Report

Lack of DREAM Protein Enhances Learning and Memory and Slows Brain Aging

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Summary

Memory deficits in aging affect millions of people and are often disturbing to those concerned. Dissection of the molecular control of learning and memory is paramount to understand and possibly enhance cognitive functions. Old-age memory loss also has been recently linked to altered Ca^{2+} homeostasis. We have previously identified DREAM (downstream regulatory element antagonistic modulator), a member of the neuronal Ca^{2+} sensor superfamily of EF-hand proteins, with specific roles in different cell compartments [1, 2]. In the nucleus, DREAM is a Ca^{2+}-dependent transcriptional repressor, binding to specific DNA signatures [1, 3], or interacting with nucleoproteins regulating their transcriptional properties [4–6]. Also, we and others have shown that dream mutant ($dream^{-/-}$) mice exhibit marked analgesia [7, 8]. Here we report that $dream^{-/-}$ mice exhibit markedly enhanced learning and synaptic plasticity related to improved cognition. Mechanistically, DREAM functions as a negative regulator of the key memory factor CREB in a Ca^{2+}-dependent manner, and loss of DREAM facilitates CREB-dependent transcription during learning. Intriguingly, 18-month-old $dream^{-/-}$ mice display learning and memory capacities similar to young mice. Moreover, loss of DREAM protects from brain degeneration in aging. These data identify the Ca^{2+}-regulated "pain gene" DREAM as a novel key regulator of memory and brain aging.

Results and Discussion

Although early studies with $dream^{-/-}$ mice did not find cognitive impairment via the Morris water maze task [7], electrophysiological analysis in hippocampal slices showed that dream mutant mice exhibit enhanced long-term synaptic efficacy (LTP) [8], electrophysiological changes implicated in learning and memory [9, 10], suggesting that $dream^{-/-}$ mice may exhibit some alterations in learning and memory. To test this hypothesis, we submitted dream mutant mice to more stringent tasks to evaluate learning and memory enhancement. The object recognition (OR) memory test is a one-trial model of learning and memory involving the perirhinal cortex and hippocampus [11, 12]. When $dream^{-/-}$ and wild-type littermate mice received a 15 min training session, no differences were observed in short-term memory (STM) assessed 1 hr after training nor in long-term memory (LTM) assayed 24 hr after training (Figure 1A). Intriguingly, with shortened training session, $dream^{-/-}$ mice exhibited remarkably increased STM as well as significantly enhanced LTM (Figure 1A). The exploration times in short and long training protocols for the OR memory test were comparable between $dream^{-/-}$ and wild-type control mice (Table S1 available online). To extend our results to associative learning, we performed trace eyeblink classical conditioning (EBCC), a hippocampal-dependent task, and fixed-interval operant conditioning tests [13], a non-hippocampal-dependent learning test. In both conditioning paradigms, $dream^{-/-}$ mice learned the two tasks significantly faster than did wild-type mice (Figures 1B and 1C). We further tested information consolidation 10 days after the last learning session in trace EBCC. Wild-type mice showed lower retention indexes than did $dream^{-/-}$ mice (Figure 1B), indicating that loss of DREAM leads to prolonged memory functions.

We next performed learning paradigms related to novel taste food intake [14] because it permits the study of different aspects of learning and memory processes, including erasure. Whereas first exposure to the new taste was characterized by neophobia in both mutant and control mice, food intake was nearly doubled at the second access in wild-type and $dream^{-/-}$ mice (Figure 1D). Another attribute of taste learning is the ability to form aversions when novel taste is paired with a malaise-inducing agent (LiCl). Intakes of the novel taste were significantly suppressed after a single LiCl administration in $dream^{-/-}$ mice relative to wild-type mice (Figure 1E). Only after a second LiCl administration was the aversion to the novel taste intake similar between wild-type and $dream^{-/-}$ mice. Finally, we assessed the time required to forget the novel taste-LiCl association. Intriguingly, whereas wild-type mice already forgot the adverse stimulus 3 days after aversion acquisition, $dream^{-/-}$ mice required 10 days to forget the novel taste-LiCl association (Figure 1F). These data show that loss of DREAM expression results in a marked and generalized improvement in learning and memory controlled by different neuronal circuits.

