knockout mice was demonstrated with both contextual fear conditioning and the standard hidden platform version of the Morris water maze. On the other hand, the impairment of working/episodic-like memory was revealed in the delayed matching-to-place (DMP) task and in the radial maze task. The reduced saving time (Figure 7B) in the DMP task strongly suggests that the mutant mice are impaired in learning the new location of the platform quickly just by one trial and at the same time suppressing the interfering memory of the previous platform location. Likewise, the inability of the mutant mice to reduce arm revisiting errors as the training proceeds in the radial maze (Figure 8A) indicates that they are impaired in this one-experience learning.

Our data revealed a clear dissociation between reference memory and working/episodic-like memory in the calcineurin knockout mice, thus suggesting an important function of calcineurin in the cellular mechanism underlying the fast one-trial learning while suppressing interfering memory.

How Forebrain Calcineurin May Subserve Selectively Working/Episodic-like Memory

Since Cre-mediated deletion of the *CNB1* gene did not occur substantially until postnatal 5 weeks of age, it is unlikely that the behavioral deficits arose from developmental abnormalities. In human and nonhuman primate studies, both the hippocampal formation and the frontal lobe have been implicated in working and episodic memory (Goldman-Rakic, 1996; Squire and Zola, 1998). Although we focused on the hippocampus for electrophysiological and behavioral studies, the knockout of calcineurin also occurred in some neocortical areas including the frontal cortex. Therefore, we cannot exclude

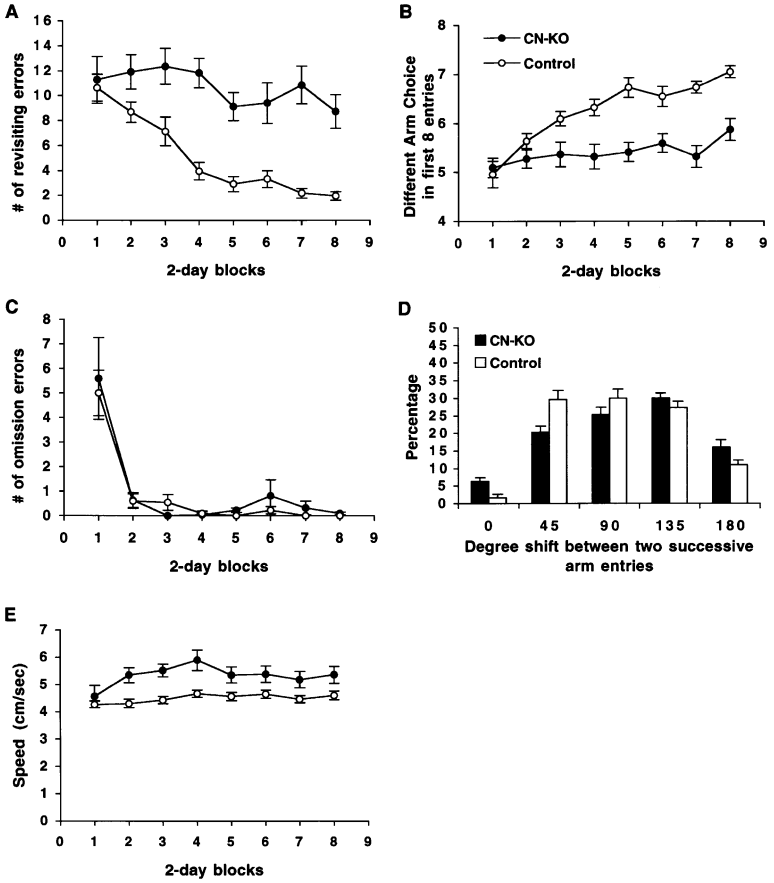

Figure 8. Spatial Working Memory in the 8/8 Version of the 8 Arm Radial Maze

Data were presented as 2 day/trial averages.
(A) Total number of revisiting errors (i.e., working memory errors) across training.
(B) The number of different arms chosen in the first eight arm visits.
(C) Number of omission errors, i.e., arm was visited but food was not taken, as an indication of motivation.
(D) Each arm visit was categorized into one of the five groups according to the angle shift between this arm and the previously visited arm, and the percentage of arm visits contained in each group was shown. This plot is intended to reveal if the animal has taken a serial (nonspatial) search strategy, in which case there would be a high percentage of 45° shift. The data were averages from the last four trials.
(E) Running speed across training.

the possibility that the behavioral deficits were caused by the absence of calcineurin in brain areas other than the hippocampus.

