Behavioral/Cognitive

Blocking Synaptic Removal of GluA2-Containing AMPA Receptors Prevents the Natural Forgetting of Long-Term Memories

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The neurobiological processes underpinning the natural forgetting of long-term memories are poorly understood. Based on the critical role of GluA2-containing AMPA receptors (GluA2/AMPARs) in long-term memory persistence, we tested in rats whether their synaptic removal underpins time-dependent memory loss. We found that blocking GluA2/AMPAR removal with the interference peptides $GluA2_{3Y}$ or G2CT in the dorsal hippocampus during a memory retention interval prevented the normal forgetting of established, long-term object location memories, but did not affect their acquisition. The same intervention also preserved associative memories of food-reward conditioned place preference that would otherwise be lost over time. We then explored whether this forgetting process could play a part in behavioral phenomena involving time-dependent memory change. We found that infusing $GluA2_{3Y}$ into the dorsal hippocampus during a 2 week retention interval blocked generalization of contextual fear expression, whereas infusing it into the infralimbic cortex after extinction of auditory fear prevented spontaneous recovery of the conditioned response. Exploring possible physiological mechanisms that could be involved in this form of memory decay, we found that bath application of $GluA2_{3Y}$ prevented depotentiation, but not induction of long-term potentiation, in a hippocampal slice preparation. Together, these findings suggest that a decay-like forgetting process that involves the synaptic removal of GluA2/AMPARs erases consolidated long-term memories in the hippocampus and other brain structures over time. This well regulated forgetting process may critically contribute to establishing adaptive behavior, whereas its dysregulation could promote the decline of memory and cognition in neuropathological disorders.

Key words: decay; forgetting; long-term memory

Significance Statement

The neurobiological mechanisms involved in the natural forgetting of long-term memory and its possible functions are not fully understood. Based on our previous work describing the role of GluA2-containing AMPA receptors in memory maintenance, here, we tested their role in forgetting of long-term memory. We found that blocking their synaptic removal after long-term memory formation extended the natural lifetime of several forms of memory. In the hippocampus, it preserved spatial memories and inhibited contextual fear generalization; in the infralimbic cortex, it blocked the spontaneous recovery of extinguished fear. These findings suggest that a constitutive decay-like forgetting process erases long-term memories over time, which, depending on the memory removed, may critically contribute to developing adaptive behavioral responses.

Introduction

The scientific study of memory set in with Ebbinghaus' forgetting curve illustrating the progressive deterioration of long-term memory as time goes by (Ebbinghaus, 1885). Since then, much has been learned about possible causes for this gradual memory loss, which has been suggested to reflect effects of interference,

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impaired retrieval, or trace decay (Wixted, 2004). However, little is known about possible neurobiological mechanisms underpinning the continuous erosion of long-term memory.

Mechanisms involved in the forgetting of long-term memory will likely be related to processes promoting long-term memory persistence. Advances in understanding long-term memory stability suggest that regulating the trafficking of synaptic AMPA receptors (AMPARs) constitutes a central process of memory storage and maintenance (Migues et al., 2010; Rao-Ruiz et al., 2011; Dong et al., 2015). Specifically, long-term memory persistence and strength correlate positively with the amount of synaptic GluA2/AMPARs (Yao et al., 2008; Migues et al., 2010; Li et al., 2011; Pauli et al., 2012; Migues et al., 2014; Dong et al., 2015). The forgetting of consolidated long-term memory over time may thus involve the gradual synaptic removal of these receptors.

Activity-dependent removal of GluA2/AMPARs relies on the interaction of different motifs on the intracellular carboxy-tail of GluA2 with various proteins, such as BRAG2, NSF, and AP2 (Wang and Linden, 2000; Kim et al., 2001; Collingridge et al., 2010; Scholz et al., 2010). The well characterized synthetic peptides $GluA2_{3Y}$ and G2CT mimic two of these regions and attenuate activity-induced, but not constitutive, GluA2-dependent synaptic removal of AMPARs (Lee et al., 2002; Ahmadian et al., 2004; Wang et al., 2004; Yoon et al., 2009; Scholz et al., 2010; Dong et al., 2015). Without affecting basal synaptic transmission, $GluA2_{3Y}$ blocks the induction of long-term depression (LTD), but not the induction of long-term potentiation (LTP), nor the acquisition or expression (i.e., retrieval), of conditioned fear memory (Ahmadian et al., 2004; Wang et al., 2004; Brebner et al., 2005; Dalton et al., 2008; Yu et al., 2008; Scholz et al., 2010; Rao-Ruiz et al., 2011; Bai et al., 2014; Dong et al., 2015). Similarly, G2CT effectively prevents GluA2/AMPAR internalization in live animals and slice preparations and blocks the formation of LTD (Griffiths et al., 2008; Yoon et al., 2009).

We used these two peptides known to interfere with GluA2 endocytosis to determine whether the forgetting of consolidated, long-term memories involves the activity-dependent removal of GluA2-containing AMPARs. We found that infusing $GluA2_{3Y}$ or G2CT into the dorsal hippocampus prevented the forgetting of consolidated object location and conditioned place preference (CPP) memories, but did not block acquisition of new object location memories. We then explored whether the ability of $GluA2_{3Y}$ to preserve established memories extends to other phenomena of time-dependent memory change. We found that infusing $GluA2_{3Y}$ into the dorsal hippocampus prevented the generalization of contextual fear over time—that is, the loss of context discrimination. Similarly, we found that infusing $GluA2_{3Y}$ into the infralimbic cortex preserved extinction memory because it prevented time-dependent spontaneous recovery of extinguished auditory fear. In summary, these findings suggest that the natural forgetting of long-term memories involves the activity-dependent removal of GluA2-containing AMPARs, which may play a role in various phenomena of time-dependent alterations of memory.

Materials and Methods

Subjects in behavioral studies. We acquired male Long–Evans rats weighing between 250 and 275 g (Charles River). Animals were housed in pairs in polyethylene cages with sawdust bedding and environmental enrichment (PVC tube, wooden gnawing block). Food and water were provided *ad libitum* in all experiments except CPP experiments, in which animals were food deprived, maintained at 85% weight of their free-feeding adult body weight, with water *ad libitum*. The lights in the colony turned on at 7:00 A.M. and off at 7:00 P.M. and all experimental procedures were performed during the lights-on phase between 9:00 A.M. and 1:00 P.M. All procedures were approved by the McGill University Animal Care and Use Committee and complied with the Animals (Scientific Procedures) Act of 1986.

Surgeries. Rats were submitted to surgery at a body weight of ~325–350 g. Anesthesia was induced with isoflurane (5% in O_2) in a transparent PVC induction chamber, where rats stayed for 5 min. Anesthesia was maintained at approximately 2–3% isoflurane in O_2 during surgery. Surgeries were performed as described in detail previously (Migues et al., 2014). Briefly, coordinates for bilateral cannula implantation relative to bregma were determined using a standard reference atlas (Paxinos and Watson, 2004). To target dorsal hippocampus, 22-gauge stainless steel cannulas (Plastics One) were implanted at an angle of 10 degrees lateral to the midsagittal plane at AP −3.60 mm, ML ± 3.10 mm, DV −2.40 mm. To target the infralimbic cortex, we implanted 22 gauge stainless steel double-guide cannulas (Plastics One) at AP 2.5 mm, ML± 0.6 mm, DV −4.40 mm. The microinjectors used for drug infusions (28 gauge; Plastics One) protruded 0.5 mm from the guide cannula. Surgeries took between 30 and 40 min (including induction). Rats remained single housed for 2 d after surgery, after which they were housed again with their original cage mate.

Drugs and infusions. Concentrations and volumes (1 μl per hemisphere) were based on previous studies (Bast et al., 2005; Hardt et al., 2009; Migues et al., 2010; Dalton et al., 2012). For cell permeability, all peptides were fused to a sequence (YGRKKRRQRRR) derived from the transduction domain of the TAT protein. $GluA2_{3Y}$ (YGRKKRRQRRR-YKEGYNVYG, 15 pmol/μl), the inactive control peptide $GluA2_{3A}$ (Ctrl) (YGRKKRRQRRR-AKEGANVAG, 15 pmol/μl) (Ahmadian et al., 2004), the G2CT peptide (YGRKKRRQRRR-KRMKLNINPS, 15 pmol/μl), the inactive control peptide for G2CT (Ctrl) (YGRKKRRQRRR-KNINKLMPRS, 15 pmol/μl), and CNQX (0.89 μg/μl) (Lee et al., 2002) were dissolved in 100 mM Tris-saline, final pH 7.2. For the electrophysiological studies, dynamin-derived peptide (Myr-QVPSRPNRAP-OH) and its scrambled version (Myr-QPPASNPRVR-OH) (Wang and Linden, 2000) were dissolved in dH_2O and diluted to a final concentration of 50 μM for intercellular infusion. The two peptides $GluA2_{3Y}$ and $GluA2_{3A}$ were synthesized and purified at the Peptide Synthesis Laboratory at University of British Columbia. The peptides G2CT and inactive control G2CT variant were purchased from AnaSpec. Dynamin-derived peptide and dynamin-scrambled were acquired from Bio-Synthesis. In the behavioral studies, drugs were infused at a rate of 0.25 μl/min into the dorsal hippocampus with a 28 gauge microinjector connected to a Hamilton syringe with plastic tubing. Injectors remained connected for an additional 90 s after the infusion stopped. Drugs were infused in the animal colony.