DREAM is expressed in the hippocampus [1], a brain area involved in learning and memory consolidation [15]. To study hippocampal electrophysiology in $dream^{-/-}$ mice, we implanted stimulating electrodes into Schaffer's collaterals and a recording electrode in apical dendrites of the CA1 field (Figure 2A). The analysis of basal synaptic transmission by paired-pulse facilitation (PPF), a short-term enhancement of glutamate synaptic efficacy, with interpulse intervals from 50 to 200 ms did not show any differences between wild-type and $dream^{-/-}$ mice (Figure 2B). We next examined the role of DREAM in LTP of synaptic transmission evoked in the CA3-CA1 synapse in behaving mice. When we used a single or three high-frequency stimulation protocols (HFS, consisting of five trains at 200 Hz, lasting 100 ms, and presented at a rate

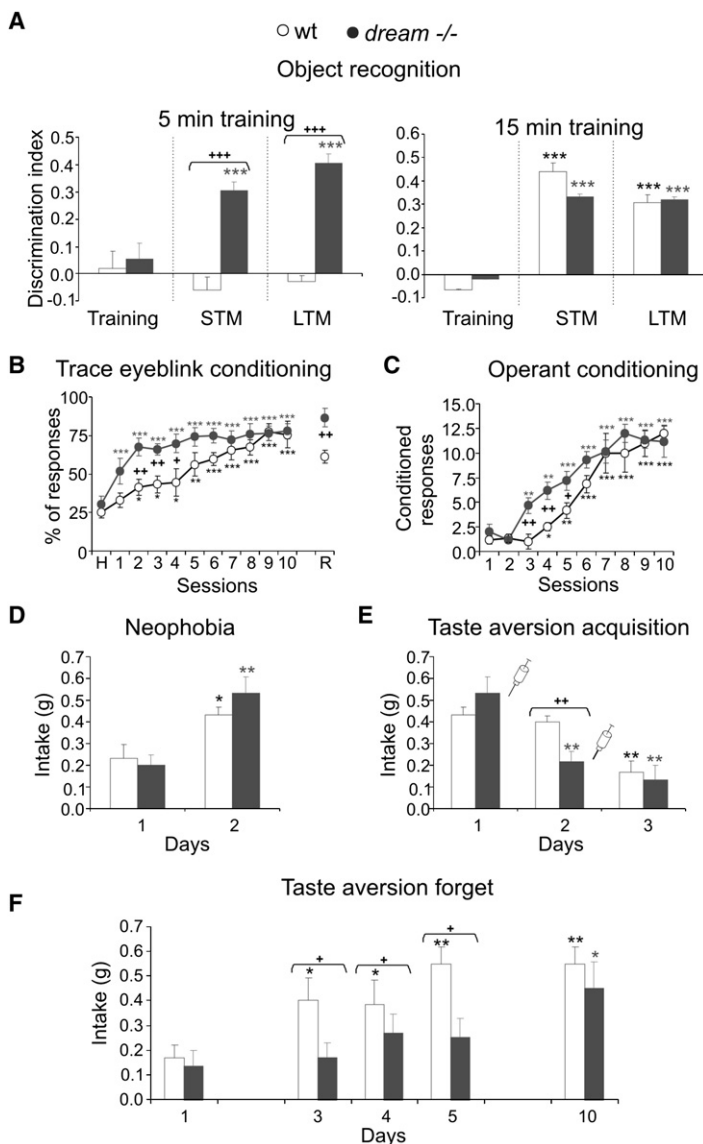*Correspondence: josef.penninger@imba.oeaw.ac.at (J.M.P.), amancar@upo.es (Á.M.C.)

Figure 1. Enhancement of Learning and Memory in $dream^{-/-}$ Mice

(A) The OR memory test was performed in $dream$ mutant and control wild-type (WT) mice after 5 and 15 min training sessions. Discrimination indices calculated as $[t_{novel} - t_{familiar}]/[t_{novel} + t_{familiar}]$ during training; short-term memory (STM) and long-term memory (LTM) (1 and 24 hr after training, respectively) are shown. n = 8 for each group.

(B) Percent of responses per session across the whole training, via a trace electrical shock-SHOCK eyeblink classical conditioning paradigm. H, habituated session. Numbers on x axis indicate different conditioning sessions. Recall sessions (R) were performed 10 days after the last conditioning session. n = 8 for each group.

(C) Number of conditioning responses per session with 12 s fixed-interval operant conditioning training. n = 10 for the two groups.

(D–F) Number of grams of coconut bar intake in each session of taste neophobia (D), taste aversion acquisition (E), and the forgetting phase (F). Syringe indicates LiCl administration. n = 8 for both groups.

