1 DREAM controls the on/off switch of specific activity-dependent transcription pathways
2
3 Britt Mellström¹*, Ignasi Sahún²*, Ana Ruiz-Nuño³*, Patricia Murtra⁴*, Rosa Gomez-Villafuertes¹, Magali Savignac¹, Juan C. Oliveros¹, Paz Gonzalez¹, Asta Kastanauskaite⁵, Shira Knafo⁵&, Min Zhuo⁶,⁷ Alejandro Higuera-Matas⁸, Michael L. Errington⁸, Rafael Maldonado¹, Javier DeFelipe⁵, John G.R. Jefferys³, Tim V.P. Bliss⁸, Mara Dierssen², Jose R. Naranjo¹#

1 National Center of Biotechnology CSIC and CIBERNED, Madrid, Spain
2 Genomic Regulation Center PRBB and CIBERER, Barcelona, Spain
3 Neuronal Networks Group, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, UK
4 University Pompeu Fabra, Barcelona, Spain
5 Cajal Institute, CSIC and CIBERNED Madrid, Spain
6 Department of Physiology, Faculty of Medicine, University of Toronto, 1 King’s College Circle, Toronto, Ontario, Canada
7 Department of Brain and Cognitive Sciences, Seoul National University, Seoul 151-746, Korea
8 MRC National Institute for Medical Research, Mill Hill, London, UK
9
10 # Corresponding author: Jose R. Naranjo
11 Centro Nacional de Biotecnologia
12 Consejo Superior de Investigaciones Científicas
13 Darwin, 3, E-28049 Madrid, Spain
14 Telephone: 34 915854682, Fax: 34 915854506
15 E-mail: naranjo@cnb.csic.es
16
17 * These authors contributed equally to the work
18 & Current address: IkerBasque Basque Foundation for Science and BioCruces, Health Research Institute.
Changes in nuclear Ca\textsuperscript{2+} homeostasis activate specific gene expression programs and are central to the acquisition and storage of information in the brain. DREAM (downstream regulatory element antagonist modulator), also known as calsenilin/KChIP3, is a Ca\textsuperscript{2+}-binding protein that binds DNA and represses transcription in a Ca\textsuperscript{2+}-dependent manner. To study the function of DREAM in the brain, we used transgenic mice expressing a Ca\textsuperscript{2+}-insensitive/CREB-independent dominant active mutant DREAM (daDREAM). Using genome-wide analysis we show that DREAM regulates the expression of specific activity-dependent transcription factors in the hippocampus, including Npas4, Nr4a1, Mef2c, JunB and c-Fos. Furthermore, DREAM regulates its own expression establishing an auto-inhibitory feedback loop to terminate activity-dependent transcription. Ablation of DREAM does not modify activity-dependent transcription because of gene compensation by the other KChIP family members. Expression of daDREAM in the forebrain resulted in a complex phenotype characterized by loss of recurrent inhibition and enhanced LTP in the dentate gyrus and impaired learning and memory. Our results indicate that DREAM is a major master-switch transcription factor that regulates the on/off status of specific activity-dependent gene expression programs that control synaptic plasticity, learning and memory.

Keywords BDNF, c-Fos, Npas4, nuclear calcium, transcriptomic analysis

Introduction
A major challenge for neuroscience is to identify the regulatory molecules underpinning the storage of information in neurons. Activity-dependent gene expression underlies neuronal plasticity and adaptive responses in the CNS to different environmental stimuli and is determinant in the formation and storage of memories. Diverse signaling pathways participate in these processes. Among them, changes in intracellular free calcium concentration is the most universal signal and the final output, in terms of adapted gene expression, is given by a specific set of proteins that decode the calcium signal according to its frequency, subcellular location and intensity (9, 15, 23). A nuclear tool-kit of Ca\textsuperscript{2+}-dependent effectors modifies the activity or the properties of specific transcription factors to regulate gene expression in response to the Ca\textsuperscript{2+} signal (for reviews see 28, 32). Despite extensive investigation, a detailed mechanistic description of Ca\textsuperscript{2+}-dependent signaling in the expression of the late, transcription-dependent component of long-term potentiation (LTP) is far from been complete (reviewed in 6). Here we examine the role of the Ca\textsuperscript{2+}-dependent transcriptional repressor DREAM in the control of activity-dependent transcription, the expression of LTP and in learning and memory.

DREAM/calsenilin/KChIP-3 belongs to a group of four genes (K\textsuperscript{+} channel interacting proteins, KChIP-1 to 4) that regulate the membrane expression and gating of Kv4 potassium channels (reviewed in 28) and also encode structurally and functionally related calcium sensors able to repress transcription in a Ca\textsuperscript{2+}-dependent manner (5, 26). Transcriptional activity of DREAM is effected by its binding to DNA and by specific interactions with other nucleoproteins, including CREM and CREB (20, 21, 44). High affinity binding of DREAM to DRE sequences in DNA is regulated by the level of nuclear Ca\textsuperscript{2+} and requires DREAM oligomerization (5, 35, 36). Unbinding of DREAM from DRE results in transcriptional derepression of target genes, as shown for prodynorphin (5, 7, 45). Mutation of the EF-hands in DREAM results in a Ca\textsuperscript{2+}-insensitive repressor that also blocks CREB binding protein
recruitment by phosphoCREB, impairing CRE-dependent transcription, since the DREAM-CREB interaction is also Ca\(^{2+}\)-dependent (20). Mutation of a leucine charged residue-rich domain (LCD) motif at the N-terminal of DREAM prevents the interaction with CREB and releases CREB-dependent transcription from basal repression by DREAM (20). Combination of LCD and EF-hand mutations generates a calcium insensitive double mutant, hereafter daDREAM (dominant active DREAM), that actively represses DREAM target genes preventing DREAM-mediated derepression in the presence of Ca\(^{2+}\)- and cAMP- stimulation. To note, the double mutant daDREAM does not impair transcription after CREB phosphorylation. Consistent with the notion of DREAM binding to the DNA as homo- or hetero-oligomers with other KChIP proteins (5, 36, 48), daDREAM acts as a cross-dominant active mutant in vivo (13, 47).

Interestingly, despite the potential role for a Ca\(^{2+}\)-dependent repressor such as DREAM in the regulation of synaptic plasticity and learning and memory processes, DREAM knockout mice are not different from wild type in paired pulse facilitation, resting membrane potential or input-output relation of fEPSPs (24). Furthermore, DREAM knockout mice do not show an obvious phenotype in a place-learning version of the Morris water maze test (7) and have only a slight increase in LTP in the dentate gyrus of the hippocampal formation (24) and slight improvement in memory in a contextual fear test (1). This is likely due to the functional redundancy among DREAM/KChIP proteins and their overlapping expression patterns (26, 48). Supporting this idea, genetic ablation of KChIP2 (19) or KChIP1 (54) also resulted in mild phenotypes.