Histology. Animals were deeply anesthetized and killed by decapitation. Removed brains were fixed in a mixture of 4% paraformaldehyde and 30% sucrose–saline and later stained with formal–thionin. A cryostat was used to obtain sections of 50 μm thickness. Cannula placement was checked under a light microscope. Animals were only included in the analyses when an experimenter blind to the treatment group detected the injector tip inside of the dorsal hippocampus (see Fig. 8 for placements of cannula tips of all animals included in the analyses reported here).

Open field to assess object location recognition memory. The open field (60 cm × 60 cm × 60 cm) was made of white laminated plywood, the floor covered with the same type of sawdust bedding used for the animal cages. The open field was placed on a wooden platform 20 cm above the floor of a 4 m × 4 m large room without windows and one door. A camera was suspended 150 cm above the open-field floor and connected to a video acquisition card in a PC. There were nine equidistant holes in the floor of the open field. Objects were glued on the outside base of glass Mason jars (65 mm high, 68 mm diameter at base, 82 mm at lid, 90 mm lid diameter; Bernardin). Holes were drilled in the middle of the metal jar lids. These lids were then secured with screws and wing nuts to the

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open-field floor at the desired position and the mason jars turned into the lid. Pilot studies determined which objects would reliably elicit sufficient exploratory activity during the first minute of exposure to provide an adequate sample of exploratory behavior. We had four copies of each object, none of which had biological significance for rats (Bordeaux-colored ceramic incense oil burner and a black PVC plumbing element of type double-Y waste drainage fitting; overall exploration time was comparable, but usually higher for the latter than the former). Each copy was assigned a unique number at a place the rat could neither see nor reach.

General procedures for object location preference. Behavioral procedures would begin 2 d after surgery, comprising 7 d of handling, 4 d of habituation, 7 d of sampling, a memory retention interval (up to 14 d, often with daily drug infusions), and finally the probe trial. Modifications to this general protocol are described for each experiment in the main text.

Daily handling during recovery period. Two days after surgery, rats were familiarized with general aspects of the training situation. Animals were placed in a green plastic box (W 40 cm × L 80 cm × H 40 cm), in which the floor was covered with ~2 cm of sawdust bedding. The box contained four differently sized plastic tubes of the type that was also placed into the home cages as part of environmental enrichment. Rats were put in groups of eight into the box, where they stayed for 30 min each day. The experimenter would pick up each rat several times during these 30 min, placing the animal briefly on the lap and then putting it back into the box to simulate the type of handling used in the actual experiment.

Habituation. Four habituation trials, 1/d, were administered beginning 8 d after surgery. Rats were transported to the holding room adjacent to the testing room 1 h before sessions would begin (this was also done for sampling and probe sessions). A habituation trial took 5 min, during which a single rat was allowed to explore the open field that contained no objects. Rats were placed into a different corner of the open field at the beginning of each habituation trial, with the sequence randomized between rats and groups. Rats were lowered into the open field with their snout facing the corner (this was also the case during sampling and probe sessions). The open field was cleaned between rats and the sawdust covering the floor swirled around to disperse possible odor traces (this was also done during sampling and probe sessions).

Sampling. Seven sampling trials were administered, 1/d, each lasting 5 min. During each session, two copies of the same object were placed in the open field at opposing positions (e.g., NW–SE, WW–EE, etc.). After each rat, objects were thoroughly cleaned with 70% ethanol.

Probe. During the probe trial, one of the objects used during sampling was moved to a new location and the other object remained at the familiar location. In each experiment, all possible combinations of new and old locations permitted by our open field were presented, with the exception that an object was never placed in the center. Open field and objects were cleaned between rats as described above.

CPP. The CPP box consisted of three compartments made out of plywood. One compartment had a black floor and ceiling and white–black vertical stripe patterns at the wall; the other compartment had a gray floor, gray ceiling, and gray walls. These two compartments were of equal size (45 cm × 45 cm × 30 cm). The front wall of each compartment was made of clear Plexiglas. The compartments were connected by a small corridor (36 cm × 18 cm × 20 cm), made of wooden walls and floor and a Plexiglas ceiling. The connecting corridor was attached to the outside of the back wall of the CPP box. Rats could access both compartments from the corridor. During each training session, rats were confined to one of the two compartments. One compartment (the "paired" compartment) was always paired with food and the other one (the "unpaired" compartment) was not. Fifty Fruit Loops were placed as food reward on the floor of the paired compartment during a training session.

A video camera placed above the connecting corridor recorded the entries and exits into the compartments and the activity in the connecting corridor. The amount of Fruit Loops consumed in the paired compartment was determined and consumption was high in all rats, ranging between 95% and 100%. Fruit Loops that were not consumed were discarded and not given to other animals. The apparatus was cleaned between rats with the antimicrobial BioClean.

General procedures for CPP. Behavioral procedures started 2 d after surgery and consisted of 5 d of daily handling using large plastic boxes as described above for the object location studies before food restriction set in. Rats received laboratory chow to maintain them at 85% of their free-feeding body weight. In the 4 d preceding the start of the CPP procedure, Fruit Loops (10 per rat) were placed in the home cage before the daily feeding of lab chow to familiarize the animals with this novel food. Rats were weighed daily and received additional lab chow to maintain the target body weight, if necessary. The first day of CPP training consisted of a 10 min habituation trial. Rats were put into the connecting corridor of the CPP apparatus and had free access to both compartments for the entire time. The conditioning training began the next day. Following an established procedure for this task (Roux et al., 2003), the paired compartment was pseudorandomly determined for each rat, ensuring that, in each experimental condition, the same number of rats received food reinforcement in the striped and gray compartment. Animals received one conditioning session per day, during which they were placed directly into either the paired or unpaired compartment and remained there for 30 min while access to the connecting corridor was blocked. The compartment into which animals were placed alternated from day to day. The sequence was counterbalanced such that, for each experimental condition, half of the rats started the conditioning sequence in the paired compartment and the other half started the sequence in the unpaired compartment. All CPP experiments consisted of 4 d of conditioning training: 2 sessions in the paired compartment and 2 sessions in the unpaired compartment (e.g., day 1: paired; day 2: unpaired; day 3: paired; day 4: unpaired; or day 1: unpaired; day 2: paired; day 3: unpaired; day 4: paired). After a delay, the length of which varied between experiments, CPP was assessed by placing the animals into the connecting corridor from which they had free access to both compartments for 10 min. During the CPP test, no food was present in any CPP compartment.

Contextual fear conditioning. Two markedly different contexts were used that varied in shape, illumination, scent, visual, and tactile features, as well as ambient sound and illumination to reduce their similarity. The training context (30 cm × 26 cm × 33 cm; Coulbourn Instruments) was housed in a sound-attenuating enclosure. Three walls were made of transparent Plexiglas and the fourth, containing a dimly lit light bulb, was made of stainless steel. A stainless steel grid (bar radius 2.5 mm, spread 1 cm apart) was used as the floor. One corner of the box was sprayed with a diluted vanilla scent before placing a rat into the box. Behavior was recorded with a digital video camera placed in front of the box. Only red light illuminated the experimental room surrounding the conditioning chamber. The testing context (Context B, 29 cm × 25 cm × 25 cm; Med Associates) was placed in a room with standard fluorescent lighting. Context B had a white, curved Plexiglas back wall; stainless steel sidewalls; a floor made of a white Plexiglas sheet; and a front plastic wall that showed a black and white striped pattern. Water-diluted peppermint essential oil was sprayed at the curved back wall before a rat was placed into the box. A fan built into the wooden cabinet surrounding the box produced constant background noise. A video camera attached to the ceiling of cabinet recorded behavior.

Procedures for contextual fear conditioning. Daily handling began 5 d before conditioning, during which time animals were familiarized with the basic aspects of the experimental situation, such as transport, being removed from the cage, and being held and constrained by the experimenter. Animals were transported to the holding area adjacent to the room containing the training context 1 h before training. The context was cleaned with the antimicrobial BioClean and 70% ethanol before a rat was placed into it. Two minutes after the rat entered the context, the first of 2 electric foot shocks (1 s, scrambled, 1 mA) was delivered and, 60 s later, the second one. Animals were removed 60 s later. To assess contextual fear memory, animals were transported to the holding area adjacent to the testing context 1 h before procedures began. The testing context was cleaned as in the training context and then a rat was placed into it and remained there for 5 min, during which no shock was delivered.

Auditory fear conditioning. The same contexts as in contextual fear conditioning were used. The training context (A) was used for auditory fear conditioning and the testing context (B) for auditory fear extinction and assessment of spontaneous recovery.