In all panels, white and gray colors represent wild-type (WT) and $dream^{-/-}$ littermate mice, respectively. All data are shown as mean values ± SEM. Plus sign indicates statistical significance of the difference between wild-type and $dream^{-/-}$ mice in the same session; asterisk indicates statistically significant differences between each session with respect to the first session in the same experimental group. Single plus sign or asterisk indicates $p \leq 0.05$; two plus signs or asterisks indicates $p \leq 0.01$; and three plus signs or asterisks indicates $p \leq 0.001$.

of 1/s), only short-term changes in synaptic efficacy were detected in wild-type mice (Figures 2C and 2D). However, the same stimulation protocols produced a sustained response in $dream^{-/-}$ mice (Figures 2C and 2D). Importantly, in the $dream^{-/-}$ mice, even a single HFS train was sufficient to produce an overall LTP that was similar to that produced by six HFS trains in $dream^{-/-}$ or wild-type mice (Figure 2E). These results indicate that long-term synaptic plasticity evoked by HFS trains is facilitated in $dream^{-/-}$ mice.

Prolonged changes in synaptic efficacy and memory involve nuclear-dependent processes [16, 17]. Administration of anisomycin, a potent protein synthesis inhibitor, before a HFS and a 5 min OR memory training session in $dream^{-/-}$ mice reduced the lasting LTP (L-LTP) induced by one HFS train and LTM facilitation, without alterations in early LTP (E-LTP; Figure S1). Thus, learning and E-LTP facilitation in $dream^{-/-}$ mice appear to be independent of protein synthesis, but enhancement in memory consolidation and L-LTP requires de novo protein synthesis. DREAM is a transcriptional repressor that is bound to the DRE site [1, 3, 4]. DREAM-dependent transcription occurs by removal of DREAM from DRE sequences. We therefore wanted to assess whether training for OR memory affects DREAM binding to DRE sites in the mouse hippocampus. Nuclear extracts from the hippocampus gave a band in electrophoretic mobility shift assays (EMSA) that could be supershifted by an DREAM antibody and was absent in nuclear extracts from $dream^{-/-}$ mice (Figure 3A). To study the effect of different training times for OR memory on DREAM activity, nuclear extracts were prepared from the hippocampus of wild-type mice 30 min after habituation (untrained) and after 5 or 15 min OR training sessions. Interestingly, animals trained for 15 min showed a marked reduction in DREAM binding activity to DRE sequences in the hippocampus nuclear extracts as compared to untrained mice. DREAM protein levels in the nuclear extracts did not change over the time course studied (Figure 3B). These results show that synaptic activity induced by learning modulates DREAM activity.