How does calcineurin in the adult forebrain selectively subserve working/episodic-like memory? Calcineurin could potentially contribute to the regulation of basal synaptic transmission by regulating the steady-state level of AMPA receptor density at postsynaptic spines (Beattie et al., 2000; Lin et al., 2000) or the properties of NMDA receptors (Lieberman and Mody, 1994; Tong et al., 1995; Shi et al., 2000). However, we have shown that the basal AMPA and NMDA receptor-mediated synaptic transmission is unaltered in the CN-KO mice as compared to controls. Pharmacological studies also suggest that calcineurin can regulate $GABA_A$ receptor-mediated transmission; however, these reports are contradictory (cf. Jones and Westbrook, 1997 and Lu et al., 2000). Although we did not directly examine $GABA_A$ receptor properties and inhibitory synaptic transmission in CN-KO mice, we observed no hyperexcitabilty (e.g., multiple population spikes) or alterations in the input-output function that would be expected from altered inhibition. Thus, taken together, our data do not support the hypothesis that a major alteration at the level of basic synaptic transmission in CN-KO mice accounts for the observed behavioral deficits.

While we cannot exclude other less well defined neuronal functions of calcineurin, such as those in transcriptional regulation (e.g., Bito et al., 1996) and cytoskeleton stabilization (Halpain et al., 1998) as the mechanisms underlying the behavioral phenotype, our favorable hypothesis is that an impairment in the rapid formation of unambiguous neural representations, caused by the reduced dynamic range of bidirectional modification, is responsible for the selective behavioral deficits in the CN-KO mice. Mathematical analysis of models of distributed matrix memory systems shows that the learning rate and the number of unambiguous activity patterns are greater in a network with bidirectional modifiability of synaptic strength than in a network with unidirectional modifiability (Willshaw and Dayan, 1990). Consequently, the information storage efficiency of a network with only LTP capability would be lower than that of a network with both LTP and LTD capabilities. In the former type of network, which is present in CA1 and presumably some other forebrain areas of CN-KO mice, ambiguity and interference are greater among the multiple memory traces. This limitation would be manifested particularly strongly in one-trial or one-experience learning required for the formation of working/episodic-like memory.

Calcineurin and LTD

How did the conditional knockout of calcineurin lead to the impaired LTD in CN-KO mice? Previous pharmacological studies suggest two possibilities: either enhanced NMDA receptor function, facilitating the processes of LTP that negate LTD, or disruption of the signaling pathway by which an elevation in postsynaptic calcium triggers LTD. Our finding of comparable NMDA

receptor-mediated EPSCs in CN-KO and control mice lead us to favor the second hypothesis.

Two potential mechanisms by which calcineurin could trigger LTD have emerged from recent studies, both related to regulation of AMPA receptors. First, a recent study showed that LTD and dedepression are associated with the dephosphorylation and phosphorylation, respectively, of a PKA phosphorylation site on the GluR1 subunit of AMPA receptors (Lee et al., 2000). The role of calcineurin in LTD, therefore, may be to dephosphorylate GluR1, directly or indirectly, at the PKA site. This model is consistent with the antagonistic effects of PKA and calcineurin suggested in other studies (Coghlan et al., 1995; Winder et al., 1998) and with recent data suggesting that PKA and calcineurin are anchored together in close proximity with the C-terminal tail of GluR1 (Coghlan et al., 1995; Colledge et al., 2000). A second possibility, not mutually exclusive with the first, is that calcineurin triggers LTD by stimulating AMPA receptor endocytosis (Beattie et al., 2000; Lin et al., 2000). Whether diminished AMPA receptor dephosphorylation or endocytosis accompanies the LTD impairment in CN-KO mice remains to be studied.