Procedures for auditory fear conditioning and extinction. Before auditory fear conditioning, rats were placed for 20 min into the testing con-

text once a day for 2 consecutive days to habituate them to general procedure and context. They were transported to the holding area adjacent to the training or testing context 1 h before habituation, conditioning, extinction, and tests of spontaneous recovery. Each context was cleaned as described above before a rat was placed into it. Twenty-four hours after the last habituation trial, rats participated in auditory fear conditioning. A conditioning session took 6 min. Two minutes after a rat had been placed into the training contex,t the first of 3 tones (5 kHz, 75 dB) was presented for 30 s. The interstimulus interval was 30 s. Each tone coterminated with an electric foot shock (0.9 mA, scrambled, 1 s). Rats were removed from the context 30 s after the last foot shock. Twenty-four hours after auditory fear conditioning, the first of three extinction sessions in the testing context began. One session was administered per day for 3 consecutive days. In each session, the tone played during conditioning was presented for a total of 12 times without shock. The interstimulus interval was 60 s. Rats were removed from the context 60 s after the last tone presentation. Extinction memory was assessed 1 d and 7 d after the last extinction session. To this end, animals were placed into the testing context and, after 2 min, the tone used during fear conditioning was played. The tone was played three times, with 30 s between each tone. Sixty seconds after the last tone presentation, the animals were removed from the context.

Electrophysiological studies. Thirty-seven hippocampal slices were prepared from 16 male Sprague Dawley rats (3–4 weeks old). Briefly, under deep anesthesia, brains were rapidly removed and coronal brain slices (400 μm thickness) containing hippocampus were cut using a vibrating blade microtome (Leica) in ice-cold artificial CSF (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 1.25 KH_2PO_4, 26 $NaHCO_3$, and 20 glucose bubbled continuously with carbogen (95% O_2/5% CO_2), adjusting pH to 7.4. Freshly cut slices were placed in an incubating chamber with carbogenated ACSF and recovered at 34°C for 1.5 h. Slices were then maintained at room temperature before recording. For electrophysiological recordings, slices were transferred to a recording chamber perfused continuously with carbogenated ACSF containing bicuculline methiodide (10 μM) to block $GABA_A$ receptor-mediated inhibitory synaptic currents. Whole-cell recordings of CA1 neurons in brain slices were performed using the "blind" method with a MultiClamp 700 A amplifier (Molecular Devices). Recording pipettes were filled with solution containing the following (mM): 132.5 Cs-gluconate, 17.5 CsCl, 2 $MgCl_2$, 0.5 EGTA, 10 HEPES, 4 ATP, and 5 QX-314, with pH adjusted to 7.2 by CsOH. EPSCs were evoked by stimulating the Schaffer collateral–commissural pathway via a constant current pulse (0.05 ms) delivered through a tungsten bipolar electrode (FHC) and recorded while CA1 neurons were voltage clamped at −60 mV. Synaptic responses were evoked at 0.05 Hz except during the induction of LTP and depotentiation. LTP was induced within 10 min after the establishment of whole-cell configuration to avoid washout of intracellular contents that is critical for the formation of long-term synaptic plasticity. LTP of AMPAR-mediated EPSCs was induced by 200 pulses of repetitive stimulations at 2 Hz while voltage clamping the recorded cells at −5 mV. After 15 min of LTP recording, depotentiation was induced by 300 pulses at 1 Hz while holding cells at −45 mV.

Data analysis. All datasets reported here tested positive for homogeneity of variance and normality. For all statistical tests, the type I error level was set to $\alpha = 0.05$.

Object location tasks. An experimenter blind to the experimental condition scored recorded videos using established evaluation standards for this task (Mumby et al., 2002; Winters et al., 2007; Hardt et al., 2010). A rat was considered exploring an object when its snout was within 2 cm of the object and oriented at an angle of at least 45 degrees toward it. Climbing and resting on the object was not considered exploratory behavior. Only the first minute of a trial was considered for the novelty preference assessment because, after the first minute, rats tend to become familiar with both locations and exploratory behavior may no longer reflect novelty preference. The novelty preference ratio d was calculated using the time spent exploring the object at the new location (t_{new}) and the time spent exploring the object at the old location (t_{old}) as follows: $d = (t_{new} - t_{old})/(t_{new} + t_{old})$. The ratio d can take any value between −1.0 and 1.0. Values significantly above $d = 0.0$ indicate that the rat explored the novelty significantly more than the familiarity, which is interpreted as revealing memory for the original object position. Values significantly below $d = 0.0$ indicate the opposite. Values not different from $d = 0.0$ denote that the rat explored both objects the same and thus expressed no object location preference, which suggests the absence of memory for their former location. To determine whether rats preferred the novelty, the observed d was compared against $d = 0.0$ using a two-tailed one-sample t test. Unpaired t tests were used to detect group differences.

CPP. The time animals spent in each CPP compartment was measured and a score to index preference for the paired side was calculated (d_{CPP}), analogous to the novelty preference ratio used in the object location studies. The preference for the paired compartment (the side reinforced with food) was calculated using the total amount of time (in seconds) spent in the paired compartment (t_{CS+}) during the 10 min long preference test and the time spent in the unpaired compartment (t_{CS-}) as follows: $d_{CPP} = (t_{CS+} - t_{CS-})/(t_{CS+} + t_{CS-})$. We determined significant place preference as described for d above. Two-tailed unpaired t tests were used to compare group differences. To determine whether a group of rats preferred the paired compartment and thus expressed CPP, a one-sample t test comparing d_{CPP} against 0.0 was used.

Contextual fear conditioning. Contextual fear memory was measured in terms of freezing (i.e., immobilization except for respiratory movements) in the testing or training context. Freezing was manually scored in blocks of 30 s by an experimenter blind to the experimental condition. Group differences were determined with mixed-design ANOVAs and significant interactions were examined with simple effects analyses.

Auditory fear conditioning. Freezing to the tone was measured manually during the tone presentation by an experimenter blind to conditions. To assess context fear, freezing during the 30 s preceding the first tone presentation was measured. Freezing data for the 12 tones presented during extinction were summarized by averaging two successive tone presentations to a total of six data points per extinction session. The three data points from each extinction memory test session were averaged into one data point. Data were analyzed the same way as for contextual fear conditioning, as described above.

Electrophysiologial studies. Series and input resistance were monitored throughout each experiment and cells were excluded from data analysis if a >20% change in the series or input resistance occurred during the course of the experiment. One-way ANOVAs for EPSC amplitude were performed at 3 time points across groups corresponding to basal transmission (5 min), LTP (20 min), and depotentiation (60 min). Holm–Šidák tests were used for multiple comparisons after significant one-way ANOVAs.

Results

Blocking synaptic removal of GluA2/AMPARs in the hippocampus prevents decay-like forgetting of long-term object location memories

We first determined at what time after training animals forget long-term memory for object locations in our novelty recognition task. We trained 5 different groups of rats by exposing them for 5 min/d for 7 d to 2 copies of the same object located at constant positions in an open field (Fig. 1*A*). We then waited for 3, 7, 10, 12, and 14 d and each time exposed 1 group again to the test arena in which 1 of the original objects had been moved to a new location. Because rats are attracted to novelty, they are more likely to explore the moved object than the one at the familiar location if they still have memory for the original object locations (Berlyne, 1950; Ennaceur and Delacour, 1988). Absence of an exploratory preference suggests that memory for the original locations is no longer available (Mumby et al., 2002; Winters et al., 2007; Hardt et al., 2010). We found that preference for the moved object decreased over time (one-way ANOVA: $F_{(4,32)} = 7.93$, $p < 0.001$). Rats preferred to explore the moved object when tested 3 and 7 d after the last training session, but not at later time points (one-sample t test: 3 d, $t_{(7)} = 4.62$, $p = 0.002$; 7 d, $t_{(6)} = 5.19$, $p = 0.002$; 10 d, $t_{(7)} = 1.33$, $p = 0.224$; 12 d and 14 d, $t < 1$; Fig 1*B*).