It has been reported that DREAM interacts with CREB [5], a pivotal transcription factor involved in memory [16, 17]. CREB binds to cAMP-response element sequences (CRE site) [18]. To attain full transcriptional activity, CREB interacts with the histone acetyl transferase enzyme CBP dependent on Ser-133 phosphorylation [19]. In vitro, unphosphorylated CREB interacts with DREAM to abolish CREB-CBP interaction in a Ca^{2+}- and PKA-dependent manner [5] (Figure S2). To study the DREAM-CREB-CBP interaction in vivo during OR memory, we performed EMSA with nuclear extracts collected from hippocampi of wild-type and $dream^{-/-}$ mice 30 min after different OR training session times in the presence or absence of antibodies against CREB, phospho-CREB (P-CREB), DREAM, and CBP (Figure 3C). In untrained and 5 min-trained wild-type mice, CREB interacted with DREAM as shown by a supershifted band in the presence of the DREAM antibody. A 15 min training provoked CREB phosphorylation and CBP recruitment whereas the association between DREAM and CREB was lost (Figure 3C). In $dream^{-/-}$ mice, the same assay
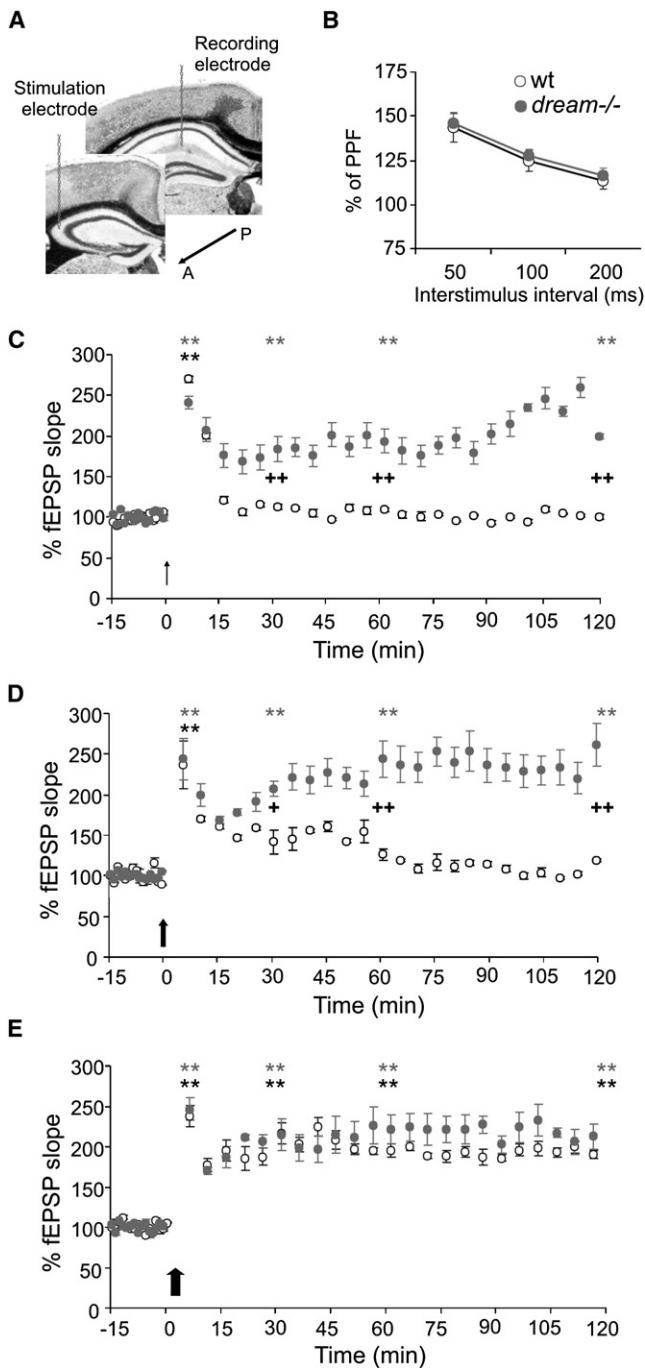
Figure 2. Loss of DREAM Facilitates Late LTP in the Hippocampus

(A) Schematic representation of sites where the stimulating and recording electrodes, aimed to activate CA3-CA1 synapses in the hippocampus, were implanted.

(B) Basal neurotransmission was measured by paired pulses with interpulse intervals from 50 ms to 200 ms. The percentage of paired-pulse facilitation (PPF) was calculated as (slope S2/slope S1) × 100.

(C and D) Changes in the slope of EPSPf provoked by single (C) and three (D) high-frequency stimulations (HFS, five trains [200 Hz, 100 ms] of pulses at a rate of 1/s). Note that the evoked L-LTP lasted for up to 2 hr in $dream^{-/-}$ mice (gray), but not in wild-type (WT) littermates (white).

(E) Six HFS trains induced L-LTP (lasting more than 2 hr) in wild-type and $dream^{-/-}$ mice. n = 6 for each group.

All data are shown as mean values ± SEM. Plus sign indicates statistically significant difference between wild-type and $dream^{-/-}$ mice at different times after HFS; asterisk indicates statistically significant differences at 5,

showed that in untrained animals, the specific band for the CRE site is formed by CREB; importantly, CREB phosphorylation and CBP recruitment to CREB required only 5 min of training (Figure 3C). These biochemical data show that loss of DREAM expression lowers the threshold for CREB phosphorylation and CREB-CBP interactions in OR learning.

To further address the functionality of CREB-DREAM-CBP interactions in learning and memory, we crossed a CRE-lacZ transgene [20], where the lacZ reporter is expressed under the control of a CREB-dependent promoter, onto a $dream$ mutant background to generate $CRE-lacZ^{+/-}$ $dream^{-/-}$ mice. First, we studied lacZ mRNA expression in the hippocampus of $CRE-lacZ^{+/-}$ $dream^{+/+}$ and $CRE-lacZ^{+/-}$ $dream^{-/-}$ mice after different new environment exploration times. Expression of lacZ in a DREAM wild-type background was detected only when mice were submitted to 15 min of exploration (Figure 3D). In the $dream$ mutant background, we detected high levels of lacZ mRNA already after 5 min of exploring the new environment (Figure 3D). These results confirmed that already a short-term exploration stimulus can trigger CREB-regulated transcription in $dream^{-/-}$ mice. We next assayed whether the CREB-regulated immediate early genes c-fos, bdnf, and c-jun, which have been previously implicated in long-term synaptic plasticity [21–24], were also induced by 5 min training in $dream^{-/-}$ mice. Semiquantitative RT-PCR to study the levels of mRNA for c-fos and bdnf (Figure 3D) and immunohistochemistry to examine c-Fos and c-Jun protein (Figure 3E) revealed that wild-type mice required 15 min of training to reach a robust increase of c-fos, bdnf, and c-jun mRNA and protein expression in the hippocampus. In $dream^{-/-}$ mice, 5 min of training was sufficient to trigger robust c-fos, bdnf, and c-jun expression in the hippocampus (Figures 3E and 3F). These data indicate that DREAM functions as a negative regulator of CREB-dependent transcription and that DREAM sets the threshold for CREB activation in learning and memory.

