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Inducible control of gene expression with destabilized Cre

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Abstract

Acute manipulation of gene and protein function in the brain is essential for understanding the mechanisms of nervous system development, plasticity and information processing. Here we describe a technique based on a destabilized Cre recombinase (DD-Cre) whose activity is controlled by the antibiotic, TMP. We show that DD-Cre triggers rapid TMP-dependent recombination of “floxed” alleles in mouse neurons *in vivo*, and validate the use of this system for neurobehavioral research.

Studies of brain development, neural circuits underlying behavior and synaptic plasticity benefit from approaches that enable rapid genetic modification of intact circuits in live animals. Two such approaches commonly used in mice rely on loxP recombination mediated by the tamoxifen-inducible Cre-ER^{1–3}, and expression of cDNAs under the control of tetracycline-dependent elements, tTA and rtTA⁴. However, these methods have drawbacks including side effects associated with Tamoxifen binding to native estrogen receptors^{5,6}, and relatively slow kinetics of induction and repression^{4,7,8}. Several elegant tools have been developed to manipulate neuronal activity in the brain with light, small molecules, and peptides^{9–13}. Most of these techniques take advantage of unique properties of specific proteins, and cannot be applied to regulate other polypeptide species. Recently, a general method for direct pharmacological control of protein stability has emerged^{14,15}. This technology utilizes mutant destabilizing (DD) forms of human FKBP12 or bacterial dihydrofolate reductase (ecDHFR), which can be attached to virtually any protein of interest (P.O.I). Newly synthesized DD-P.O.I.s containing the FKBP12 or ecDHFR tags are degraded through the proteosomal pathway, but their decay can be blocked with the ligands Shield-1 and Trimethoprim (TMP), respectively^{14,15}. TMP-inducible protein stabilization is particularly attractive for *in vivo* applications because TMP can be easily delivered to laboratory animals, exhibits a high rate of diffusion in peripheral tissues and nervous system, and does not have endogenous targets in mammals^{14,16}.

Since TMP penetrates the blood-brain barrier, we sought to test the utility of ecDHFR tags for neurogenetic and behavioral research. Supporting previous studies in rats¹⁶, we found that TMP reaches a peak concentration in the mouse brain within 10 minutes after intra-

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AUTHOR CONTRIBUTIONS A.M. and R.S. conceived hypotheses and designed the experiments. R.S. generated expression constructs and characterized mutant mice. M.M. and K.B. examined TMP pharmacokinetics in the brain. K.B., S.P. and N.T.R. contributed to imaging and behavioral analyses. T.W. provided DD tags and assisted with interpretation of results. A.M. wrote the manuscript.

peritoneal (i.p.) injection and declines below detection threshold by 30 minutes (see Online Methods). In cultured neurons, TMP acutely restored the levels and biological activity of various soluble and membrane DD-P.O.I.s localized to cytoplasm, neurotransmitter vesicles and nucleus (Supplementary Fig. 1 and data not shown). We then fused ecDHFR to Cre recombinase (DD-Cre, Supplementary Fig. 2) considering this regulatory element could be used in a wide range of experimental systems. When introduced into cortical cultures with a lentivirus, DD-Cre was rapidly stabilized by the drug. Side-by-side comparison of DD-Cre and Cre-ERT2 showed similar dependency on lentiviral titers of ligand-inducible loxP recombination and background recombination levels (Supplementary Fig. 3). Likewise, both Cre forms exhibited comparable background upon lentiviral expression in the cortex of Rosa26 Ai9 tdTomato reporter mice. However, DD-Cre activated the expression of the reporter in a larger number of neurons in response to i.p. drug injections, suggesting efficient recombination can be achieved by a transient DD-Cre stabilization *in vivo* (Supplementary Fig. 4).