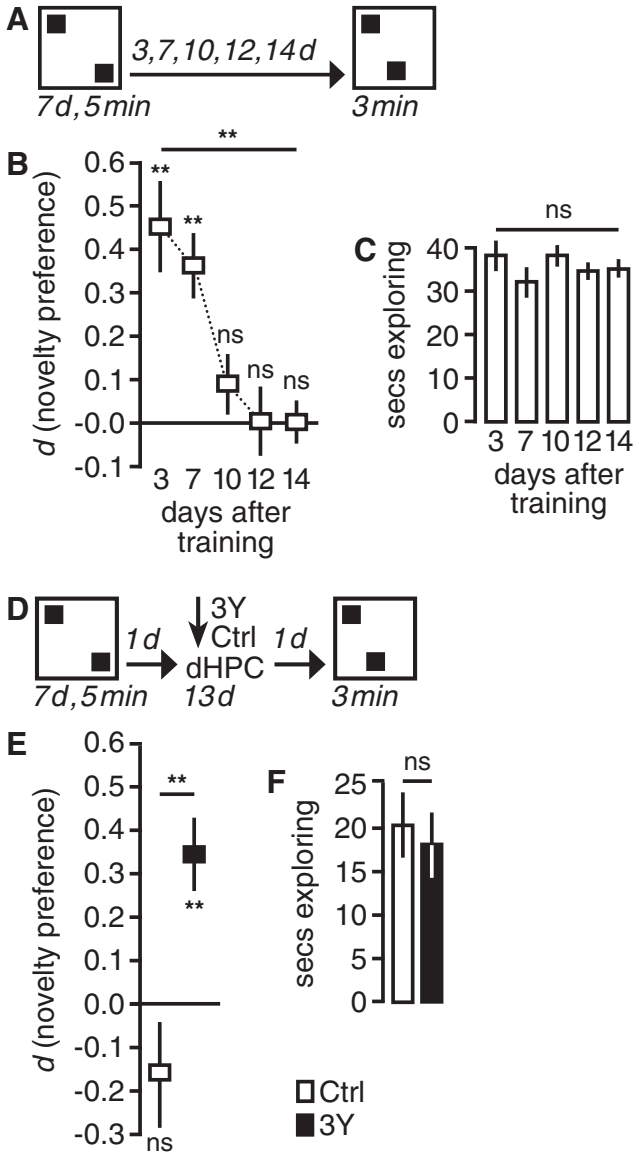

Figure 1. Infusing $GluA2_{3Y}$ into the dorsal hippocampus to restrict synaptic internalization of GluA2/AMPARs prevents forgetting of long-term object location memories. ***A–C***, Long-term object location memories are naturally forgotten within 10 d. ***A***, Rats were exposed for 5 min each day for 7 d to two identical copies of a junk object located in an open field at fixed positions. Different groups of rats were tested for object location memory 3 d, 7 d, 10 d, 12 d, or 14 d after the last training session ($n = 8, 7, 8, 6, 8$, respectively). During the test, one object was moved to a new place. ***B***, Rats expressed novelty preference ($d > 0$) when tested 3 and 7 d after learning, but treated both object locations the same ($d = 0$) at later time points (10 d, 12 d, 14 d). ***C***, Total exploratory activity directed at the two objects was the same in all groups, indicating that differences in activity levels do not account for time-dependent loss of novelty preference. ***D–E***, Infusing $GluA2_{3Y}$ into the dorsal hippocampus during the 13 d retention interval prevents forgetting of object location memories (see Fig. 8*A* for cannula placements). ***D***, Rats were trained as before for 7 d (Fig. 1*A*). Twenty-four hours after the last training session, rats were infused twice daily (A.M. and P.M.) with $GluA2_{3Y}$ (3Y, $n = 7$) or its inactive control variant $GluA2_{3A}$ (Ctrl, $n = 7$) into the dorsal hippocampus (dHPC) for 13 d to block synaptic removal of GluA2/AMPARs (here and elsewhere, downward arrows symbolize drug infusions). Twenty-four hours after the last infusion; that is, 14 d after training (i.e., when these memories normally would have been forgotten, see Fig. 1*B*), retention of object location memories was assessed. ***E***, Only rats infused with $GluA2_{3Y}$ preferred exploring the object moved to the novel location and expressed a significantly stronger novelty preference than the rats infused with the inactive control peptide. ***F***, There were no differences in overall exploratory activity.

There were no differences between the groups in overall exploration of objects, suggesting that changes in novelty preference were unlikely due to altered motivation or motility (one-way ANOVA: $F_{(4,32)} = 1.45$, $p = 0.240$; Fig. 1*C*). These findings suggest that, given our training protocol, rats no longer expressed long-term object location memories between 8 and 10 d after training.

We next explored whether interfering with the activity-dependent synaptic removal of GluA2/AMPARs during the memory retention interval could prevent forgetting of long-term object location memories over time. Activity-dependent removal of GluA2/AMPARs requires dynamin- and clathrin-mediated endocytosis (Carroll et al., 1999). Specifically, the binding sites for BRAG2 and the clathrin-adaptor protein AP2 on the carboxy-tail of GluA2 have been implicated in the synaptic removal of GluA2/AMPARs (Lee et al., 2002; Yoon et al., 2009; Scholz et al., 2010). We thus used the well characterized peptide $GluA2_{3Y}$ to interfere competitively with the binding of BRAG2 to GluA2 and to attenuate synaptic GluA2/AMPAR removal. Beginning 24 h after the last training session, we infused either $GluA2_{3Y}$ (3Y) or its inactive control variant $GluA2_{3A}$ (Ctrl), in which alanine was substituted for tyrosine, directly into the dorsal hippocampus twice daily (morning and afternoon) for 13 d (Fig. 1*D–F*). We assessed memory retention 24 h after the last infusion; that is, 14 d after the last training session. We found a significant group difference in location novelty expression (unpaired *t* test: $t_{(13)} = -3.81$, $p = 0.002$; Fig. 1*E*). The animals that were infused with $GluA2_{3Y}$ preferentially explored the object moved to a new place, whereas the animals that had received the inactive $GluA2_{3A}$ explored both locations the same (one-sample *t* tests: $GluA2_{3Y}$, $t_{(7)} = 4.17$, $p = 0.004$; $GluA2_{3A}$, $t_{(7)} = -1.61$, $p = 0.16$; Fig. 1*E*). There were no group differences in terms of overall exploratory activity (unpaired *t* test: $t < 1$; Fig. 1*F*) and, during training, both groups explored the 2 object locations for the same amount of time (Table 1). Therefore, the preserved novelty preference after $GluA2_{3Y}$ infusions during the retention interval is unlikely to have resulted from inherent group differences in exploratory behavior, motivation, or motility. Rather, the results suggest that infusing $GluA2_{3Y}$ into the dorsal hippocampus during the retention interval prevented the time-dependent forgetting of object location memory.

We sought to find additional support for a possible role of the synaptic removal of GluA2/AMPARs in time-dependent forgetting and, to this end, targeted a different region on the GluA2 C terminus that is involved in activity-dependent internalization of GluA2/AMPARs (Fig. 2*A–C*). We used the peptide G2CT, which interferes competitively with the binding of the clathrin-adaptor protein AP2 to GluA2, a mechanism involved in the activity-dependent synaptic removal of GluA2/AMPARs (Griffiths et al., 2008). We repeated the previous experiment, but infused G2CT or its inactive control peptide (Ctrl) into the dorsal hippocampus for 13 d after training (Fig. 2*A*). Memory for the object locations was tested 24 h after the last infusion; that is, 14 d after the end of training. Similar to the previous experiment, we detected a significant difference in location novelty preference between the groups (unpaired *t* test: $t_{(12)} = 4.23$, $p = 0.001$; Fig. 2*B*). Rats infused with G2CT preferred to explore the object moved to the novel location, whereas rats that had received the inactive peptide variant (Ctrl) explored both object locations the same (one-sample *t* test: G2CT, $t_{(6)} = 3.85$, $p = 0.008$; Ctrl, $t_{(6)} = -1.78$, $p = 0.13$; Figure 2*B*). Overall exploratory activity was not different between the groups (unpaired *t* test: $t < 1$; Fig. 2*C*) and both groups explored both object locations the same during training

Table 1. Exploratory behavior during training sessions

		Day 1		Day 7		Statistical comparisons	
Figure	Group	O1	O2	O1	O2	Day 1 vs day 7	All other effects
1*E*	Ctrl	13.81 ± 1,78	15.99 ± 2.46	9.19 ± 2.11	12.90 ± 3.77	$F_{(1,13)} = 11.8, p = 0.004$	NS
	3Y	16.36 ± 1.00	13.03 ± 1.50	9.88 ± 1.50	10.23 ± 1.80		
2*B*	Ctrl	15.13 ± 1.57	14.98 ± 1.16	10.37 ± 2.44	8.73 ± 2.22	$F_{(1,11)} = 9.86, p = 0.009$	NS
	G2CT	13.60 ± 2.27	15.27 ± 1.28	10.36 ± 1.47	8.73 ± 0.90		
2*E*	Ctrl	12.70 ± 1.72	15.22 ± 1.07	9.37 ± 1.67	8.46 ± 2.03	$F_{(1,11)} = 13.2, p = 0.004$	NS
	3Y	13.25 ± 0.91	13.13 ± 0.84	9.59 ± 1.05	9.72 ± 1.32		
3*B*	PI	13.67 ± 0.77	13.37 ± 1.08	9.50 ± 1.71	9.86 ± 1.19	$F_{(1,12)} = 22.5, p = 0.0005$	NS
	RI	10.57 ± 1.73	12.20 ± 1.83	10.06 ± 0.66	9.53 ± 1.38		
3*E*	Ctrl	11.15 ± 1.18	10.96 ± 0.80	7.72 ± 0.78	8.22 ± 1.04	$F_{(1,14)} = 14.8, p = 0.002$	NS
	3Y	11.05 ± 1.18	9.97 ± 1.17	7.38 ± 0.93	7.56 ± 0.90		

Analyses of training behavior for the object-location experiments presented in the main text. Each line shows how many seconds rats explored each object (O1, O2) during the first minute of the first (day 1) and last (day 7) training session. Three-way repeated-measures ANOVAs, with session (day 1 versus day 7) and object (O1 versus O2) as repeated factors and group (e.g., $GluA2_{3Y}$ vs Ctrl, etc.) as the between-subjects factor, were used to detect differences in the time animals spent exploring objects. These analyses revealed the same principal pattern for all experiments. First, animals showed greater exploratory activity during the first training session than during the last. Second, animals explored both objects the same; there was no preference for an object location during training.