In the present study we used transgenic mice expressing a Ca\(^{2+}\)-insensitive/CREB-independent dominant active mutant DREAM (daDREAM) to reveal the functional involvement
of DRE-mediated transcription in hippocampal function. We found that the expression of several activity-dependent transcription factors is affected in daDREAM mice and the expression of their downstream targets is modified in transgenic hippocampal neurons. Notably, down regulation of Npas4 in daDREAM mice resulted in a severe alteration of GABAergic transmission leading to a reduction in recurrent inhibition and enhanced LTP in the dentate gyrus in vivo. Associated with these physiological abnormalities, DREAM transgenic mice showed significant impairments in learning and memory.

**Materials and methods**

**Mice**

Generation of DREAM transgenic mice has been reported previously (13, 45). Of the different transgenic lines generated, in this study we used transgenic line 26 that shows specific expression of the transgene in the telencephalon. Expression of the transgene in different brain areas was quantified by real-time qPCR as described (48). DREAM knockout mice have been reported previously (7).

**Microarray**

RNA from whole hippocampus from wild type and transgenic mice was prepared using TRIzol (Invitrogen) and the RNAeasy Mini Kit (Qiagen). RNA was quantified and the quality was assessed with a 2100 Bioanalyzer (Agilent technologies). cDNA was synthesized from 4 µg of total RNA using one-cycle target labeling and control reagents (Affymetrix) to produce biotin labeled cRNA. The cRNA preparation (15 µg) was fragmented at 94°C for 35 min into 35-200 bases in length. Labeled cRNAs were hybridized to Affymetrix chips (GeneChip Mouse Genome
Each sample was added to a hybridization solution containing 100 mM 2-(N-morpholino) ethanesulfonic acid, 1 M Na+, and 20 mM of EDTA in the presence of 0.01% of Tween-20 to a final cRNA concentration of 0.05 µg/ml. Hybridization was performed for 16 h at 45°C. Each microarray was washed and stained with streptavidin-phycoerythrin in a Fluidics station 450 (Affymetrix) and scanned at 1.56 µm resolution in a GeneChip® Scanner 3000 7G System (Affymetrix).

**Microarray data analysis**

Three biological replicates were independently hybridized for each transcriptomic comparison. GeneChip intensities were background-corrected, normalized and summarized by the RMA method (16) using the “Affy” package (12) from Bioconductor. For each comparison, moderated \( t \)-test was applied to identify differentially expressed genes as implemented in the “Limma” package (49) from Bioconductor. Raw \( P \)-values were adjusted for multiple hypothesis testing using the false discovery rate (FDR) method (3). Genes with FDR < 0.1 and Fold Changes > 1.6 or < -1.6 were included in the list of induced or repressed candidates, respectively. FIESTA viewer was used to facilitate the application of these numerical filters and the selection of candidate genes in each comparison (33). For the biological classification of candidate genes, Gene Ontology terms included in the “generic GO slim set” (S. Mundodi and A. Ireland; http://www.geneontology.org/GO.slims.shtml) were used to group candidate genes into a reduced number of biological categories. Terms from “Molecular function” name space were taken into account. The Gene Ontology terms associated to the genes were obtained from the original Affymetrix annotation files available at http://www.affymetrix.com.

**Primary neuronal culture**
Serum-free cortico-hippocampal neurons from mouse E14 wild type, homozygous transgenic or DREAM deficient embryos were cultured as described (27) in Neurobasal medium supplemented with B27, 2 mM Glutamax and 100 μg/ml penicillin/streptomycin (Invitrogen). Fully differentiated neurons after 10 days in culture were stimulated with 60 mM KCl and harvested after 30 or 90 min for RNA or chromatin preparation, respectively. For knockdown of KChIP proteins, neuronal cultures were transduced 24 h after plating using lentiviral vectors encoding antisense-KChIP-2, antisense-DREAM, GFP or empty vector prepared as described (13). The virus concentration was estimated by measuring the amount of p24 protein (Perkin-Elmer). Cultures received 5 μg of p24/10^6 neurons and medium was changed 8 h after the addition of the virus. Neurons were harvested 7 days after infection. Typical infection efficiency was about 90% as assessed using viral delivery of GFP.

**Real-time quantitative PCR**

RNA was isolated from whole tissues or cell suspensions using TRIzol (Invitrogen), treated with DNase (Ambion) and reverse transcribed using hexamer primer and Moloney murine leukemia virus reverse transcriptase. To confirm the absence of genomic DNA, each sample was processed in parallel without reverse transcriptase. Real-time quantitative PCR (qPCR) for endogenous DREAM and daDREAM was performed as described (48). Validation of microarray up or down regulated genes was with specific primers and TaqMan MGB probes (Applied Biosystems) or SYBR Green based technology (Table S1) and with specific assays from Applied Biosystems (Table S2). The results (triplicates) were normalized as indicated by parallel amplification of HPRT, β-actin or GPDH (Table S1).
Chromatin immunoprecipitation was performed as described (50). Briefly, nuclei from primary cultured neurons were collected and chromatin was sonicated to an average length of 500-1000 bp. The sheared chromatin was precleared with blocked protein A/G Sepharose beads (Pierce) and used for immunoprecipitation, with 6 µg of affinity-purified polyclonal antibody Ab731 against aa 22-42 in the DREAM protein (46). Immunoprecipitated DNA was subjected to semiquantitative PCR with specific primers (Table S3). The PCR was performed within the linear range using 20-25 cycles of amplification and trace amounts of $^{32}$P-dCTP. Scanned autoradiograms were quantified using the QuantityOne software (Biorad).

Western blot

Whole cell extracts from neuronal cell cultures were prepared by incubation of cell pellets on ice for 45 min with occasional vortexing with lysis buffer [50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X100, 0.5% deoxycholate, 0.1% SDS] supplemented with protease inhibitor cocktail (Roche). Extracts were cleared by centrifugation at 14,000 g for 20 min and protein content quantified by DC Protein Assay (Bio-Rad). The affinity-purified rabbit polyclonal antibodies used were the DREAM-specific Ab730 (46) or the pan DREAM/KChIP Ab1014 (48). Antibodies used were, for PSD95 (7E3-1B8, Affinity BioReagents), GABA A receptor β3 (ab 4046, Abcam) and β-actin (AC-15, Sigma). Proteins were visualized with HRP-conjugated secondary antibody (Jackson) followed by ECL (SuperSignal West Femto, Thermo Scientific). Blots were quantified using the Quantity One software (Biorad).

Seizures
Wild type and transgenic adult mice were injected intraperitoneally with pentylenetetrazol (45 mg/kg) or with PBS. The behavioral score according to a modified (0 to 6, where 0 = no response and 6 = generalized tonic-clonic convulsions) Racine’s scale (40) was recorded every 5 min during 30 min following drug administration. Median score for each animal was calculated and the data were analyzed using Mann Whitney U-test for difference between groups.