To examine performance of DD-Cre throughout the forebrain, we generated transgenic mice driving DD-Cre under the control of excitatory neuron-specific CamKII α promoter. Two independent founder lines were mated with an Ai9 strain (Fig. 1a) and the resulting offspring were analyzed after postnatal week 4, when CamKII α promoter activity reaches steady-state levels (Supplementary Fig. 5). We initially subjected DD-Cre/Ai9 mutant to either a single or seven sequential daily TMP injections (170 μ g/gm body weight) to test if multiple exposures to the drug boost recombination efficiency. A single TMP injection was sufficient to drive recombination in one of the lines, producing robust reporter expression (Fig. 1b and Supplementary Fig. 6). The densities of tdTomato-positive neurons varied in distinct brain regions, most likely reflecting the pattern of transgenic DD-Cre expression rather than its non-uniform stabilization across different areas. Indeed, repetitive injections resulted in no improvement of recombination rates, and >80% of cells with detectable DD-Cre mRNA became tdTomato-positive in all domains following a single TMP dose. With exception of the Dentate Gyrus (DG) where 17% of mature granule cells expressed the reporter in the absence of TMP, constitutive DD-Cre activity was sparse in the cortex and hippocampus (Fig. 1c, and Supplementary Fig. 6 and Supplementary Table 1). This background leak may be due to the unique properties of granule cells, high Ai9 reporter sensitivity and excessive DD-Cre copy number as other transgenic strains generated in our laboratory showed an enrichment of CamKII α -driven transcripts in the DG¹⁷. The second DD-Cre founder line (LE-DD-Cre) had virtually no leaky activity but only displayed TMP-dependent recombination in a small fraction of cells, perhaps due to mosaicism. While unsuitable for biochemical and behavioral studies, these mice are useful for imaging projections and synaptic connectivity of isolated neurons (Supplementary Fig. 7).

To define the DD-Cre pharmacokinetics in the brain, we calculated the densities of neurons tagged with tdTomato in cortices of DD-Cre/Ai9 animals that were treated with one dose of TMP at different concentrations (between 8 and 170 μ g/gm). Under these conditions we observed a linear TMP dose-response (Figs. 1d, f). We also analyzed the time course of reporter expression in mice subjected to 170 μ g/gm of TMP. The yield of modified genomic DNA as assessed by PCR and the numbers of cells with detectable tdTomato fluorescence reached plateaus at ~24 and 48 hours following drug administration, respectively (Figs. 1e, g). This delay in tdTomato induction is not surprising given that it requires protein synthesis.

The Rosa26 locus has a relaxed chromatin structure which makes it easy for Cre to access (this also likely contributes to the observed background), so we asked if DD-Cre is capable of recombining other DNA substrates. To this end, we injected adeno-associated viruses encoding a “floxed” GFP reporter (AAV2.5 DIO-GFP) into the cortices of DD-Cre mice. TMP potentially triggered recombination in this case as well, with no apparent increase in the

density of GFP-positive neurons after repetitive drug treatments (Figs. 1h, i and Supplementary Table 1). We also found that drug-mediated recombination with minimum background can be achieved by adjusting the viral titers (Supplementary Fig. 8).

To evaluate the applicability of DD-Cre for behavioral studies, we generated a mouse strain carrying DD-Cre and a Cre-inducible form of Tetanus toxin (TeNT), which inhibits neurotransmitter release by cleaving the vesicular SNARE protein VAMP/Syb2 (R26^{flloxstop}-TeNT, Fig. 2a)¹⁸. We treated DD-Cre/TeNT and their control littermates with TMP, and examined their brains by immunostaining for TeNT, Syb2 and Zif268/Egr1, a product of an immediate early gene whose transcription is induced by synaptic excitation. Drug-injected animals had decreased Zif268/Egr1 and Syb2 immunoreactivity (Figs. 2b–e). Immunoblotting revealed that Syb2 cleavage starts to occur ~6 hours after drug administration, reflecting the acute induction of TeNT mediated by DD-Cre (Figs. 2f,g). Electrophysiological recordings from brain slices confirmed a TMP-dependent loss of excitatory synaptic strength in DD-Cre/TeNT mice (Fig. 2h and Supplementary Table 2).