Unexpectedly, the CN-KO mice provided a clear genetic dissection of the mechanisms of LTD and depotentiation. A number of previous studies of LTD and depotentiation had revealed many similarities. For example, both forms of plasticity are induced by similar types of low-frequency stimulation, depend on NMDA receptor activation for induction, and are sensitive to inhibitors of protein phosphatases (reviewed by Bear and Abraham, 1996). However, recent data suggest mechanistic distinctions. For example, while LTD and dedepression are associated with changes in phosphorylation of Ser845 on GluR1, a PKA substrate, depotentiation and LTP are associated with changes in phosphorylation of Ser831, a CaMKII/PKC substrate (Lee et al., 2000). These and other results suggested a model in which different kinases are responsible for LTP and the reversal of LTD. The current findings now suggest that different phosphatases are responsible for LTD and the reversal of LTP. The important conclusion is that distinct signaling cascades regulate bidirectional synaptic modifications, depending on the initial state of the synapse.

A Comparison with Other Genetically Modified Mice

Previously, it was reported that a global knockout of PSD95 caused a severe impairment of hippocampal LTD and an enhanced LTP across the whole range of stimulation frequency (Migaud et al., 1998). In contrast to our conditional calcineurin knockout mice, these knockout mice exhibited a profound impairment of spatial reference memory in the Morris water maze task. It is possible that the reference memory deficit observed in the PSD95 knockout mice is attributable to the robust upward shift of the LTD/LTP modification function; perhaps the basal level of synaptic transmission is so enhanced that the memory encoding system may be generally disrupted in these mice. Unlike in the PSD95 mice, a correlation between enhanced hippocampal LTP and impaired reference memory did not occur in the dominant-negative calcineurin transgenic mice (Malleret et al., 2001). Instead, in these mice an enhanced ability to induce LTP with one train of 100 Hz stimulation was correlated with enhanced spatial reference memory and recognition memory.

The phenotype of the dominant-negative calcineurin transgenic mice differed from what we report here for the forebrain-specific CN-KO mice. While the calcineurin protein was undetectable in the CA1 pyramidal neurons of our knockout mice by immunohistochemistry (Figure 2), substantial endogenous calcineurin activity remained in the dominant-negative calcineurin transgenic mice (Malleret et al., 2001), which may be related to the impaired and normal LTD in the knockout and transgenic mice, respectively. The different level of inhibition of calcineurin and the different LTD phenotypes may, in turn, explain the different behavioral phenotypes of their transgenic mice and our knockout mice (enhanced versus normal reference memory in the Morris water maze task and normal versus impaired working memory in the 8 arm radial maze task). On the basis of the observations that both LTP and reference memory are enhanced, Mansuy and colleagues focused on the possible role of calcineurin as a negative regulator of LTP and memory. However, they could not assess the role of calcineurin in memory as a positive regulator of LTD.

In this study, we addressed a broader role of calcineurin in the forebrain by using a conditional null mutant. While our data do not support the hypothesis that augmented LTP correlates with enhanced reference memory, we demonstrated a positive correlation between working/episodic-like memory and calcineurin's functions in LTD and bidirectional plasticity. Bidirectional synaptic modification seems to be critical for one-trial learning-based working/episodic-like memory, although unidirectional synaptic modification may be sufficient for multi-trial learning-based reference memory. In this view, LTD is not a mechanism that constrains memory. On the contrary, in conjugation with LTP, it constitutes the basis for an effective distributed memory system.

Experimental Procedures

More detailed description of experimental procedures is available in the supplemental data at http://www.cell.com/cgi/content/full/107/5/617/DC1.