Electrophysiology in vivo

Adult male mice homozygous for the transgene and wild type littermates aged three to six months were anaesthetized with urethane (1.8 g/kg, i.p.). Experiments were performed under the Animals (Scientific Procedures) Act (1986), UK. Animals were placed in a head-holder and a glass micropipette was positioned in the ipsilateral hilus of the dentate gyrus to record the field responses evoked by stimuli delivered through a concentric bipolar stimulating electrode placed in the ipsilateral perforant path. In order to assess changes in feed-forward and feed-back inhibition, pairs of stimuli were delivered at interpulse intervals varying from 10-500 ms, at an intensity sufficient to evoke a population spike of ~1mV. For each animal, the average of three responses was obtained at each of nine intervals between 10 and 50 ms. For LTP experiments, single test stimuli were delivered at a frequency of 0.033 Hz; intensity 70-300 µA, pulse width 60 µs. Pulse width was doubled during the tetanus (six series of six trains of six pulses at 400 Hz, 200 ms between trains, 20 s between series). The slope of the field EPSP (fEPSP) was normalized with respect to the mean response in the 10 min before the tetanus. Data are presented as mean ± SEM. For comparisons of the magnitude of LTP between groups, the normalized values of the fEPSP 50-60 min after the tetanus were averaged for each animal, and groups compared using Student’s unpaired two-tailed t-test.
Electrophysiology in vitro

Transgenic and wild type littermates were anaesthetized by intraperitoneal injection of a mixture of medetomidine (1 mg/kg) and ketamine (76 mg/kg), before being killed by cervical dislocation. The brains were rapidly removed and chilled (< 3°C). Parasagittal slices were cut from the dorsal hippocampus using a Vibroslice (Campden Instruments, Loughborough, UK). A detailed description of the protocols used for in vitro electrophysiology can be found in Supplementary Material.

Behavioral analysis

Experiments were performed in adult male mice homozygous for the transgene and wild type littermates. Mice were initially housed five per cage in a temperature (21 ± 1°C) and humidity (65 ± 10%) controlled room with a 12-/12- h light/dark cycle (lights on from 0800 to 2000 hours) with ad libitum food and water. Experiments took place during the light phase. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/609 EEC; National Institutes of Health 1995) and approved by the local ethical committee (CEEA-PRBB). All behavior experiments were carried out in blind conditions. A detailed description of the protocols used for Morris water maze, open field, active avoidance, object recognition task and fear conditioning can be found in Supplementary Material.

Results

Genome-wide analysis in daDREAM hippocampus

daDREAM has been shown to function as a dominant active mutant for the transcriptional repressor function of endogenous DREAM/KChIP proteins (13, 46, 48). We used transgenic
mice expressing daDREAM in telencephalic areas of the brain (Fig. 1), where endogenous DREAM is expressed (42), to search for DREAM transcriptional targets that could reveal a potential role for DREAM in hippocampal synaptic plasticity, learning and memory. Comparison of basal gene expression in wild type and transgenic adult hippocampus, using cDNA microarrays, identified more than 250 genes whose expression was altered in daDREAM transgenic mice (Gene Expression Omnibus accession number GSE17844 and Fig. S1). Up and down regulated genes were categorized according to Gene Ontology terms and are presented in Tables S4-S6.

Among the genes with modified expression, we focused our attention on those predicted to encode transcription factors that could initiate gene expression programs governing changes in synaptic plasticity, learning and memory. We found that several genes encoding early-induced activity-dependent transcription factors including Npas4, Nr4a1, Mef2C, JunB and c-Fos were down regulated in transgenic hippocampus (Fig. 2A). For these targets, the microarray results were confirmed in transgenic hippocampus by real-time qPCR (Fig. 2B). Importantly, expression of other factors known to mediate activity-dependent changes in gene expression like SRF, Atf3 and c-Jun (18, 51, 55) was not modified in transgenic hippocampus, supporting the specificity of the changes induced by daDREAM. Taken together, these results implicate Ca²⁺-dependent DREAM-mediated derepression of specific immediate early gene transcription in the function of hippocampal neurons.

**DREAM directly down-regulates transcription from the Npas4 promoter**

Among target proteins with a function in the nucleus, we focused our attention on Npas4, a bHLH-PAS transcriptional activator (34, 37). Contrary to other immediate early genes like c-Fos, JunB, Egr2 or Nr4a1, Npas4 is selectively induced by rises in nuclear Ca²⁺, binds to
numerous enhancers in an activity-dependent manner (17) and has been shown to transactivate the BDNF gene and to control the formation of GABAergic inhibitory synapses (25). Reduced basal level of Npas4 mRNA was found in transgenic hippocampus (Fig. 2B) but also in other areas with transgene expression, such as the cerebral cortex and striatum, and was not modified in cerebellum where daDREAM is not expressed (Fig. S2).

Sequence analysis of the Npas4 proximal promoter identified several DRE sites for DREAM binding (Fig. S3A). To determine if Npas4 is a direct target for DREAM repression we used chromatin immunoprecipitation with DNA isolated from primary cultured cortico-hippocampal neurons, a system where the DREAM and other KChIP proteins are expressed (Fig. 3A). Basal expression of Npas4 was reduced in primary daDREAM neuronal cultures compared to wild type cultures (Fig. 3B) confirming the results obtained in adult tissues. Importantly, membrane depolarization by stimulation with 60 mM K+ resulted in strong induction of Npas4 expression in wild type neurons, while only a slight induction was obtained in transgenic neurons (Fig. 3B).

Chromatin immunoprecipitation with a DREAM specific antibody showed that in basal conditions binding of DREAM to the Npas4 promoter was stronger in transgenic cultures, while potassium depolarization resulted in the Ca\(^{2+}\)-dependent unbinding of DREAM from the Npas4 promoter in wild type neurons but had no effect in transgenic cultures (Fig. 3C). As negative controls for these assays, omission of the DREAM antibody or chromatin immunoprecipitation from cultured DREAM-deficient neurons resulted in failure to amplify the Npas4 promoter (Fig. 3C). These results indicate that DREAM directly binds to and controls expression of the Npas4 gene in neurons. As a positive control for these experiments we analyzed the expression of c-Fos, a bona fide target for DREAM (5). Basal and activity-dependent expression of c-Fos was reduced in cultured daDREAM neurons compared to wild type neurons (Fig. 3D) and chromatin
immunoprecipitation confirmed the direct binding of DREAM to the c-Fos promoter (Fig. 3E and S3B).