Next, we asked how TMP-inducible synaptic silencing, residual DD-Cre activity and TMP itself impact information processing in the forebrain. To accomplish this, we interrogated vehicle and drug-injected DD-Cre/TeNT mutants and their Cre-negative littermates using a battery of behavioral tests. No genotypic and drug-dependent changes were detected in locomotion and vision (Fig. 3a and Supplementary Fig. 9 and Supplementary Table 3). However, administration of TMP to DD-Cre/TeNT mice abolished recognition and spatial memory, as evidenced by their lack of interest in novel objects, loss of preference for target quadrants in the Barnes maze probe test, and increased frequency of errors made during reversal test. In contrast, these memory forms were intact in vehicle-injected DD-Cre/TeNT and TeNT as well as TMP-treated TeNT strains. (Figs. 3b, c and Supplementary Fig. 9 and Supplementary Table 3). Interestingly, all four groups of animals had normal associative memory (data not shown). Hence, neither TMP alone nor constitutive DD-Cre activity in the brain of DD-Cre/TeNT mice affects the behavior in the open field and memory acquisition/retrieval, whereas inducible blockade of neurotransmission interferes with specific cortical- and hippocampal-dependent tasks.

The rapid kinetics, wide dynamic range, and lack of detectable TMP side effects make DD-Cre system an attractive tool for a broad spectrum of neurogenetic applications. The temporal control of DD-Cre *in vivo* is faster than Cre-ERT2 and tTA/rtTA, which usually require repetitive exposure to the inducing drugs^{3,7,8,19,20}. DD-Cre's strong TMP dose-dependence also makes it an ideal tool for simultaneous perturbation and tagging of small populations of cells for the study of cell-autonomous mechanisms. Similar to other chemical-genetic methods, DD-Cre may exhibit constitutive background. Although our analyses indicate no behavioral consequences of such background in adult DD-Cre/TeNT mice and suggest that tight regulation can be attained at the cellular level *in vitro* and *in vivo*, promoter strength, developmental profile of DD-Cre expression and sensitivity of its downstream targets should be taken into consideration when designing new mouse strains.

Viewed in a broader scope, our results provide a framework for future development of animal models carrying destabilized proteins in specific tissues and cell types. For example, attaching DD tags to transcription factors, membrane receptors and signaling molecules may enable wide control over different protein functions and facilitate research of cellular differentiation and metabolism. Furthermore, these tags can potentially be used in combination with other common elements such as FLP and tTA/rtTA (for dual pharmacological control). The performances of DD-P.O.I.s., may be influenced by rates of decay, recruitment of stabilized ligand-bound fusions to appropriate subcellular domains,

and interference of DD tags with protein function. Thus, new DD systems should always be validated in cultured cells prior to committing to *in vivo* experiments.

ONLINE METHODS

Lentivirus shuttle vectors and *in vitro* studies

Sequences encoding DD-YFP, DD-Cre, Cre-ERT2, Cherry-H2B, GFP and floxstop-GFP were subcloned into lentivirus shuttle vectors containing a synthetic neuronal Synapsin promoter. KD+DD-Syb2 vector was composed of H1 promoter driving a hairpin specific for mouse Syb2 (GTGCAGCCAAGCTCAAGCG) followed by the Synapsin promoter and DD-Syb2 rescue cDNA sequence containing non-coding mutations that disable RNA interference. Methods for preparation of neuronal cultures, lentivirus production and infection, immunostaining, immunoblotting and electrophysiology were described previously^{17,21}.

Mice

Constitutive CamKII α :Cre driver line, R26 Ai9 Cre reporter and R26^{floxstop-TeNT} allele were previously characterized^{18,22,23}. To generate CamKII α :DD-Cre transgenic strains, a sequence encoding DD-Cre (Supplementary Fig. 2) was inserted downstream of the 8 kb CamKII α promoter in a targeting vector that also included 5' and 3' introns flanking the cDNA and a 3' SV40 polyA signal. The targeting construct was linearized and used for pronuclear microinjection at the TSRI Mouse Genetics Core. Positive founders were identified by PCR with the following primer pairs:

GGCAATTGAGATCTACCATGATCTCTCTGATTGCCG

CGCCGCTAGCTAATCGCCATCTTCCAGCAG; (1.3 kb product specific for DHFR-Cre) and CTCCGTTTGCACCTCAGGAGCAC /

GCGCGGCGCGCCTCTCCTTTCCAGGATCTCAAAG (0.9 kb product specific for CamKII α promoter and ecDHFR). Founders were then mated with mice carrying Ai9 or R26^{floxstop-TeNT} alleles (mixed C57/Bl6 and 129/sv background) and their offspring was genotyped and used for experiments.