Production of Mouse Lines

The fCNB1-targeting vector was constructed such that the three major coding exons, E2, E3, and E4, were flanked by a loxP site and an LFNT cassette (Figure 1A). The LFNT cassette consisted of a pair of loxP sites, a pair of FRT sites, and pgk-*neo* and hsv-*tk* selection markers. A C57BL/6 ES cell line (Kontgen et al., 1993) was transfected with this targeting vector to obtain homologously recombined clones. Two such ES clones were then transfected with either Cre (pMC1-Cre) or Flp (pOG44, Stratagene, in which a mutation at the 70th residue [Phe] was corrected) to delete the *neo*/*tk* marker genes, in order to eliminate any effects the marker genes may have on the normal expression of CNB1. After this, only a single loxP site and FRT site remained at the locus. Seven of the resulting ES clones were then injected into Balb/c blastocysts, and several chimeric mice from two independent ES clones were obtained. The chimeras were bred with C57BL/6 mice, and progeny (F1) containing the recombinant allele (fCNB1) were obtained. The CW2 transgenic line was produced by pronuclear injection of the αCaMKII-Cre DNA construct (Tsien et al., 1996) into C57BL/6 zygotes and was selected for use from a number of similar transgenic lines based on its most restricted recombination pattern as assayed by crossing to a LSL-

lacZ reporter line. One of the two independently derived fCNB1 lines was crossed with CW2 to obtain the conditional knockout mice for all studies in this paper.

In Situ Hybridization

Fresh frozen sections of mouse brains were fixed with 4% paraformaldehyde and hybridized with a ^{33}P-labeled cRNA probe transcribed from a pSP72 plasmid (Promega) containing a CNB1 cDNA fragment, which consists of exons 3 and 4.

Immunohistochemistry

Paraffin sections were made from 5-month-old male mice perfused with Bouin's fixative (Sigma). The sections were stained with a mixture of rabbit anti-CNB antibody (Upstate Biotechnology, 1:20 dilution) and two types of mouse anti-CNA antibodies (Transduction Laboratories, 1:20 dilution; and PharMingen, 1:50 dilution), both of which can recognize both CNAα and CNAβ subunits, and then with a mixture of secondary antibodies, Alexa 594-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG (both from Molecular Probes).

Extracellular Recordings

Hippocampal slices from 12- to 15-week-old male mice were prepared as described (Lee et al., 2000). Slices were placed in an interface chamber maintained at 32°C. Synaptic responses were evoked by stimulating Schaffer collaterals and were recorded extracellularly in CA1 stratum radiatum. Baseline responses were obtained by stimulating at 0.033 Hz using an intensity that yielded a half-maximal field-potential slope. All conditioning stimulation was at the same intensity as baseline. For the theta-burst LTP before depotentiation, four episodes of TBS were delivered at 0.1 Hz. TBS consists of ten stimulus trains delivered at 5 Hz, with each train consisting of four pulses at 100 Hz. Average values of conditioned fEPSP slope changes, expressed as the percentage of initial baseline response, are measured 1 hr after conditioning stimulation, except for 45 min after 10 Hz stimulation, and averaged across the last 10 min.

Intracellular Recordings

Hippocampal slices, with CA3 removed, were prepared from 3-month-old male mice and recorded as described (Philpot et al., 2001). Slices were placed in a submersion chamber maintained at 30°C. Voltage-clamp recordings were performed in the whole-cell configuration using a patch-clamp amplifier (Axoclamp 1D, Axon Instruments), and data were acquired and analyzed using a system from DataWave Technologies. EPSCs were evoked from a stimulating electrode placed in the Schaffer collaterals, and stimulation was given for 200 μs every 6 s. Recording properties were similar in cells from control and CN-KO mice (data not shown). The deactivation kinetics of NMDAR EPSCs was calculated as the current duration (τ_w) as described (Philpot et al., 2001).