GABAergic transmission is affected in daDREAM mice

Npas4 regulates the establishment of GABAergic synapses (25). Thus, to assess whether Npas4 repression in the daDREAM hippocampus has functional consequences, we measured by real-time qPCR the expression of several postsynaptic GABAergic markers. Expression of the α1, α2, β2 and β3 GABA receptor subunits was markedly reduced in the hippocampus of daDREAM mice (Fig. 4A). Of note, down regulation of the α2 subunit was already noticeable in the results of the genome-wide analysis (Table S4-S6). The down regulation of GABA receptor subunits in daDREAM hippocampus was specific and did not affect the expression of presynaptic markers of inhibitory synapses such as GABA transporters (GAT1 and VIAAT), glutamic acid decarboxylase (GAD) or calbindin, which did not show any difference between genotypes (Fig. 4B). Immunostaining for parvalbumin or GAT1 did not show cellular deficits in hippocampal GABAergic interneurons in transgenic mice (Fig. S4). Furthermore, levels of PSD95 protein (Fig. 4C), a marker of excitatory synapses, were also not affected. For comparison, reduced level of β3 GABA receptor protein in transgenic hippocampus is shown (Fig. 4C).

Impairment of GABA receptor signaling associated with specific mutations in GABA_A receptor subunits is linked to epilepsy in humans and genetic ablation of specific subunits reproduces a phenotype prone to spontaneous or pharmacologically induced seizures (11). To investigate whether reduced mRNA levels of specific GABA_A receptor subunits had a functional correlate, we analyzed the susceptibility to convulsants in daDREAM mice. Administration of
sub-convulsive doses of pentylenetetrazol or kainate induced a significantly increased response in transgenic mice compared to wild type (Fig. 4D). These results indicate that the reduction in specific GABA_\text{A} receptor subunits in daDREAM mice has a functional correlate at the receptor protein level that in turn may anticipate changes in the electrophysiological properties of transgenic neurons.

**Paired pulse inhibition is abolished and LTP is enhanced in daDREAM mice**

To investigate changes in synaptic plasticity in daDREAM mice, we analyzed paired-pulse inhibition and long-term potentiation (LTP) in vivo. The simultaneous discharge of granule cells evoked by a strong stimulus to perforant path fibers sets up a powerful recurrent inhibition mediated by inhibitory interneurons, particularly basket cells (2). As a result, the amplitude of the population spike elicited by the second of a pair of strong stimuli is greatly reduced when the inter-stimulus interval is less than ~30 ms. Indeed, in wild type mice the population spike in response to the second stimulus was abolished at intervals of less than 20 ms (Fig. 5A). Conversely, in daDREAM mice, due to reduced GABAergic synaptic strength, the feedback inhibition was completely absent and, even at the shortest intervals; the population spike to the second pulse was enhanced rather than inhibited (Fig. 5A). At longer intervals, when paired pulse depression gives way to potentiation and a massive enhancement of the population spike, no significant differences were observed between wild type and daDREAM mice (Fig. 5A). The impairment of paired pulse inhibition was replicated in vitro, where whole cell recording confirmed the substantial weakening of inhibition (Fig. S5A, B). Inhibitory postsynaptic currents (IPSCs) recorded from wild type neurons with a holding potential of 0 mV, were evoked by stimuli as weak as 1 mA, and grew progressively with increasing stimulus strength. IPSCs were also evoked in transgenic cells, but were substantially and significantly smaller for all but the
largest stimuli (Fig. S5B). To localize the source of the weakening of inhibition we measured the frequency and amplitude of spontaneous IPSCs at holding potential of 0 mV (Fig. S5C). The mean amplitude of the spontaneous IPSCs did not differ between genotypes, nor did time constants and half width, but the frequency of the spontaneous IPSCs was significantly lower in transgenic mice, at about half the frequency in the wild type cells (Fig. S5C). To test the strength of excitatory input to granule cells, plots of the mean slope of the field excitatory postsynaptic potential (EPSP) as a function of stimulus strength were collected for wild type and transgenic mice. No difference was seen between the two genotypes, suggesting that excitatory drive was not grossly affected in transgenic mice (Fig. S5D). Spontaneous EPSCs were not statistically significant different, but there was a tendency to increased frequency and amplitude (Fig. S5E). The magnitude of early LTP measured 50-60 min after induction was greater in transgenic than in wild type littermates (Fig. 5B), presumably as a result of impaired recurrent inhibition at the time of induction, leading to greater depolarization and enhanced activation of NMDA receptors. Importantly, modified synaptic plasticity in daDREAM mice was not related to changes in A-type currents that were identical in transgenic and wild type hippocampal neurons (Fig. S6). Similar results were previously shown in spinal cord neurons (45).

**DREAM directly regulates BDNF expression**

Activity-dependent expression of the mouse BDNF gene is induced by several mechanisms. These include transactivators, like CREB, Npas4 and CaRF, that bind to promoter regions located immediately before exon II and IV (Fig. S3C) and de-repression mechanisms operated by REST, MeCP2 and DREAM (14, 29, 39, 45). Consistent with the expression of daDREAM and the reduced levels of the Npas4, a significant decrease in BDNF mRNA (Fig. 6A) was noticeable in the hippocampus of daDREAM mice. Binding of DREAM to DRE sites in the rat BDNF...
promoter have been shown in vitro and reduced levels of BDNF mRNA and protein have been reported in spinal cord from a different transgenic line that has ubiquitous daDREAM expression (45). To investigate a direct regulation of BDNF expression by DREAM we performed chromatin immunoprecipitation. Using specific primers for mouse promoter IV, we could detect the Ca\(^{2+}\)-dependent binding of wild-type DREAM to this promoter and the stronger interaction of daDREAM both in basal conditions and after depolarization (Fig. 6B). Since BDNF expression has been related to structural plasticity, learning and memory, blockade of activity-dependent BDNF expression in daDREAM hippocampus may predict an impaired cognition in these mice.

**Impaired learning and memory in daDREAM mice**

Ca\(^{2+}\)-dependent gene expression underpins changes in synaptic plasticity and learning and memory processes. To assess a potential phenotype in daDREAM mice we used three learning and memory tests; the Morris water maze, the object recognition test and an active avoidance task. In the water maze, transgenic mice showed a clear defect in hippocampal learning. During acquisition sessions (A1-A10), performance was impaired (Fig. 7A and S7A). Swimming speed is unlikely to contribute to this phenotype, since daDREAM mice showed no differences with respect to wild type mice (inset in Fig. 7A). However, in the removal session no impairment was detected in reference memory, once learning was established after extensive training (Fig. S7B).

In the reversal-learning session, daDREAM mice had difficulties in learning the new platform position (Figure S8). Thus, a repeated reversal learning experiment was performed, which showed that transgenic mice have a significant deficit in both reference (odd trials; Fig. 7B and Table S7) and working memory (even trials; Fig. 7C and Table S7), being less efficient than wild type mice. In the object recognition task, novelty recognition was not affected when analyzed one hour after training and transgenic mice exhibited a similar discrimination index as wild type
Conditioned learning in the active avoidance procedure showed similar learning performance during the first five training sessions in both genotypes (Fig. S9). In the last sessions, however, performance was significantly decreased in daDREAM mice (Fig. S9), suggesting deficits in associative learning in these mice.