In vivo drug injection

Trimethoprim (Sigma, T7883) was reconstituted in DMSO at the saturating concentration of 100 mg/ml. This solution was prepared fresh for every experiment, and diluted in 0.9% saline immediately before injections. Intra-peritoneal (i.p.) injections were carried out using a 29g needle to deliver TMP doses ranging between 8 and 170 μ g/gm body weight. Vehicle solution containing saline and DMSO was used as a control. TMP pharmacokinetics was examined in wildtype C57/BL6 mice using an *in vitro* bioassay based on inhibition of bacterial growth. TMP reached peaked concentrations in brain extracts of 1 μ g/ml at 10 minutes following a single injection (50 μ g/gm). The maximum daily TMP doses did not exceed 170 μ g/gm to avoid potential distress and precipitation of the drug. Mutant animals were analyzed at various time-points after drug treatments, as indicated in figure legends. Tamoxifen was reconstituted in corn oil (10 mg/ml) and injected at 100 μ g/gm body weight.

In vivo virus injection

Concentrated lentiviruses were produced in house. AAV2.5 DIO-GFP was generated at the Salk vector core using a shuttle vector composed of 1.26 kb EF1 α promoter and inverted GFP sequence flanked by two pairs of loxP sites. Viral titers were determined by PCR. Newborn pups were anesthetized on ice and injected with 0.5 μ l of the viral stock using a glass micropipette (10 μ m tip diameter). Pups were then warmed for 1–2 min under an incandescent lamp and returned to home cages until experiments.

Immunohistochemistry

Mice were anesthetized with isoflurane and perfused with 4% PFA. The brains were incubated overnight in 0.5% PFA, and sliced in PBS using a vibratome. The 90 μ m thick brain sections were briefly boiled in 0.1M citrate solution for antigen retrieval, and subsequently placed for 5 minutes into citrate buffer (40.5 ml of 0.1M sodium citrate, 9.5 ml of 0.1M citric acid, pH 6.0). Sections were then washed in PBS, blocked for 3 hours in 4% BSA, 2% horse serum, 0.5% Triton, and incubated overnight with primary antibodies diluted in blocking solution, followed by washes in PBS and incubation with DAPI and/or corresponding fluorescently labeled secondary antibodies.

Antibodies

Monoclonal anti-Syb2 (Cl69.1) and polyclonal anti-Syt1 antibodies were a kind gift of Dr. Thomas Südhof. Anti- β Tubulin, anti-Zif268/Egr1 and anti-SPO antibodies were purchased from Sigma (Cat# T2200), Cell Signaling Technologies (Cat# 4153S) and Synaptic Systems (Cat# 102-002), respectively. All antibodies were used at 1:1000 dilution for immunoblotting and immunohistochemistry.

In situ hybridization

Probes for DD-Cre and tdTomato transcripts were synthesized using the Roche RNA labeling kit, validated by electrophoresis and sequencing, and tagged with either FITC or DIG. Brain sections were prepared as described above, and acetylated in the buffer containing triethanolamine and acetic anhydride. Sections were then pre-hybridized at 65°C in the buffer composed of 50% formamide, 5X saline-sodium-citrate, 5X Denhardts, 250 μ g/ml yeast tRNA, 500 μ g/ml herring sperm DNA, 50 μ g/ml heparin, 2.5mM EDTA, 0.1% Tween-20, 0.25% CHAPS followed by overnight incubation with heat-denatured probes diluted in the same buffer. Labeled sections were washed in 0.2X SSC, equilibrated in 100mM Tris-HCl pH7.5, 150mM NaCl, and blocked in 0.5% Perkin-Elmer Blocking Reagent in TN buffer. Sections were then washed in 0.05% Tween20 followed by Tyramide amplification (Perkin-Elmer TSA Plus system), and sequential treatments with anti-FITC-POD and FITC-Tyramide. Upon quenching peroxidase activity with 3% hydrogen peroxide, sections were treated with anti-DIG-POD (1:500), washed, and mounted for analysis.

Image acquisition and data analysis

Images were collected under the Nikon C2 confocal microscope using 10 $\times$, 20 $\times$ and 60 $\times$ oil immersion objectives. Digital images were initially processed with the Nikon Elements software package. Digital manipulations were applied equally to all pixels. The densities of reporter-positive neurons were calculated automatically using Metamorph software. Three-dimensional reconstructions of GCs shown in Supplementary Fig. 7 were performed with the NeuroLucida package.