Fear Conditioning

On the training day (day 1), each mouse was placed in a shocking chamber (Coulbourn Instruments) (Box A), and 160 s later, three tone-shock pairs were given at 1 min intervals. Each tone-shock pair consists of a 20 s white noise tone (CS) followed by a 1 s foot shock (US) at 0.75 mA. Afterwards, the mouse remained in the chamber for 60 s before being returned to home cage. On day 2, each mouse was placed back in Box A for 6 min for the measurement of freezing to the context. On day 3, each mouse was put in a white plexiglass chamber (Box B), and 160 s later, three 20 s tones were delivered at 1 min intervals. Freezing during the first 160 s was "pretone" in Box B, i.e., response to an unconditioned context, and freezing in the next 3 min and 20 s was the response to the tone. Context extinction trials were conducted on days 4–7 in which each mouse was placed back to Box A for 6 min per day. Freezing, i.e., complete lack of mobility in any parts of the body during a 1 s period, was scored every 3 s by the experimenter who was blind to the genotype of the mice. For this and all the following behavioral experiments, 3- to 5-month-old male mice were used.

Morris Water Maze Task

The pool was 160 cm in diameter and was made opaque by covering the water surface with tiny resin beads (Hanna Resin Distribution, MA). Water temperature was kept at room temperature (19°C). The platform was 15 cm in diameter. Each mouse was trained four trials per day with intertrial intervals of 30–60 min. In each trial the mouse was allowed to swim until it found the platform, or until 90 s had elapsed, at which point the mouse was guided to the platform. The mouse was then allowed to sit on the platform for 30 s before being picked up. Each probe trial was for 60 s. No pretraining was given in this reference memory task.

Delayed Matching-to-Place (DMP) Task

The DMP task was conducted as described (Chen et al., 2000), with minor modifications. The mice were first pretrained to a visible platform for 3 days, with four trials per day and intertrial intervals of 20–40 min. The mice were then trained repeatedly to navigate to a hidden platform of a fixed location until reaching a rigorous criterion of three consecutive trials with an average escape latency of less than 20 s, or until completing a maximum of 24 trials. Each mouse was given up to eight trials per day with intertrial intervals of 10 min. If a mouse reached criterion in fewer than five trials, it was continually trained to complete five trials, so that a complete set of latency data of all mice could be obtained for the first five trials. After the training was completed (which took up to three days), starting with the next day, the mice were trained to a new hidden platform location in the same manner as the training to the first location except that the maximal number of trials given was reduced to 16. This protocol was repeated four more times until a total of six platform locations were learned. All water maze experiments, including this DMP task and the above reference memory task, were videotaped, and the tapes were later digitized and analyzed with the Image WM software (O'Hara & Co).

8 Arm Radial Maze Task

The 8 arm radial maze task was conducted as described (Miyakawa et al., 2001). The maze was surrounded by prominent distal cues. During training, all eight arms were baited with one hidden food pellet each. A mouse was placed on the central platform and allowed to enter any one of the eight arms to get food. Automatically controlled guillotine doors for all eight arms were shut down as soon as the mouse had visited one arm and returned to the central platform. The mouse was confined in the central platform for 5 s before the doors were reopened for the next arm choice. This 5 s delay forces the mouse to use spatial strategy to remember which arms have been visited instead of adopting a simpler serial searching strategy. A trial was terminated immediately after all eight pellets were consumed or 15 min had elapsed. The animals went through one trial per day for a total of 16 trials. Data acquisition, control of guillotine doors, and data analysis were performed by Image RM software (O'Hara & Co).

Animal Handling, Experimental Design, and Data Analysis

All procedures involving mice were performed in compliance with National Institutes of Health, Massachusetts Institute of Technology, and Brown University guidelines. All experiments were conducted in a blind fashion. The genotypes were decoded only after all data had been collected and, in electrophysiological studies, after the data was analyzed for each individual slice. Statistical analyses were conducted using Excel, StatView, or SAS. Values were expressed as mean ± SEM.

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