**Gene compensation accounts for the mild phenotype in DREAM-/- mice**

The strong phenotype due to daDREAM expression in the hippocampus contrasts the mild phenotype of DREAM knockout mice (7, 24). Functional redundancy among DREAM/KChIP proteins and their overlapping expression patterns in different organs and brain areas, including the hippocampus (26), might account for the lack of phenotype. Supporting this idea, genetic ablation of KChIP2 (19) or KChIP1 (54) also resulted in mild phenotypes. In fact, we found that expression of the transcription factors Npas4, Mef2C, Nr4a1, Jun B and c-Fos were not significantly modified in DREAM^{-/-} hippocampus (Fig. 8A). Moreover, we observed no change in BDNF or in GABAergic postsynaptic markers in DREAM^{-/-} hippocampus (Fig. 8B, C). These data are in agreement with the lack of increase in prodynorphin gene expression originally reported in the hippocampus of DREAM knockout mice (7). To assess that gene compensation by other KChIP family members is responsible for the absence of changes in gene expression in DREAM^{-/-} hippocampus, we aimed to knock down expression of other KChIPs expressed in primary cultured cortico-hippocampal neurons from DREAM^{-/-} embryos (Fig. 8D), using antisense lentiviral expression vectors. As expected, transduction of primary cultured DREAM^{-/-} neurons with the antisense RNA for full-length KChIP-2 resulted in a reduction in KChIP-2 mRNA levels (Fig. 8E). Unexpectedly, however, levels of KChIP-1 and KChIP-4 mRNA were also reduced after antisense KChIP-2 expression (Fig. 8E). Importantly, further reduction in
KChIPs levels in the DREAM−/− background was associated to increased mRNA levels of the activity-dependent target genes Npas4 and c-fos compared to expression in untransduced DREAM−/− or wild type neurons (Fig. 8F). The infection with the antisense KChIP-2 lentiviral vector and the knock down of KChIP expression did not produce a nonspecific generalized effect on the cultured neurons, since no effect was observed in the level of CREB mRNA (Fig. 8F), an activity-dependent transcription factor whose activity is mostly controlled at the post-translational level and did not appear as a target for DREAM in the genome-wide analysis. These results support the notion of a compensatory effect by other KChIPs as an explanation for the absence of a transcriptional phenotype in DREAM−/− mice.

**DREAM regulates its own expression**

To further understand the mechanism of DREAM regulation of activity-dependent gene expression we sought evidence for a mechanism that could switch off activity-dependent transcriptional cascades once initiated. Of note, microarray results showed a significant downregulation of endogenous DREAM expression in daDREAM hippocampus and qPCR analysis confirmed reduced levels of endogenous DREAM in daDREAM transgenic hippocampus (Figure 9A). The reduction was significant also in other telencephalic areas where the transgene is expressed as well as in cultured transgenic neurons (Fig. 9A, B). Thus, we investigated whether DREAM could regulate its own expression and in this way close a self-regulatory loop. Chromatin immunoprecipitation analysis substantiated this hypothesis and disclosed the Ca²⁺-dependent binding of DREAM to DRE sites present in the promoter region of the DREAM gene (Fig. 9C and S3D). These results indicate that activity-dependent derepression of endogenous DREAM leads to an increase in DREAM levels, simultaneous to the increase in other DREAM targets, that in turn will contribute to restore basal repression of DREAM and its...
target genes in wild type hippocampus, in this way leading to the transient induction of many activity-dependent genes.

Discussion

Activation of calcium-dependent kinases and phosphatases has been proposed as a universal mechanism to drive activity-dependent gene expression (reviewed in 14, 28). Identification of the calcium-dependent transcriptional repressor DREAM disclosed an alternative mechanism to link changes in nuclear calcium concentration directly with enhanced expression of the immediate early gene c-Fos (5). In the present work, the genome-wide analysis has identified several additional immediate early genes as targets for DREAM derepression indicating that DREAM is a permissive transcriptional switch for activity-dependent transcription. Furthermore, the self-regulatory function of DREAM identifies a loop to shut down transcription of immediate early genes shortly after initiation by the upregulation of DREAM protein.

Genome-wide expression analysis was performed using the whole hippocampus, and therefore the results do not discriminate between hippocampal subareas where DREAM could differentially regulate early transcriptional activation in different neuronal subpopulations. This limitation of the present study is particularly relevant since the results showed that DREAM controls activity-regulated transcription factors that contribute in opposite directions to experience-dependent synapse development and remodeling. Thus, the down regulation of Mef2c in DREAM transgenic mice may result in a deficient elimination of the excess of excitatory synapses during development (10) in a subset of neurons, whereas the repression of Npas4 in other sub-areas results in reduction of the number of GABAergic synapses that form on excitatory neurons (25). Nevertheless, expression analysis of post-synaptic markers in whole
hippocampus confirmed the down regulation of GABA receptor subunits participating in inhibitory mechanisms, while no change was observed neither in the levels of PSD95, a marker of excitatory synapses nor in the expression of several glutamate receptors (53).

Analysis of activity-induced genes in cultured hippocampal neurons (55) revealed a set of 169 genes that are more than 2-fold up-regulated in response to bicuculline-induced action potential bursting and whose induction is blocked by at least 40% in the presence of CaMBP4, a nuclear fusion protein containing four repeats of the M13 calmodulin binding peptide that binds to and inactivates the nuclear calcium/calmodulin complex (52). To categorize activity-dependent genes according to the mechanism of induction, we compared the results from Zhang et al. with the results from our transcriptomic analysis. Although these two studies used different experimental conditions, in vivo versus cultured neurons and basal repression versus activity-dependent induction, the comparison established three useful gene categories. The first includes genes that are significantly down regulated in daDREAM hippocampus but are not or only slightly affected by inhibition of the nuclear calcium/calmodulin complex in hippocampal neurons. The most relevant genes from the point of view of transcriptional cascades are shown in supplementary Table S8A and correspond to “specific” DREAM targets, whose activity-dependent early induction in wild type neurons is mediated mainly by DREAM derepression. The precise molecular mechanisms leading to gene transactivation once DREAM has detached from the promoter of each target gene are not yet fully understood. The second category encompasses genes that are significantly down regulated in daDREAM hippocampus and strongly dependent on the nuclear calcium/calmodulin complex. The most important genes are shown in supplementary Table S8B and correspond to genes in which DREAM has a permissive function at the initial on/off switch but whose full induction is dependent on the activity of calcium/calmodulin-regulated kinases. The third category includes genes whose activity-
dependent induction is strictly independent of DREAM. A short list (Table S8C) includes those genes induced in a first or a second wave of gene expression after membrane depolarization and that are not modified in daDREAM hippocampus. Thus, in normal conditions, endogenous DREAM has a permissive effect on the early activation of a set of genes some of which will be further activated by parallel pathways involving the nuclear calcium/calmodulin complex. Importantly, it has been shown that post-translational modifications of DREAM, such as sumoylation or change in the redox state, which are associated to increased nuclear localization and repressor capability, respectively (37, 43), could render a DREAM protein with increased repressor activity to block to a higher extent activity-dependent learning and memory formation. In this scenario, it is tempting to speculate that changes in the post-translational processing of DREAM could participate in the cognitive decline associated to aging or to pathological conditions of the brain.