Adult (6-month-old) $dream^{-/-}$ mice exhibit marked improvements in cognitive capacities. Because learning and memory deficits are common in aging, we wanted to test whether DREAM might also confer improved cognition in aged mice (18 months old). With a 15 min training session for OR memory, only aged $dream^{-/-}$ mice remembered the information both 1 hr and 24 hr after training, reflecting STM and LTM (Figure 4A). The exploration times in each session of the OR memory test were not different between aged wild-type and $dream^{-/-}$ mice (Table S2). Thus, loss of DREAM not only improves learning and memory in young mice but also confers improved cognition in old age.

At the cellular level, deficiencies in learning and memory processes in aged mice correlate with morphological and functional changes in the hippocampus [25, 26]. Normal aging process in wild-type mice results in a decrease in the hippocampus:brain area ratio characterized by loss of NeuN-positive neurons and reduced expression of synaptic proteins such as MAP2 and synaptophisin (Figures 4B and 4C). Furthermore, aging results in impaired glutamatergic neuronal transmission as indicated by reduced expression of NMDA receptors (Figure 4C). As reported previously [27], aging mice also develop gliosis as detected by an increase in GFAP-expressing astrocytes (Figure 4D) and the expansion of cd11b-positive

30, 60, and 120 min after HFS with respect to baseline values in the same group. Single plus sign or asterisk indicates p ≤ 0.05; two plus signs or asterisks indicates p ≤ 0.01.
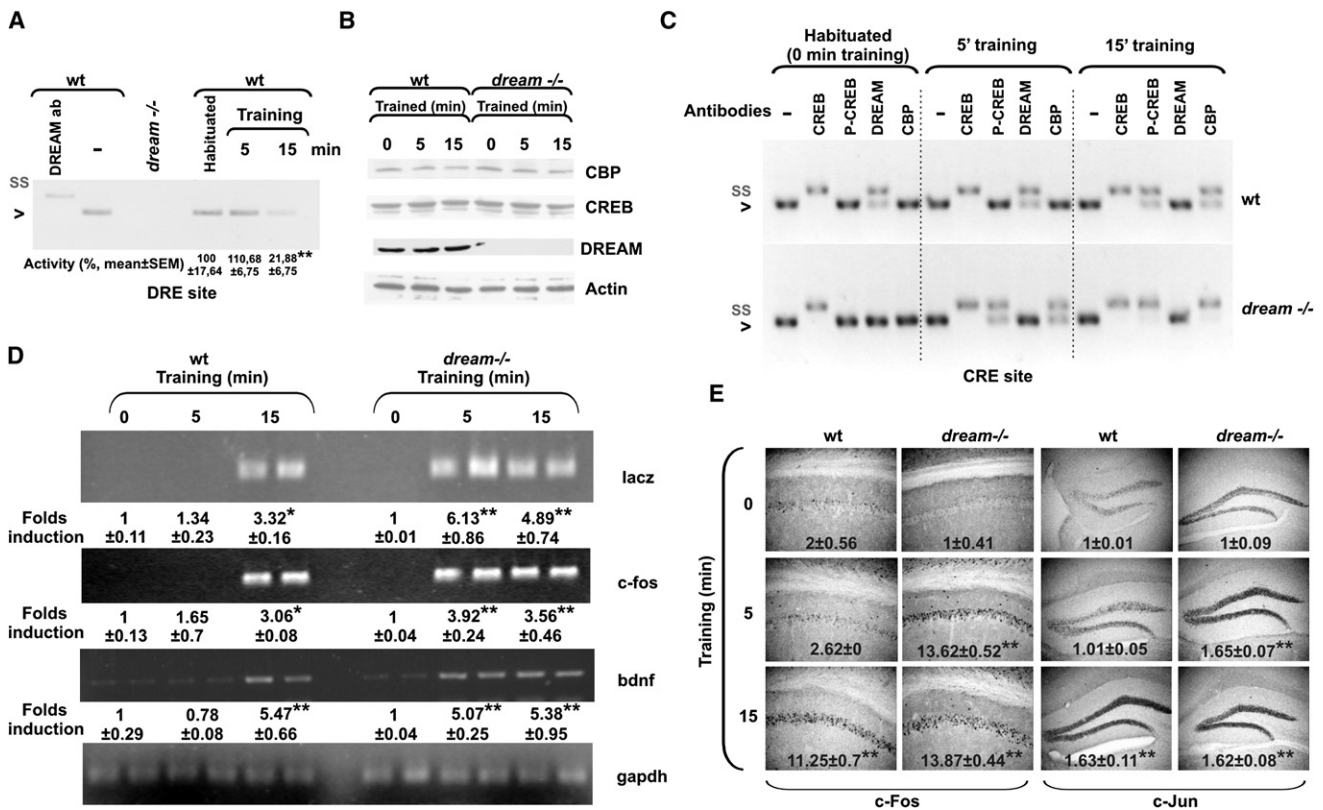
Figure 3. DREAM Regulates CREB-Dependent Gene Expression in Learning