PCR

Genomic DNA was isolated from the cortices of DD-Cre/TeNT mice at different time points after TMP injection and analyzed by PCR with the following primer pairs specific for the recombined allele: CGTGCTGGTTATTGTGCTGTCTCATC / CCTCGGCGCGGGTCTTGTAGTTGCC.

Electrophysiology

Anesthetized mice were decapitated into oxygenated Ca^{2+} -free aCSF solution composed of 124mM NaCl, 2mM KCl, 1.25mM KH_2PO_4 , 2mM MgSO_4 , 26mM NaHCO_3 , 10mM dextrose, pH7.4. 400 μ m brain sections were prepared using a vibratome. Slices were transferred to aCSF containing 2mM Ca^{2+} and allowed to recover for 1 hr at 30°C prior to

recordings. Excitatory field potentials were monitored from DG granule cells using a glass electrode filled with 2M NaCl (~3MΩ). Synaptic responses were elicited by an extracellular electrode (Concentric bipolar electrode, FHC) placed near perforant path, and recorded in the presence of 20μm Gabazine. Data was sampled with Multiclamp700B amplifier and analyzed off-line using Clampfit10 and Origin8 software packages.

Behavioral studies were conducted at TSRI core facility according to protocols approved by the IACUC committee. Mice were injected once with either vehicle or TMP solution (170 μg/gm) 48 hours prior to initiation of behavioral trials. All experiments were performed by an experimenter who was unaware of genotypes. Locomotor activity was measured for 2 hours in polycarbonate cages (42 × 22 × 20 cm) placed into frames (25.5 × 47 cm) mounted with two levels of photocell beams at 2 and 7 cm above the bottom of the cage (San Diego Instruments, San Diego, CA). These two sets of beams allow for the recording of both horizontal (locomotion) and vertical (rearing) behavior. Vision was assessed in a stationary elevated platform surrounded by a drum with black and white striped walls. Mice were placed on the platform to habituate for 1 minute and then the drum was rotated at 2rpm in one direction for 1 minute, stopped for 30 sec, and then rotated in the other direction for 1 minute. The total number of head tracks (15 degree movements at speed of drum) was recorded. In our hands, mice that have intact vision track 5–25 times, whereas blind mice do not track at all. Novel object test assays recognition memory while leaving the spatial location of the objects intact. Mice were individually habituated to a 51cm × 51cm × 39cm open field for 5 min. Mice were then tested with two objects placed in the field (Playmobil, Geobra Brandstatter GmbH & Co. KG, Zirndorf, Germany). The objects chosen (e.g. unicorn, cow, boy and queen) were made of durable non-toxic plastic and are fixed to 10 cm × 7 cm × 0.5 cm square clear Plexiglas bases to prevent mice from moving the objects during testing²⁴. For the familiarization trials, two different objects were placed in the open field and an individual animal was allowed to explore for 5 min. After four familiarization trials (separated by 1 minute in a holding cage), the mouse was tested in the object novelty recognition test in which a novel object replaced one of the familiar objects. All objects and the arena were thoroughly cleaned with 70% ethanol between trials to remove odors. Behavior was video recorded and then scored for contacts (touching with nose or nose pointing at object and within 0.5 cm of object). Habituation to the objects across the familiarization trials (decreased contacts) is an initial measure of learning and then renewed interest (increased contacts) in the new object indicates successful object memory. The Barnes maze test is a spatial learning and memory test originally developed in rats²⁵, but also adapted for mice²⁶. It consists of an opaque Plexiglass disc 75 cm in diameter elevated 58 cm above the floor by a tripod. Twenty holes, 5 cm in diameter, are located 5 cm from the perimeter, and a black Plexiglass escape box (19 × 8 × 7 cm) is placed under one of the holes. Distinct spatial cues are located around the maze and are kept constant throughout the study. Acquisition: On the first day of testing, a training session was performed, which consisted of placing the animals in the escape box for one minute. At the beginning of each session, mice were then placed in the middle of the maze in a 10 cm high cylindrical black start chamber. After 10 seconds the start chamber was removed, a buzzer (80 dB) and a light (400 lux) were turned on, and mice were set free to explore the maze. The session ended when mice entered the escape tunnel or after 3 min elapsed. When mice entered the escape tunnel, the buzzer was turned off and animals were allowed to remain in the dark for one minute. If a mouse did not enter the tunnel by itself it was gently put in the escape box for one minute. The tunnel was always located underneath the same hole (stable within the spatial environment), which was randomly determined for each mouse. Mice were tested once a day for 4 days for the acquisition portion of the study. Probe test: For the 5th test (probe test), the escape tunnel was removed and mice were allowed to freely explore the maze for 3 min. The time spent in each quadrant was determined and the percent time spent in the target quadrant (the one originally containing the escape box) was compared with the