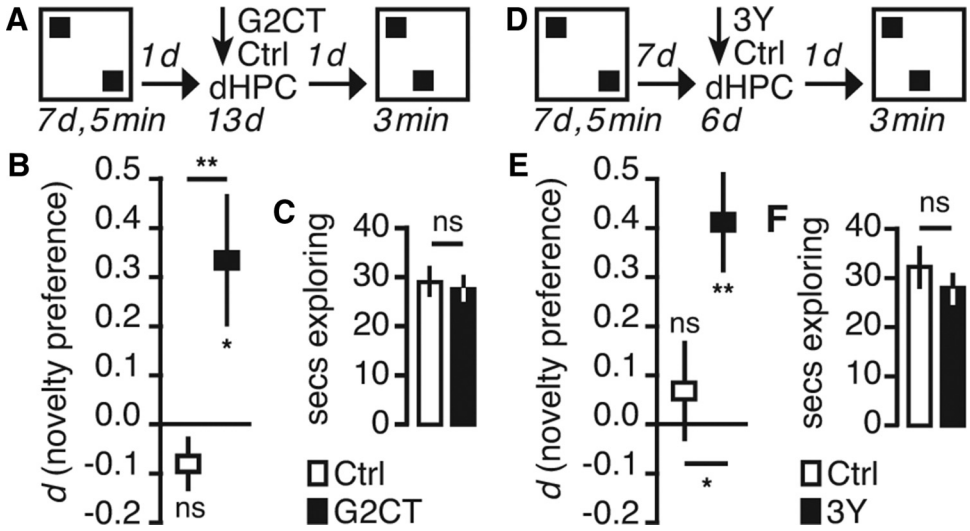

Figure 2. Interfering with AP2-dependent GluA2/AMPAR removal and delayed onset of $GluA2_{3Y}$ infusions prevent forgetting of long-term object location memories. ***A–C***, Inhibiting AP2-dependent synaptic removal of GluA2/AMPARs prevents forgetting (see Fig. 8*B* for cannula placements). ***A***, Animals were trained as before (Fig. 1*D*), but instead of $GluA2_{3Y}$, the peptide G2CT ($n = 7$, inactive control peptide, Ctrl, $n = 7$) was infused to interfere with the binding of AP2 to GluA2 to attenuate activity-dependent synaptic removal of GluA2/AMPARs. ***B***, Only animals infused with G2CT preferred exploring the object at the new location, expressing significantly stronger novelty preference than the animals infused with the inactive control peptide (Ctrl). ***C***, There were no group differences in exploratory activity. ***D–F***, Infusing $GluA2_{3Y}$ can prolong remote memories shortly before they would be naturally forgotten (see Fig. 8*C* for cannula placements). ***D***, Seven days after the last training session (i.e., on day 8 after training), shortly before rats normally would forget the location memory (see Fig. 1*A–C*), animals received $GluA2_{3Y}$ infusions into the dorsal hippocampus twice daily for 6 d. Twenty-four hours after the last infusion (or 14 d after the end of training), memory for object location was assessed by moving one of the objects to a novel location. ***E***, Animals infused with $GluA2_{3Y}$ ($n = 7$) preferred to explore the object at the new location, whereas animals that had received the inactive control variant (Ctrl, $n = 6$) explored both objects for an equal amount of time. The group difference was significant. ***F***, Exploratory activity was the same for both groups.

(Table 1). Together with the results reported above, these findings suggest that interfering with the activity-dependent synaptic removal of GluA2/AMPARs in the dorsal hippocampus can prevent the forgetting of long-term object location memory.

We next tested whether infusing $GluA2_{3Y}$ could also preserve memories shortly before they would be forgotten; for example, 1 week after learning. We trained animals as in the preceding experiments (Figs. 1*A*, *D*, 2*A*). After a delay of 7 d (i.e., on day 8 after learning), shortly before memory loss would normally occur (Fig. 1*B*), daily infusions of $GluA2_{3Y}$ (3Y) or its inactive control variant $GluA2_{3A}$ (Ctrl) set in for 6 d (Fig. 2*D*). We assessed memory 24 h after the last infusion; that is, 14 d after the end of training, as in the previous experiments. There was a significant difference in novel location preference between the 2 groups (unpaired *t* test: $t_{(11)} = 2.33$, $p = 0.04$; Fig. 2*E*). Only rats infused with $GluA2_{3Y}$ expressed novelty preference and explored the repositioned object more so than the object that had remained at its original place, whereas rats that had received the inactive $GluA2_{3A}$ (Ctrl) explored both object locations the same (one-sample *t* test: $GluA2_{3Y}$, $t_{(6)} = 3.764$, $p = 0.009$; $GluA2_{3A}$, $t < 1$; Fig. 2*E*). There were no group differences in either exploratory activity ($t < 1$; Fig. 2*F*) or exploratory preference during training (Table 1). Therefore, infusing $GluA2_{3Y}$ into the dorsal hippocampus can preserve remote memories shortly before they are normally forgotten.

Infusing $GluA2_{3Y}$ during the retention interval could have preserved memory in the previous experiments because it impaired learning and the formation of new, competing memories during the retention interval that could cause interference. To explore this alternative explanation, we first sought to determine whether in our paradigm new learning would indeed cause interference, reasoning that the acquisition of similar memories would present a strong source of interference (Wixted, 2005; Bartko et al., 2010; Dewar et al., 2010). As in the first experiment (Fig. 1*D*), we trained 2 groups of rats for 7 d without drug infusions. Starting the next day, we trained these now no longer naive

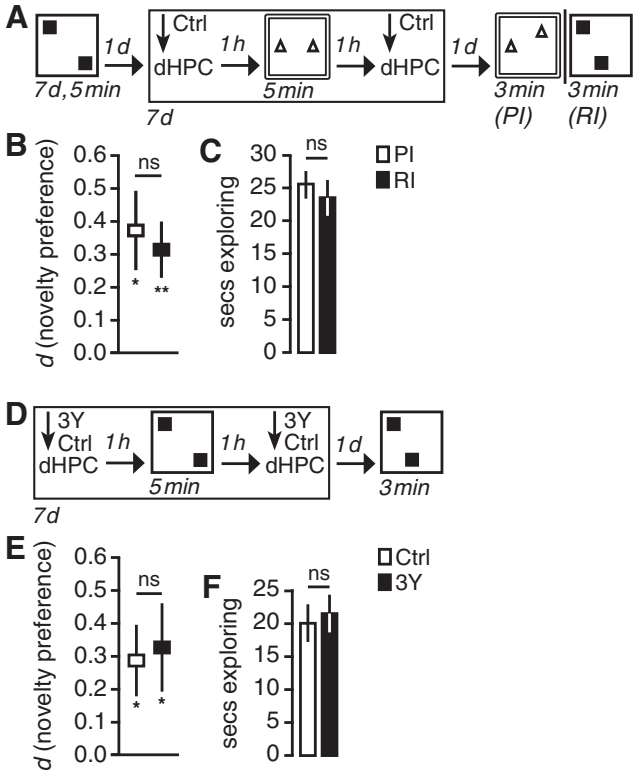

Figure 3. Long-term object location memories are not vulnerable to interference arising from new learning. ***A–C***, Prior learning of object location memories does not impair acquisition of new object location memories (proactive interference, PI, $n = 8$). Similarly, learning of new object locations does not impair existing long-term object location memories (retroactive interference, RI, $n = 7$; see Fig. 8*D* for cannula placements). ***A***, Rats were first trained for 7 d as in the previous studies. The next day, they were trained for another 7 d in a different open field with different objects located at different positions. Before and after each second training session, rats received infusions of the inactive $GluA2_{3A}$ (Ctrl). Twenty-four hours later, 8 d after the last training session, rats were either tested for recent (PI) or remote (RI) object location memory. ***B***, Rats expressed robust novelty preference for recent and remote long-term object location memories, suggesting the absence of proactive and retroactive interference in this task. ***C***, There were no differences in exploratory activity during the memory probe between the PI and RI groups (see Fig. 8*E* for cannula placements). ***D–F***, Infusing $GluA2_{3Y}$ ($n = 8$, Ctrl $n = 8$) before and after object location learning does not block memory formation in naive rats. ***D***, Animals were trained as before (i.e., Fig. 1). One hour before and immediately after each training session, $GluA2_{3Y}$ or its inactive version $GluA2_{3A}$ was infused into the dorsal hippocampus. Twenty-four hours after the last training session, object location memory was assessed. ***E***, $GluA2_{3Y}$ infusions did not affect memory because both groups of rats preferred to explore the object at the novel location. There were no group differences in novelty preference. ***F***, Both groups of rats showed the same overall exploratory activity.

rats for another 7 d in a new spatial context with new objects placed at different locations (Fig. 3*A*). We infused the inactive control peptide $GluA2_{3A}$ 1 h before and immediately after each training session to keep the experience similar to the previous experiments in which all animals received infusions during the retention interval. Twenty-four hours after the last infusion; that is, 8 d after the end of the first training, we probed memory for the locations of objects encountered during the first (assessing retroactive interference) or the second training session (proactive interference). Both groups of rats preferred exploring the object moved to the novel location (one-sample *t* tests: retroactive interference group: $t_{(5)} = 3.07$, $p = 0.027$; proactive interference group: $t_{(6)} = 5.70$, $p = 0.001$; Fig. 3*B*) and there were no group differences in novelty preference (unpaired *t* test, $t < 1$). There was no difference in overall exploratory activity during the probe trial (unpaired *t* test, $t < 1$; Fig. 3*C*), nor during sampling (Table 1). Therefore, the long-term object location memories that rats acquire in our paradigm seem not to be affected by the acquisition of new location memories during the retention interval.