(A) DREAM DNA-binding activity is modulated by recognition memory training. Values are compared with those of untrained animals (100% activity). n = 3 mice per group.

(B) Western blot for CBP, CREB, and DREAM in nuclear hippocampal extracts collected from untrained wild-type (WT) and $dream^{-/-}$ mice and 30 min after 5 or 15 min of training. As internal control, β-Actin is shown.

(C) Composition of CRE-binding complexes in hippocampal nuclear extracts of untrained WT and $dream^{-/-}$ mice and extracts obtained 30 min after 5 and 15 min OR memory training sessions. Complexes were assayed by supershift with the indicated antibodies. > indicates specific DNA-binding complexes and SS indicates supershift DNA-binding complexes. Data are representative of three different experiments with similar results.

(D) Semiquantitative RT-PCR analyses of lacZ, c-fos, and bdnf mRNA from hippocampus of untrained and 5 and 15 min OR-trained $CRE\text{-}lacZ^{+/-}$ $dream^{+/+}$ and $CRE\text{-}lacZ^{+/-}$ $dream^{-/-}$ mice. gapdh mRNA served as internal control. Values illustrate the densitometric quantification of lacZ, c-fos, and bdnf gene expression in each experimental group with respect to untrained animals at time point zero (0). n = 5 mice per group.

(E) c-Fos and c-Jun protein expression in hippocampi of untrained (time point 0) WT and $dream^{-/-}$ mice and mice submitted to 5 or 15 min OR training. Representative micrographs are shown. Magnifications ×20. n = 5 mice per group. Data are shown as mean values ± SEM of c-Fos-positive cells/ 100 μm² in the hippocampal CA1 field or c-Jun densitometric quantification of the hippocampal CA1 region and dentate gyrate, respectively. Asterisk indicates statistical significance comparing trained and untrained groups within the same genetic cohort. *$p \leq 0.05$; **$p \leq 0.01$.

microglia cells (Figure 4E). Importantly, all of these age-related changes in the hippocampus did not occur in 18-month-old $dream^{-/-}$ mice (Figures 4B–4E). Of note, $dream$ mutant mice showed increased GFAP immunoreactivity at a younger age, but this did not change during aging (Figure 4D). Thus, loss of DREAM expression not only allows for improved cognition but also markedly slows "brain aging."

Our results demonstrate that DREAM is a modulator of neuronal plasticity that determines the stimulation threshold required for establishing long-term changes in synaptic efficacy. DREAM DNA binding activity, as well as interaction with CREB, is regulated by synaptic activity induced by exploratory training. Importantly, in $dream^{-/-}$ mice, even short-term synaptic activity is sufficient to trigger CREB-dependent gene expression required for LTP and memory. Moreover, $dream^{-/-}$ mice did not show neuronal degeneration and cognitive deficiencies associated with aging. Mice that express a constitutive active CREB [28] develop extensive neurodegeneration, so these findings suggest that DREAM might control additional transcriptional programs and possibly nontranscriptional

targets to regulate survival and maintenance of neurons in the aging brain. Recent data suggest that Ca^{2+} leakage might be an important feature of neurodegeneration and memory loss in old age [29]. For instance, presenilin1 and 2 have been proposed to form low-conductance Ca^{2+} leak channels, a function disrupted by familial Alzheimer's disease-linked mutations [30]. DREAM functions are regulated by Ca^{2+}, so reduced Ca^{2+} flux in old-age dementia could result in enhanced DREAM activities whereas Ca^{2+} signals might reduce DREAM functions and thereby improve cognition and brain aging.