average percent time in the other three quadrants. This is a direct test of spatial memory as there is no potential for local cues to be used in the mouse's behavioral decision. ***Retention test:*** Two weeks later the mice were tested for long-term memory with the escape box in the original position (retention test). ***Reversal test:*** Finally, on the day after retention test, the escape tunnel was moved to a new location (90 degrees from the original position) and the behavior of the mouse was recorded. This test allows for the examination of perseveration at the old hole as well as the working memory strategies the mice adopt to locate the new tunnel location. Each session was videotaped and scored by an experimenter blind to the genotype of the mouse. Measures recorded include the number of errors made per session and the latency to locate and enter the escape tunnel. Errors are defined as nose pokes and head deflections over any hole that did not have the tunnel beneath it. ***Associative memory.*** Mice were allowed to explore the fear conditioning boxes for 3 min and were then presented with four 20s periods of white noise, each paired with an electric foot shock (0.6 mA) during the last two seconds (1-min inter-tone/shock intervals). Contextual memory testing consisted of one 3 min exposure to the training box (Med Associates SD) and was followed 2-hrs later by cued memory testing. Mice were introduced to an alternative box and presented with four 20 sec periods of white noise at 1 min intervals. Freezing was determined in 0.75s bouts and expressed as percent time in the context or of tone presentation. Contextual freezing was normalized by subtracting the freezing before the first white noise presentations during conditioning. Cued freezing was normalized by subtracting freezing before the first white noise presentation during cued memory testing. All memory acquisition/retrieval tests were performed with groups of equal size containing equal numbers of males and females.

Statistical analyses were performed using ANOVA and Student t-test. Data are represented as Mean±S.E.M., unless indicated in the text. Whenever possible, sample sizes were chosen to ensure an appropriate amount of statistical power. Raw data is shown for experiments utilizing less than 3 mice per TMP concentration or time point after induction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. TMP-dependent loxP recombination in the brain of DD-Cre mice

a, Schematic representation of CamKIIα:DD-Cre and R26 Ai9 Cre reporter alleles.

b, Images of whole brains and DAPI-stained sections isolated from vehicle- and TMP-injected DD-Cre/Ai9 mutants. SSC=somato-sensory cortex (layers II–IV).

c, Background and TMP-dependent recombination in the SSC and hippocampal CA1, CA3 and Dentate Gyrus (DG). Mice were treated with either single or seven sequential daily TMP doses (170 μg/gm/day) starting at P30 and analyzed at P37. Percentages of tdTomato-positive cells were calculated using DAPI as a reference. $n = 3–4$ mice per group and brain region (for all regions: Vehicle versus TMP, $P < 0.0001$; LII–IV: 1x TMP versus 7x TMP, $P = 0.76$; LV–VI: 1x TMP versus 7x TMP, $P = 0.66$; CA1: 1x TMP versus 7x TMP, $P = 0.82$; CA3: 1x TMP versus 7x TMP, $P = 0.28$; DG: 1x TMP versus 7x TMP, $P = 0.11$).

d–g, DD-Cre TMP dose-response and time-course. **d**, Images of cortical sections of animals that were injected once with indicated amounts of TMP. **e**, Pattern of reporter expression in the SSC at different time points after drug administration (P30, single treatment, 170 μg/gm). **f**, TMP dose-dependence of DD-Cre activity in the SSC (layers II–IV). $n = 3$ mice per dose. **g**, Time-course of recombination across SSC was assessed by tdTomato imaging and PCR with primers specific for R26 locus. $n = 2–3$ mice per time-point.

h and **i**, Activation of a GFP reporter introduced in the brain of DD-Cre mice with an adeno-associated virus (AAV2.5 EF1:DIO-GFP, 0.5 × 10¹² GC/mL). Images of cortical sections and quantifications of induction efficiency in animals subjected to a single injection protocol (170 μg/gm of TMP) are shown. $n = 4–5$ mice per group (Vehicle versus TMP, $P < 0.0001$). All data are plotted as Mean ± S.E.M. See also Supplementary Figs. 3–8 and Table 1.