Ablation of the DREAM gene in DREAM−/− mice resulted in minor or no changes in learning and memory (7, 24), possibly due to compensation by other KChIPs expressed in the hippocampus. Our results after knockdown of KChIP proteins in cultured neurons supported this idea and confirmed the repressor activity of DREAM/KChIP proteins on the expression of the transcription factors Npas4, Mef 2C, Nr4a1, Jun B and c-Fos. Interestingly, the hypoalgesic phenotype described in DREAM−/− mice (7) is associated with increased expression of the prodynorphin gene, a bona fide target for DREAM/KChIP repression, in the spinal cord. These data suggest that there is no gene compensation at this level, which could be related to the low expression level of KChIP1 and KChIP2 in the spinal cord (J. R. Naranjo, unpublished data) or to additional mechanisms not yet characterized. Previous work using a mammalian expression vector for full-length antisense KChIP-2 successfully reduced KChIP-2 expression without affecting DREAM mRNA levels, after transfection in lymphocytes (48). Here, using a lentiviral
vector for antisense KChIP-2 we observed the down regulation of KChIP2 but also of KChIP1 and 4. High degree of sequence homology might account for cross-down regulation among KChIP mRNAs, and higher infection rate and transduction efficiency of lentiviral vectors compared to mammalian expression vectors might contribute to manifest the cross-activity observed in the present study.

In contrast, expression of daDREAM in transgenic hippocampal neurons results in a tonic basal repression of important transcriptional cascades with traceable consequences at the electrophysiological level. Thus, reduced Npas4 content in daDREAM hippocampal neurons resulted in a reduction of the mean frequency, i.e. increase in the mIPSC inter-event interval, of the spontaneous IPSC to about half that in wild type neurons, as previously shown after transient or permanent Npas4 ablation (25). The mean amplitude, time constants and half widths of the spontaneous IPSCs did not differ between genotypes (Figure S5C), as reported for hippocampus of Npas4 knockout mice (25), while these parameters were decreased only after transient Npas4 knockdown (25). Permanent versus transient Npas4 down regulation might account for developmental compensatory mechanisms responsible for the distinct effects on IPSCs amplitude. Compensatory events during development in daDREAM hippocampus might mask changes in mIPSC amplitude despite of a net reduction in the expression of GABA receptors through different mechanisms involving, for instance, the insertion and clustering of membrane receptors as shown in mice deficient of the scaffolding protein gephyrin at hippocampal inhibitory synapses. In these mice, the frequency of mIPSCs is the same as in wild type but the amplitude is significantly reduced (22). In addition, it has been recently shown that the Npas4-mediated transcriptional program differentially regulates inhibitory inputs in distinct neuronal compartments (4). Thus, increased somatic inhibition or decreased dendritic inhibition could be
recorded upon Npas4 activation. Whether these or yet other unknown mechanisms are operating in daDREAM hippocampus is not presently known. Reduced expression of GABA_A receptors and decreased GABAergic transmission could account for the reduction in recurrent inhibition and enhanced LTP in transgenic dentate gyrus.

To relate specific deficits in transcriptional cascades in daDREAM hippocampal neurons and changes in behavior is more complex. Nevertheless, the reduction in the activity-dependent response in transgenic neurons in culture is in line with the impaired ability in learning and memory tests shown by daDREAM mice. Overall, the cognitive profile suggests a complex defect probably specific for the cortico-hippocampal circuit, which mainly affects spatial and associative learning, but also working and reference memories. These results agree with the impairment in long-term recognition memory shown in Npas4 deficient mice (8, 41).

Conversely, short-term object recognition memory is not affected in daDREAM mice. Interestingly, daDREAM mice showed increased early LTP in the hippocampus that is considered to be a cellular analogue of learning. This is more likely determined by the level of inhibition, rather than by other factors like the level of BDNF that, nevertheless, was markedly reduced in daDREAM mice. BDNF has a role in activity-dependent neuroplasticity in the hippocampus that could explain the spatial learning defects in daDREAM mice with BDNF deficiency. In this regards, heterozygous BDNF knockout mice reportedly under-express BDNF and have reduced late LTP, but their spatial memory and search strategy assessed with Morris water maze (31) is preserved, suggesting that the relationship between LTP and memory is not always straightforward. Since it is always the case that early LTP transits smoothly into late LTP, daDREAM may also affect long-lasting forms of memory that are generally believed to be mediated by new protein synthesis, leading to synaptic growth and requirement of gene transcription and new protein synthesis (30, 38). In a more broaden view; the present results also
suggest a role of DREAM throughout development in the establishment and refinement of neuronal circuitries which, indisputably, will result in the changes in brain function in the adult animal.

In conclusion, the results from the genome-wide analysis of hippocampus from daDREAM mice indicate that Ca\(^{2+}\)-dependent unbinding from DNA of DREAM/KChIP proteins is an initial regulatory step necessary to switch on and off gene expression cascades that control the inhibition-excitation balance and ultimately allow learning and memory to occur. Central to these cascades and immediately downstream from DREAM, the Npas4 protein emerges as a critical early regulator of activity-dependent learning and memory. The master-switch regulatory role of DREAM makes this repressor protein a novel target for drug development of new generation molecules, not based on phosphodiesterase inhibition and independent of the CREB pathway, which could improve cognition.

**Acknowledgements**

This work was supported by grants from Spanish Ministry of Health and Science, Madrid Community, La Marató, La Caixa, Reina Sofia and Areces Foundations, the EU 6th Framework Program (NeuroNE, CureFXS), the ERA-NET Program (Neuron and E-Rare) and the Medical Research Council. S.K. has a postdoctoral contract from the Ramón y Cajal Program of the Ministry of Science and Innovation.

We thank Xose M. Dopazo for technical assistance.

**Ethical standards**

The experiments comply with the current laws of the country in which they were performed.
Conflict of interest

The authors declare that they have no conflict of interest.

References


33. **Oliveros, J. C.** 2007, posting date. FIESTA at BioinfoGP. [Online.]