This study for the first time identifies a transcriptional repressor that is critically involved in long-term hippocampal-dependent synaptic potentiation and memory formation in mice. Mechanistically, DREAM controls CREB-dependent transcriptional activity, thereby directly coupling to a central regulator of cognition. Intriguingly, loss of DREAM also results in improved memory functions in old age and slows hippocampal brain aging. DREAM is also a central regulator of pain perception [7], and chronic pain has been known for long time to worsen memory and learning in humans [31,
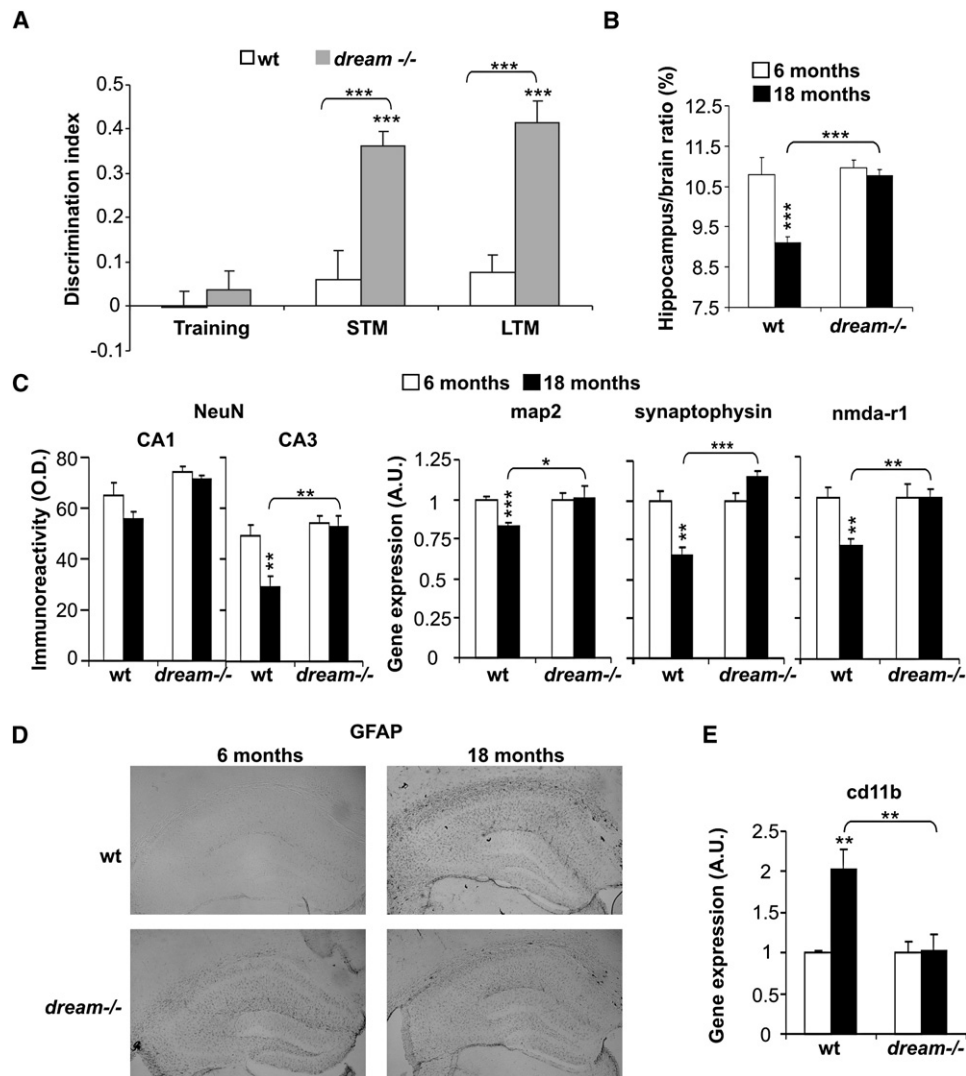
Figure 4. Lack of DREAM Slows Brain Aging

(A) The OR memory test was performed in 18-month-old control wild-type (WT) and *dream*^{−/−} mice after 15 min training sessions. Discrimination indices during training, short-term memory (STM; 1 hr after training), and long-term memory (LTM; 24 hr after training) are shown. n = 8 for both groups.