Nevertheless, we next explored whether $GluA2_{3Y}$ could have blocked new encoding and learning, which might have interfered retroactively with established long-term object location memories. To test this possibility, we trained animals in the novelty preference task as before (Fig. 1*D*), but infused $GluA2_{3Y}$ or the inactive $GluA2_{3A}$ (Ctrl) 1 h before and immediately after each of the 7 training trials (Fig. 3*D*). Twenty-four hours after the last infusion, we assessed location memory and found no difference in novel location preference between the 2 groups (unpaired *t* test: $t < 1$; Fig. 3*E*). Rats that had received $GluA2_{3Y}$, as well as those infused with $GluA2_{3A}$ (Ctrl), expressed exploratory preference for the object moved to the novel location (one-sample *t* test, $GluA2_{3Y}$, $t_{(7)} = 2.78$, $p = 0.003$; $GluA2_{3A}$, $t_{(7)} = 3.18$, $p = 0.016$; Fig. 3*E*). There were no differences in overall exploratory activity (unpaired *t* test, $t < 1$; Fig. 3*F*) and behavior during training was the same (Table 1). Therefore, $GluA2_{3Y}$ did not affect the learning of object locations and the formation of corresponding long-term memories. Together, these results suggest that interference from new learning does not significantly contribute to the forgetting of long-term object location memories in our protocol.

Blocking synaptic removal of GluA2/AMPARs in the hippocampus prevents forgetting of CPP

Location memories in the novelty preference task are acquired with nonassociative forms of learning in the sense that no explicit reinforcement rewards exploratory responses. Learning, however, often involves Pavlovian or instrumental conditioning, so we next investigated whether $GluA2_{3Y}$ can also prevent the time-dependent forgetting of a conditioned response. We used CPP, an appetitive Pavlovian conditioning task in which one of two spatial contexts is paired with food while the other one is not. The subsequent bias of rats in spending time in the paired (rewarded) rather than the unpaired (nonrewarded) context reveals CPP. On the first day of the conditioning procedure, animals were allowed to freely explore the two visually distinct compartments of the CPP apparatus for 10 min. Beginning 24 h later, rats were confined once per day for 4 d to 1 of the 2 compartments for 30 min in an alternating fashion (twice to each compartment in total). Rats encountered the food reward only in the paired compartment. First, we determined whether expressing the conditioned response requires AMPAR-mediated synaptic transmission in the dorsal hippocampus. Twenty-four hours after the conditioning procedure, rats received infusions of the AMPAR antagonist CNQX or its vehicle into the dorsal hippocampus. Fifteen minutes later, they were allowed to freely explore the CPP apparatus for 10 min. Expression of the conditioned response was different between the groups (vehicle, $d = 0.29 \pm 0.04$; CNQX, $d = 0.01 \pm 0.06$; unpaired *t* test: $t_{(6)} = 3.70$, $p = 0.01$) and only rats infused with vehicle displayed a preference for the paired compartment (one-sample *t* test: vehicle, $t_{(3)} = 6.83$, $p = 0.006$; CNQX, $t < 1$). There were no differences in the total amount of time animals spent in the compartments, which suggests that motivation and motility were not different between the groups (unpaired *t* test: $t < 1$). These findings indicate that expression of CPP requires AMPAR activation in the dorsal hippocampus.

We next determined at what time point after learning rats would no longer express the conditioned response. In different animals, we assessed CPP either 1 d (i.e., 24 h) or 10 d after training (Fig. 4*A–C*). CPP was different between the groups

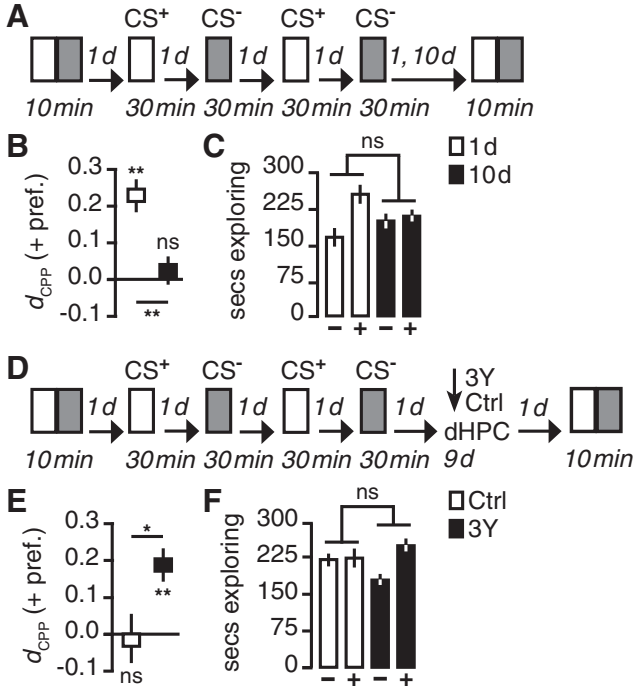

Figure 4. Forgetting of associative memories acquired with Pavlovian conditioning requires synaptic removal of GluA2/AMPARs. ***A–C***, Rats forget CPP within 10 d. ***A***, On the first day of training, food-deprived animals had free access to the two compartments of the CPP box that were connected to each other with a corridor. Conditioning began the next day. Rats participated in 1 30 min training session each day for 4 d. During these 4 d of conditioning, in an alternating fashion, animals were confined to either the compartment that always contained food reinforcement (CS+) or to the compartment that never contained food reinforcement (CS−). Expression of CPP was assessed either 1 d (i.e., 24 h; $n = 6$) or 10 d ($n = 6$) after training. ***B***, Rats tested 24 h after conditioning preferred the compartment that was paired with food ($d_{CPP} > 0$), but rats tested after 10 d no longer expressed this bias ($d_{CPP} = 0$). ***C***, Both groups spent the same overall time in the compartments during the test, indicating that compartment preference was neither due to differences in motility nor to changes in the amount of time spent in the connecting corridor. ***D–F***, Infusing $GluA2_{3Y}$ into the dorsal hippocampus blocks the forgetting of CPP (see Fig. 8*F* for cannula placements). ***D***, Rats were trained as before (***A–C***). Twenty-four hours after the last conditioning session, animals received 2 daily infusions of $GluA2_{3Y}$ ($n = 7$) or of the inactive control variant $GluA2_{3A}$ (Ctrl; $n = 8$) into dorsal hippocampus for 9 d. Twenty-four hours after the last infusion, 10 d after the last training session, the food-deprived animals had free access to the two compartments of the CPP box for 10 min. ***E***, Only animals infused with $GluA2_{3Y}$ preferred the side that had been paired with food; animals that had received the inactive peptide (Ctrl) spent the same amount of time in both compartments. The difference in preference for the paired side was significantly stronger in the $GluA2_{3Y}$-infused animals than in the animals that had received the control peptide (Ctrl). ***F***, Both groups spent the same total amount of time in the compartments.

(unpaired t test: $t_{(10)} = 4.35$, $p = 0.001$) in that animals tested 1 d after training expressed robust preference for the paired compartment, whereas rats tested 10 d after training did not (one-sample t test: 1 d, $t_{(5)} = 6.13$, $p = 0.002$; 10 d, $t < 1$; Fig. 4*B*). Both groups spent the same amount of time exploring the compartments, suggesting that differences in motivation or motility did not moderate differences in CPP (unpaired t test: $t < 1$; Fig. 4*C*). Therefore, CPP was forgotten within 10 d after training.