Figure 2. TMP-induced synaptic silencing in the brain of DD-Cre/TeNT mice

a, Schematic representation of CamKII α :DD-Cre and R26^{loxstop}-TeNT alleles.

b–e, P30 mice of indicated genotypes were injected with either vehicle or TMP (170 μ g/gm) and analyzed 7 days after treatments by immunostaining for Zif268/Egr1, TeNT and Syb2.

b, Typical images of coronal brain sections show the global pattern of Zif268/Egr1 expression. **c–e**, Analyses of Zif268/Egr1 and Syb2 immunoreactivity in the SSC. Graphs represent averaged intensities of Zif268/Egr1 staining per neuron (Vehicle versus TMP, $P < 0.0001$) and densities of Syb2-positive synaptic puncta (Vehicle versus TMP, $P = 0.001$). Each quantification was performed with 3 pairs of Vehicle and TMP-treated mice. Color coding applies to both panels.

f and **g**, Cortices of vehicle- and TMP-injected mice were isolated at various time points after drug administration and probed by immunoblotting for Syb2 and β Tubulin (as a loading marker). Raw immunoblot and Syb2/ β Tubulin band intensity ratios from one of two independent experiments are shown.

h, Evoked excitatory postsynaptic potentials (EPSPs) were monitored from DG granule cells (as shown on the cartoon) in brain slices of vehicle or TMP-treated DD-Cre/TeNT animals 48 hours post injection (170 μ g/gm). EPSP amplitudes are plotted as a function of stimulus intensity. $n = 3$ mice per group (Vehicle versus TMP, P s = 0.4 (0.1 mA); 0.32 (0.2 mA); 0.042 (0.3 mA); 0.008 (0.4 mA); 0.009 (0.5 mA); 0.015 (0.6 mA); 0.045 (0.7 mA); 0.11 (0.8 mA)).

Data are represented as Mean $\pm$ S.E.M.. See also Supplementary Table 2.

Figure 3. DD-Cre/TeNT mice exhibit a TMP-dependent loss of recognition and spatial memory Mice of indicated genotypes were tested for locomotor activity and memory acquisition/retrieval. Animals were treated once with either vehicle or TMP (170 $\mu\text{g}/\text{gm}$) 48 hours prior to initiation of behavioral trials.

a, Total horizontal locomotor activity was assessed with photocell beams for 2 hours over 5 min time intervals.

b and **c**, Novel object recognition and spatial memory tests. **b**, Averaged numbers of contacts initiated by each group of mice with old (O) and novel (N) objects (O versus N: TeNT+Vehicle, $P = 0.035$; TeNT+TMP, $P = 0.038$; DD-Cre/TeNT+Vehicle, $P = 0.007$; DD-Cre/TeNT+TMP, $P = 0.94$). **c**, Averaged percentages of time spent in correct target (T) and other (O) quadrants in the Barnes maze probe test (DD-Cre/TeNT+TMP versus DD-Cre/TeNT+Vehicle, $P = 0.026$; DD-Cre/TeNT+TMP versus TeNT+TMP, $P = 0.019$; DD-Cre/TeNT+TMP versus TeNT+Vehicle, $P = 0.024$; DD-Cre/TeNT+Vehicle versus TeNT+TMP, $P = 0.9$; DD-Cre/TeNT+Vehicle versus TeNT+Vehicle, $P = 0.98$; TeNT+TMP versus TeNT+Vehicle, $P = 0.92$). All measurements were performed with P60 animals ($n = 7-9$ mice per group) and represented as Mean $\pm$ S.E.M. See also Supplementary Fig. 9 and Table 3 for additional behavioral data.