Figure legends

Figure 1 Expression of daDREAM in transgenic brain

(A) Comparative real time qPCR analysis of the expression levels of daDREAM in cerebral cortex (Cx), hippocampus (H), striatum (St) and cerebellum (Cb) from transgenic mice. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM from 8-12 mice in two independent experiments. (B) Coronal brain section from daDREAM mice showing the distribution of β-galactosidase activity in hippocampus (upper panel) and cerebral cortex (lower panel). Bars represent 250 μm.

Figure 2 Genome-wide analysis in daDREAM and wild type hippocampus

(A) Functional clustering (Molecular Function, Gene Ontology) of up- and down regulated genes encoding transcription factors or DNA-binding proteins. Biological triplicates of wild type (wt) and transgenic (tg) mice are presented as a “heat map”. F. Change; differential expression based on the ratio between averaged intensities (linear scale), FDR; adjusted P values, Gene Symbol | Gene Title; probe annotations as provided by Affymetrix. (B) Real time qPCR analysis of indicated transcripts in the hippocampus from wild type and transgenic mice. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM from 8-12 mice in two independent experiments. **P <0.01 ***P <0.001 (two-tailed, unpaired t-test) relative to wt.

Figure 3 DREAM directly regulates Npas4-mediated transcription
(A) Western blot analysis of cortex-hippocampal neuronal cultures from wild type (wt), transgenic daDREAM (tg) and DREAM knockout (ko) embryos. A DREAM-specific immunoreactive band (empty arrowhead), detected by the peptide specific antibody Ab730 against DREAM, was increased in transgenic daDREAM neurons and absent in knockout cultures. Appearance of a non-specific band is marked with an asterisk. Use of the pan-KChIP antibody Ab1014 on the same samples showed different immunoreactive bands (full arrowheads) corresponding to the different KChIP isoforms expressed in these cultured neurons.

Quantification of the specific DREAM and the total KChIP immunoreactivities as relative ratios vs loading control β-actin is shown at the bottom. (B) Real-time qPCR analysis of Npas4 in cortico-hippocampal primary cultured neurons from wild type (wt) and transgenic (tg) embryos in basal conditions and 30 min after stimulation with 60 mM K+. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM from three separate cultures in triplicates. **** P < 0.0001 (One-way ANOVA, Tukey's multiple comparison). (C) Chromatin immunoprecipitation assay of the Npas4 promoter using chromatin isolated from primary cultured neurons from wild type (wt) and transgenic (tg) embryos before and after potassium depolarization. Cultured neurons from DREAM knockout (ko) embryos and exclusion of the antibody (-Ab) in the immunoprecipitation of wild type chromatin were included as negative controls. Autoradiogram of the semiquantitative PCR is shown. After densitometric quantification, the results shown are the mean ± SEM from four experiments. Input is 1%. ****

P < 0.0001 (n = 8, two-tailed, unpaired t-test) vs wt nonstimulated. (D) and (E) Real-time qPCR analysis of c-Fos mRNA and chromatin immunoprecipitation assay of the c-fos promoter, respectively, as in B and C. qPCR, **** P < 0.0001 (One-way ANOVA, Tukey's multiple comparison). ChIP, * P = 0.0380 (n = 5, two-tailed, unpaired t-test) vs wt nonstimulated.
Figure 4 GABAergic inhibitory transmission is reduced in daDREAM hippocampus

(A) and (B) Real-time qPCR analysis of indicated GABAR subunits (A) and GABAergic markers (B), in wild type (wt) and transgenic (tg) hippocampus. Results are the mean ± SEM from 8-12 mice in two independent experiments. * \( P < 0.05 \), *** \( P < 0.001 \) (One-way ANOVA, Tukey's multiple comparison). (C) Western-blot analysis of PSD95 and \( \beta \)3 GABA receptor proteins in wild type and transgenic hippocampus. \( \beta \)-actin content was used as loading control. Representative autoradiograms are shown. Densitometric quantification of the specific immunoreactive band vs loading control \( \beta \)-actin, from a total of 4 (PSD95) or 8 (GABARA\( \beta \)3) samples from each genotype, is shown. ** \( P = 0.0039 \) (two-tailed, unpaired \( t \)-test,).

(D) Seizure severity after convulsant administration (PTZ, 45 mg/kg) in wild type (wt, \( n = 7 \)) and transgenic mice (tg, \( n = 8 \)), ** \( P = 0.0083 \) (Mann Whitney test).

Figure 5 Loss of paired-pulse inhibition and enhanced LTP in DREAM mutant mice

(A) For paired-pulse studies, the amplitude of the population spike evoked by the second stimulus, normalized to the amplitude of the population spike evoked by the first stimulus, is plotted as a function of the interstimulus interval for transgenic (tg, \( n = 6 \)) and corresponding wild type littermates (wt, \( n = 11 \)). Note the complete suppression of the second population spike at short intervals in wild type mice and its absence at all intervals in transgenic mice. Stimulus intensity was set so that the first stimulus of the pair evoked a population spike of \( \sim 1\text{mV} \). Representative responses to paired stimuli for each group are displayed on the right. Calibration 3 mV, 4 ms. (B) LTP was measured at perforant path-granule cell synapses in anaesthetized transgenic and wild type mice. LTP, measured 50-60 min after the tetanus (arrow), was
significantly enhanced. The magnitude of LTP was 32.3 ± 2.6% for transgenic and 13.7 ± 4.0% for wild type mice, \( P < 0.005 \).

**Figure 6** DREAM directly regulates activity-dependent BDNF gene expression

(A) Real-time qPCR of BDNF levels in wild type (wt) and transgenic (tg) hippocampus. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM of 8-12 mice. *** \( P < 0.001 \) (One-way ANOVA, Tukey's multiple comparison). (B) Chromatin immunoprecipitation assay of the promoter IV of the BDNF gene using chromatin isolated from primary cultured neurons from wild type (wt) and transgenic (tg) embryos before and after potassium depolarization. Cultured neurons from DREAM knockout (ko) embryos or exclusion of the antibody (-Ab) in the immunoprecipitation of wild type chromatin were included as negative controls. Autoradiogram of the semiquantitative PCR is shown. After densitometric quantification, the results shown below are the mean ± SEM from four experiments. ** \( P = 0.0022 \) (n = 3, two-tailed, unpaired \( t \)-test) vs wt nonstimulated.