We then explored whether $GluA2_{3Y}$ could prevent the loss of CPP. In two different groups of rats, $GluA2_{3Y}$ (3Y) or its inactive control variant $GluA2_{3A}$ (Ctrl) was infused twice daily into dorsal hippocampus for 9 d beginning 24 h after the last day of the conditioning procedure (Fig. 4*D*). We assessed CPP 24 h after the last infusion; that is, 10 d after the last conditioning session, and found a significant difference in conditioned responding between the groups (unpaired t test: $t_{(13)} = 2.47$, $p = 0.03$; Fig. 4*E*). Animals infused with $GluA2_{3Y}$ still expressed CPP, whereas rats that had received the inactive $GluA2_{3A}$ treated both compartments the same (one-sample t test: $GluA2_{3Y}$, $t_{(6)} = 4.66$, $p = 0.004$; $GluA2_{3A}$, $t < 1$; Fig. 4*E*). There were no differences in the overall time that the two groups spent in the two compartments, suggesting that $GluA2_{3Y}$ did not affect general exploratory behavior (unpaired t test: $t < 1$; Fig. 4*F*). Therefore, suppressing synaptic removal of GluA2/AMPARs with $GluA2_{3Y}$ infusions in the dorsal hippocampus prevented the forgetting of a conditioned response.

$GluA2_{3Y}$ prevents contextual fear generalization and spontaneous recovery of auditory fear

Progressive memory loss over time could contribute to phenomena of time-dependent memory change (Hardt et al., 2013), such as the generalization of contextual fear memory. Shortly after contextual fear conditioning, animals express less fear in a novel than in the conditioning context; however, 2 weeks after training, the fear expressed is the same in both (Riccio et al., 1984; Biedenkapp and Rudy, 2007; Winocur et al., 2007). This change in spatial specificity of the fear response is accompanied by a disengagement of the hippocampus, which is critical for contextual fear memory expression shortly after conditioning, but not at later time points (Kim and Fanselow, 1992; Wiltgen and Silva, 2007). We therefore explored whether infusing $GluA2_{3Y}$ into the dorsal hippocampus could prevent contextual fear memory generalization.

First, we established that context discrimination decreases over time (Fig. 5*A*,*B*). Different groups of rats received two unsignaled foot shocks in a conditioning chamber (context "old") and then were returned first to a new chamber and 1 d later to the old one, or vice versa, beginning either 24 h or 14 d later (Fig. 5*A*; testing sequence did not affect freezing, unpaired t tests $t < 1$, and groups were collapsed for analyses). Fear expression in the new compared with the old context varied over time (mixed two-way ANOVA: $F_{(1,14)} = 5.43$, $p = 0.04$; Fig. 5*B*). Shortly after training, animals expressed less fear in the new than in the training context (simple effects analysis: $F_{(1,14)} = 21.99$, $p = 0.001$), but 14 d after training, fear expression was the same in both (simple effects analysis: $F_{(1,14)} = 1.94$, $p = 0.19$). Fear expression in the training context was the same at both time points (simple effects analysis: $F_{(1,14)} = 1.08$, $p = 0.32$), but higher in the new one during the later test (simple effects analysis: $F_{(1,14)} = 16.81$, $p = 0.001$).

We then tested the effects of $GluA2_{3Y}$ on contextual fear generalization in this protocol (Fig. 5*C*,*D*). During the 13 d retention interval, beginning 24 h after conditioning, animals received infusions of $GluA2_{3Y}$ or the inactive control peptide $GluA2_{3A}$ (Ctrl) twice daily (A.M. and P.M.) into the dorsal hippocampus. Twenty-four hours after the last infusion, that is, 14 d after conditioning, animals were exposed to a new context and 24 h later to the original training context or vice versa (Fig. 5*C*; as before, context sequence did not affect fear expression, $t < 1$, and groups were collapsed for analyses). The groups showed different contextual fear expression in the new compared with the old context (mixed two-way ANOVA: $F_{(1,13)} = 5.07$, $p = 0.04$; Fig. 5*D*): rats infused with $GluA2_{3Y}$ expressed less fear in the new than in the old context compared with animals in the control group (simple effects analysis: $F_{(1,13)} = 8.52$, $p = 0.012$), which feared both contexts the same (simple effects analysis: $F < 1$). Furthermore, the fear that $GluA2_{3Y}$-infused animals expressed in the new context was lower than the fear that animals receiving the inactive

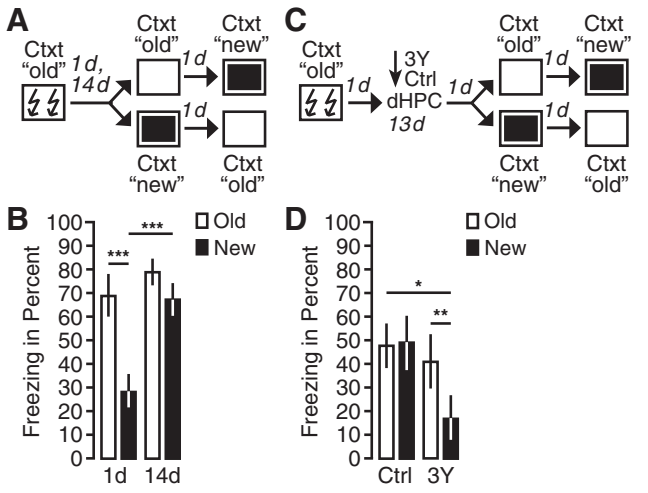

Figure 5. Infusing $GluA2_{3Y}$ into the dorsal hippocampus to block synaptic removal of GluA2/AMPARs prevents generalization of contextual fear (see Fig. 8*G* for cannula placements). ***A***, ***B***, Contextual fear generalizes to a novel context within 14 d. ***A***, Animals received two unsignaled electric foot shocks in the training context (old) and were then exposed either to a new or the old context 24 h or 14 d later ($n = 8$ for all four groups; sequence had no effect on performance and groups were collapsed for analyses). ***B***, Twenty-four hours after contextual fear conditioning, rats froze significantly more in the training (old) than in the novel context. However, they expressed the same amount of fear to both contexts when tested 14 d after conditioning; that is, fear expression in the novel context after 14 d was as high as fear expression in the training context after 24 h. ***C***, ***D***, Infusing $GluA2_{3Y}$ during the retention interval prevents contextual fear generalization. ***C***, Animals received two unsignaled foot shocks in the training context (old) and, beginning 24 h later, received 2 daily infusions of $GluA2_{3Y}$ (3Y, $n = 7$) or the inactive $GluA2_{3A}$ (Ctrl, $n = 8$) into the dorsal hippocampus for 13 d. Twenty-four hours after the last infusion, 14 d after training, contextual fear expression was assessed in either the training (old) or a novel context (new). As before, the order of context testing had no effects on behavior and groups were collapsed for analyses. ***D***, Rats infused with the inactive $GluA2_{3A}$ (Ctrl) feared both contexts the same, thus presenting with contextual fear generalization, as expected. Rats that received $GluA2_{3Y}$ expressed the same fear in the training context as the rats in the control group; however, they froze significantly less in the novel context, which suggests that they were able to better discriminate between the training and other contexts.

control peptide expressed (simple effects analysis: $F_{(1,13)} = 5.95$, $p = 0.03$). In the old context, there was no such difference between the groups (simple effects analysis: $F < 1$). These results suggest that $GluA2_{3Y}$ preserves hippocampal contextual fear memories, which normally would change over time, thus leading to contextual fear generalization.

In a similar way, progressive weakening of memory could underpin spontaneous recovery of extinguished responses (Pavlov, 1927). During extinction training, animals learn that the unconditioned stimulus no longer follows upon presentation of the conditioned stimulus (CS). They acquire an inhibitory memory that restrains expression of the conditioned response (Bouton, 2004; Rescorla, 2004). Some time after the end of extinction training, however, the suppressed response can spontaneously return, which could reflect loss of the inhibitory memory. Therefore, we tested whether infusing $GluA2_{3Y}$ into the infralimbic cortex, an area critically involved in the maintenance of extinction memory (Quirk et al., 2000; Lebrón et al., 2004; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011), could prevent the spontaneous recovery of extinguished fear. Rats first received auditory fear conditioning (Fig. 6*A*), during which we paired a tone (CS) with foot shock. Twenty-four hours later, animals were randomly divided into two groups and then the first of three extinction sessions began. For 3 consecutive days, the CS was presented 12 times/d. This procedure significantly reduced fear expression to the CS across the 3 d in both groups (repeated-measures ANOVA: effect of day, $F_{(2,20)} = 62.80$, $p < 0.0001$; effect of group, $F < 1$; Fig. 6*B*). During each extinction session, both groups also showed low levels of fear before the first CS was presented (between 0% and 19% freezing, data not shown), which did not change across extinction sessions (repeated-measures ANOVA: effect of day, $F_{(2,20)} = 2.05$, $p = 0.16$; effect of group, $F < 1$), demonstrating the absence of contextual fear conditioning. Twenty-four hours after the last extinction session, we assessed inhibition of the fear response, presenting the CS three times in a single session. Beginning 24 h later, we infused one group of rats twice daily (A.M. and P.M.) with $GluA2_{3Y}$ and the other one with the inactive $GluA2_{3A}$ (Ctrl) for 6 successive days. Twenty-four hours after the last infusion, we again presented the CS three times in a single session. Fear expression to the CS was different in the first compared with the second test between the groups (repeated-measures ANOVA: $F_{(1,10)} = 23.57$, $p = 0.0007$, Fig. 6*C*). During the first test, both groups expressed the same low levels of fear to the CS, thus showing good retention of extinction memory (simple effects analysis: $F_{(1,10)} = 1.88$, $p = 0.20$). The animals that were infused with $GluA2_{3Y}$ retained this inhibition in the second test (simple effects analysis: $F < 1$). In contrast, fear expression was different to the group that had received the inactive $GluA2_{3A}$ (simple effects analysis: $F_{(1,10)} = 50.33$, $p < 0.0001$), which showed a significant increase in fear expression from the first to the second test (simple effects analysis: $F_{(1,10)} = 53.63$, $p < 0.0001$).