(B) Hippocampus:brain area ratios in 6- and 18-month-old WT and *dream*^{−/−} mice. n = 8 mice per group. Areas were determined with ImageJ software.

(C) Expression of the general neuronal marker NeuN (determined by immunohistochemistry) and map2, synaptophisin, and nmda-r1 (assessed by semiquantitative RT-PCR) in hippocampi collected from 6- (white bars) and 18- (black bars) month-old WT and *dream*^{−/−} mice. n = 6 animals per group.

(D and E) Lack of age-dependent hippocampal gliosis in 6- and 18-month-old *dream*^{−/−} mice.

(D) Control WT and *dream*^{−/−} mice were assayed for GFAP protein via immunoreactivity.

(E) cd11b mRNA expression was detected by semiquantitative RT-PCR.

In all panels, mean values ± SEM are shown. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

32], so DREAM could be a missing link between pain responses and cognition. Thus, DREAM could be a potential new target to not only reduce pain but also to increase cognition and to slow the decline of neurons and memory functions in aging.

Experimental Procedures

Transgenic Mice

Both *dream* mutant mice and CRE-lacZ transgenic mice have been previously described [7, 20]. *dream*^{−/−} CRE-lacZ mice were generated by interbreeding and crossed onto a C57Bl/6 background. All behavioral and electrophysiological studies were performed with male mice conducted in accordance with the guidelines of the European Union Council (86/609/EU) and according to Spanish regulations for the use of laboratory animals

in chronic experiments (BOE 67/8509-12, 1988). All experiments were approved by the institutional animal care committee.

Behavioral Assays

The OR memory test, fixed operant conditioning, trace eyeblink classical conditioning, and taste aversion conditioning were performed as described [13]. Taste aversion conditioning was performed as described in [14]. We also included a forgetting test, where different cohorts were exposed for 10 min to a coconut bar in the absence of a malaise-inducing agent (LiCl) at different times (3, 4, 5, or 10 days) after aversion acquisition. Details on behavioral tests are described in the Supplemental Data.

Electrophysiology

Surgery and free-moving mice electrophysiological recordings were performed as described [10, 13] and described in detail in the Supplemental Data.

Immunohistochemistry and Histological Analysis

For immunohistochemistry (IHQ), antibodies against c-Fos (sc-52, Santa Cruz), c-Jun (sc-45, Santa Cruz), the specific neuronal-specific nuclear protein NeuN (Chemicon, Temecula, CA), and glial fibrillary acidic protein (GFAP; DakoCytomation, Carpinteria, CA) were used. All antisera were used at 1:1000 dilutions. In all of the cases, antibody staining was developed with H_2O_2 and diaminobenzidine. Tissue sections were examined with dark-field microscopy. To minimize variability, at least five sections per animal were analyzed with a bright-field DMRB RFY HC microscope (Leica, Bensheim, Germany). For IHQ quantification, sections corresponding to stereotaxic coordinates −1.34 mm to −2.80 mm with respect to the Bregma were analyzed. The hippocampus:brain ratio was analyzed as described [33].

Electrophoretic Mobility Shifts

Nuclear extracts were prepared as described [34]. Double-stranded oligonucleotide probes corresponding to human DRE (downstream regulatory element) (5′-GAAGCCGGAGTCAAGGAGGCCCCTG-3′) or CRE (cAMP-response element) (5′-CTTGGCTGACGTCAGAGA-3′) were labeled with [γ-^{32}P]ATP. Nuclear proteins (5–10 μg) were mixed with specific antibodies against CREB (X-12, Santa Cruz), phospho-CREB (New England Biolabs), CBP (A-22, Santa Cruz), or DREAM (FL-214, Santa Cruz) for 12 hr at 4°C followed by incubation with the radioactive-labeled probes. Protein-DNA complexes were resolved in 5% nondenaturing polyacrylamide gels and visualized by autoradiography.

mRNA Analysis by Reverse Transcription-PCR

Total RNA from brain tissue was extracted with Tripure reagent (Roche Products, Hertfordshire, UK). A minimum of six animals per group, collected from at least two different experimental sessions, were used for each experiment. Detailed primer information can be found in Supplemental Experimental Procedures. Arbitrary units were calculated and compared to gapdh expression.

Statistical Analyses

The results were processed for statistical analysis with the SPSS for Windows package (SPSS, Chicago, IL). Unless otherwise stated, data are represented as mean values ± SEM. Data were analyzed with one-way and two-way ANOVA tests. The Tukey test was used for post hoc comparisons.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01614-X.

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