**Figure 7** Impaired learning and memory in daDREAM mice

(A) Escape latency across acquisition sessions is increased in transgenic (tg, n=11) with respect to wild type (wt, n=10) mice (Repeated measures ANOVA \( F_{(1,19)} = 9.052, P = 0.007 \)). No difference in swimming speed was detected between genotypes (Repeated measures ANOVA \( F_{(1,19)} = 3.633, P = 0.072 \); Inset). (B) and (C) In the repeated reversal learning paradigm, transgenic mice (n=10) showed an increase in escape latency on odd (B) and even (C) trials, related with impairment in reference and working memory, respectively, across the eight acquisition sessions as compared to wild type mice (n=10). Data are expressed as mean ± SEM. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \). For statistical details see Supporting Table S7.
**Figure 8** Activity-dependent gene expression is not modified in the hippocampus of DREAM knockout mice

(A) to (C) Real-time qPCR analysis of indicated transcripts in the hippocampus from wild type (wt, n = 10) and DREAM deficient (ko, n = 10) mice. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM of two separate experiments. No statistically significant differences were found. (D) Real-time qPCR analysis of KChIP transcripts in primary cultured cortico-hippocampal DREAM−/− neurons in basal conditions. (E) Real-time qPCR analysis of KChIP transcripts in primary cultured cortico-hippocampal DREAM−/− neurons in basal conditions or after transduction with empty (LV) or antisense-KChIP-2 lentiviral vector (AS). The result is expressed relative to the basal expression level of respective KChIP. * P < 0.05 vs respective basal (One-way ANOVA, Dunnett’s multiple comparison test). (F) Real-time qPCR analysis of Npas4, c-fos and CREB mRNA in cortico-hippocampal primary cultured neurons from DREAM−/− embryos in basal conditions (-) or 6 days after infection with the indicated lentiviral vector. The increase in Npas4 and c-fos levels following KChIP knockdown is not observed in CREB mRNA levels. For comparison, no significant changes in Npas4, c-fos or CREB mRNA levels are observed in basal conditions in DREAM−/− vs wt cultured neurons. Values are normalized with respect to HPRT or β-actin mRNA content. Results are the mean ± SEM from three separate cultures in triplicates. * P < 0.05, ** P < 0.01, vs respective basal (One-way ANOVA, Dunnett’s multiple comparison test).

**Figure 9** DREAM directly regulates its own activity-dependent expression
(A) Real-time qPCR analysis of endogenous DREAM mRNA in different telencephalic areas from wild type (wt) and transgenic (tg) mice. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM of 8-12 mice. *** P < 0.001 (unpaired t-test) (B) Real-time qPCR analysis of endogenous DREAM mRNA in primary cortico-hippocampal cultures from wild type and transgenic embryos in basal conditions and after potassium depolarization. Values are normalized with respect to HPRT mRNA content. Results are the mean ± SEM from three separate cultures in triplicates. * P <0.05, *** P <0.0001 (One-way ANOVA, Tukey's multiple comparison). (C) Chromatin immunoprecipitation assay of the mouse DREAM promoter using a DREAM-specific antibody and isolated chromatin from primary cultured neurons from wild type (wt) and transgenic (tg) embryos before and after potassium depolarization. Cultured neurons from DREAM knockout (ko) embryos or exclusion of the antibody (-Ab) in the immunoprecipitation of wild type chromatin were included as negative controls. Autoradiogram of the semiquantitative PCR is shown. After densitometric quantification, the results shown below are the mean ± SEM from four experiments. ** P = 0.0031 (n = 3, two-tailed, unpaired t-test) vs wt nonstimulated.
### Figure 2

**A**

<table>
<thead>
<tr>
<th>F.change</th>
<th>FDR</th>
<th>Gene Symbol</th>
<th>Gene Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2.23</td>
<td>0.000077158</td>
<td>Ssbp2</td>
<td>single-stranded DNA binding protein 2</td>
</tr>
<tr>
<td>+2.14</td>
<td>0.01302366</td>
<td>Zic1</td>
<td>zinc finger protein of the cerebellum 1</td>
</tr>
<tr>
<td>+1.70</td>
<td>0.00115802</td>
<td>Hist1h1c</td>
<td>histone cluster 1, H1c</td>
</tr>
<tr>
<td>-1.61</td>
<td>0.00392722</td>
<td>Zfp423</td>
<td>zinc finger protein 423</td>
</tr>
<tr>
<td>-1.62</td>
<td>0.00516801</td>
<td>Smarcc1</td>
<td>SWI/SNF related protein</td>
</tr>
<tr>
<td>-1.68</td>
<td>0.00445360</td>
<td>Mef2c</td>
<td>myocyte enhancer factor 2C</td>
</tr>
<tr>
<td>-1.69</td>
<td>0.00804135</td>
<td>Hmx box1</td>
<td>homeobox containing 1</td>
</tr>
<tr>
<td>-1.77</td>
<td>0.01005704</td>
<td>Dbp1 D site albumin promoter binding protein</td>
<td></td>
</tr>
<tr>
<td>-1.83</td>
<td>0.04096959</td>
<td>Egr2</td>
<td>early growth response 2</td>
</tr>
<tr>
<td>-1.84</td>
<td>0.00180015</td>
<td>Junb</td>
<td>Jun-B oncogene</td>
</tr>
<tr>
<td>-1.93</td>
<td>0.00030301</td>
<td>Nfia</td>
<td>nuclear factor I/A</td>
</tr>
<tr>
<td>-2.04</td>
<td>0.00612617</td>
<td>Tshz2</td>
<td>tashirt zinc finger family member 2</td>
</tr>
<tr>
<td>-2.09</td>
<td>0.00928037</td>
<td>Nrrd1</td>
<td>nuclear receptor subfamily 4, group A, member 1</td>
</tr>
<tr>
<td>-3.11</td>
<td>0.00001513</td>
<td>Mbd4</td>
<td>methyl-CpG binding domain protein 4</td>
</tr>
<tr>
<td>-3.31</td>
<td>0.00001477</td>
<td>Npas4</td>
<td>neuronal PAS domain protein 4</td>
</tr>
<tr>
<td>-4.20</td>
<td>0.00163377</td>
<td>Fos</td>
<td>FBJ osteosarcoma oncogene</td>
</tr>
</tbody>
</table>

**B**

![Graphs showing gene expression levels](image)

- **Npas4**: wt > tg
- **Nr4a1**: wt < tg
- **c-Fos**: wt < tg
- **Mef2c**: wt > tg
- **Per3**: wt < tg
- **JunB**: wt < tg
- **Sox11**: wt > tg
- **Egr2**: wt > tg
- **Mbd4**: wt > tg
Figure 4

A

[BAR] (GABA/actin) *10^6

<table>
<thead>
<tr>
<th></th>
<th>α2</th>
<th>α1</th>
<th>β2</th>
<th>β3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

B

(GABA marker/actin) *10^6

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAT1</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>VIAAT</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>GAD2</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

C

PSD95

β-actin

wt | tg |

0.5 | 1.0 |

GABARβ3

β-actin

wt | tg |

0.5 | 1.0 |

D

Seizure score

PTZ

0 | 5 |

0.5 | 1.0 |

** | **
Figure 5

A

Spike height, % change

Inter-pulse interval (ms)

B

EPSP slope, % change

Min