Blocking synaptic removal of GluA2/AMPARs prevents depotentiation

Overall, these findings suggest that activity-dependent synaptic removal of GluA2/AMPARs underpins forgetting of long-term memories in the hippocampus. Two physiological forms of neural activity, LTD and depotentiation, which lead to reductions of synaptic potentiation, have been described. Both LTD and depotentiation could therefore provide candidate mechanisms involved in this type of forgetting. It has been shown in various stimulation protocols that $GluA2_{3Y}$ blocks the induction of LTD (Ahmadian et al., 2004; Brebner et al., 2005; Scholz et al., 2010); whether it also impairs depotentiation has not yet been tested. We therefore assessed whether $GluA2_{3Y}$ could block depotentiation of LTP (Figs. 7, 8). In a hippocampal slice preparation, EPSCs were evoked in CA1 by stimulating the Schaffer collateral–commissural pathway. Once stable EPSCs were obtained, we induced LTP of AMPAR-mediated EPSCs by 200 pulses of repetitive stimulation at 2 Hz while voltage clamping the recorded cells at −5 mV. This method reliably induced LTP that lasted >45 min, the longest time measured in our study. Fifteen minutes into LTP recording, we induced depotentiation by delivering 300 pulses at 1 Hz while holding the cells at −45 mV. This reduced the previously potentiated EPSPs down to the basal level observed before LTP induction (Fig. 7*A*, *D*). Perfusing the postsynaptic cell with $GluA2_{3Y}$ (3Y; Fig. 7*B*) or with a dynamin-derived peptide (Dyn; Fig. 7*C*) to block clathrin-mediated endocytosis prevented depotentiation without affecting basal transmission or LTP induction (one-way ANOVA: basal transmission, $F < 1$; LTP induction, $F < 1$); the inactive $GluA2_{3A}$ ($Ctrl^{3Y}$) or the inactive dynamin variant ($Ctrl^{Dyn}$) had no effect on depotentiation (one-way ANOVA comparing EPSC amplitudes at the end of recording, 45 min after delivering low-frequency stimulation to induce DP: $F_{(4,30)} = 11.67$, $p < 0.001$; Holm–Šidák multiple *post hoc* comparisons: DP vs 3Y, $p < 0.001$; DP vs Dyn, $p < 0.001$; DP vs $Ctrl^{3Y}$, $p = 0.95$; DP vs $Ctrl^{Dyn}$, $p = 0.95$; Fig. 7*D*). These

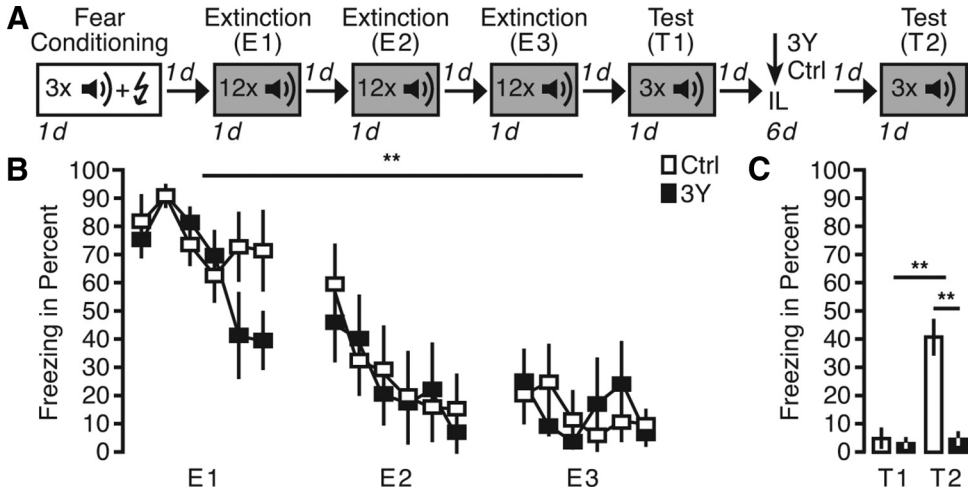

Figure 6. Infusing GluA2$_{3Y}$ into the infralimbic cortex to block internalization of GluA2/AMPARs prevents spontaneous recovery of extinguished auditory fear (see Fig. 8*H* for cannula placements). ***A***, Rats received three tone-shock (CS– unconditioned stimulus, US) pairings in the conditioning context. Twenty-four hours later, extinction training began. For three consecutive days, rats received 12 CS presentations in the test context each day. Twenty-four hours after the last extinction session, extinction memory was assessed (Test 1) in the test context, and, beginning 24 h later, rats received for 6 consecutive days either 2 daily infusions of GluA2$_{3Y}$ ($n = 6$) or of the inactive GluA2$_{3A}$ (Ctrl, $n = 6$). Auditory fear was assessed the day after the last infusion (test 2), 7 d after test 1, by presenting the CS 3 times in the testing context. ***B***, Both groups presented with significantly decreased fear expression over the three extinction sessions. ***C***, Rats that received infralimbic infusions of GluA2$_{3Y}$ during the 6 d retention interval maintained the same level of robust fear suppression during both tests (test 1 and test 2). In contrast, rats that had received the inactive GluA2$_{3A}$ showed spontaneous recovery, significantly increasing their fear expression from test 1 to test 2.

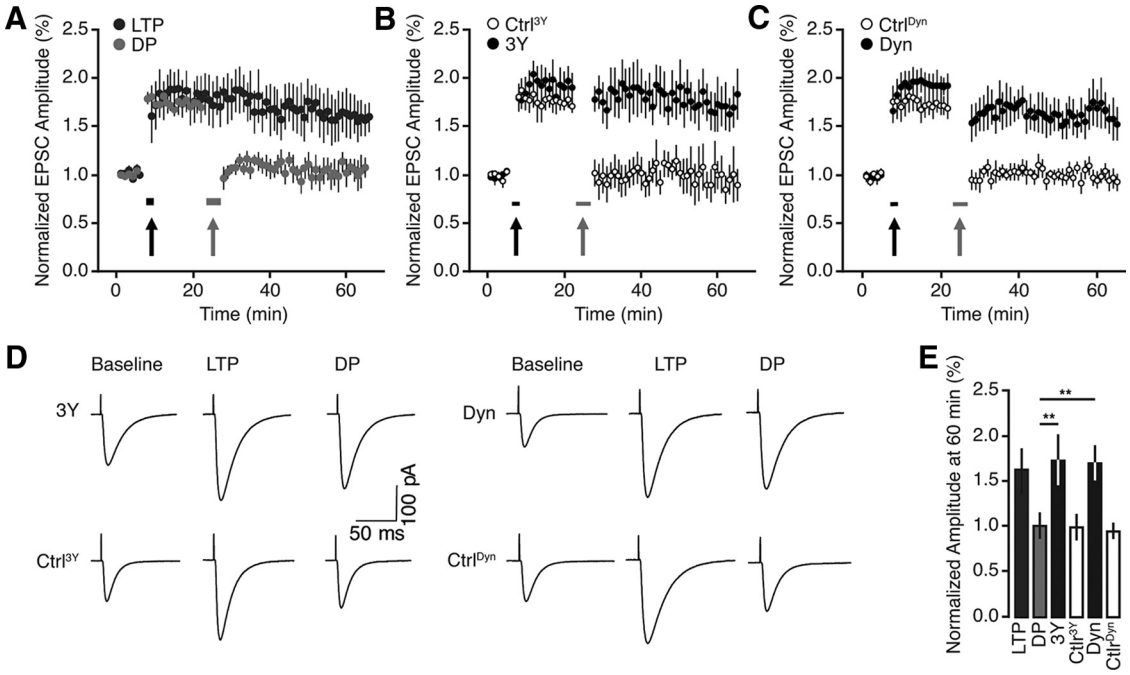

Figure 7. Rapid synaptic AMPAR removal is required for depotentiation of hippocampal LTP in CA1. ***A***, LTP was induced by a pairing protocol (200 pulses at 2 Hz while holding at −5 mV). LTP could be depotentiated by a low-frequency stimulation protocol (300 pulses at 1 Hz, holding at −45 mV). Black arrows here and in the two other panels indicate time point of high-frequency stimulation (200 pulses at 2 Hz); gray arrows mark delivery of low-frequency stimulation (300 pulses at 1 Hz). ***B***, Intracellular delivery of GluA2$_{3Y}$, but not the inactive control peptide GluA2$_{3A}$ (Ctrl ^{3Y}), blocked depotentiation. ***C***, Intracellular delivery of dynamin-derived peptide (Dyn), but not its inactive control variant (Ctrl ^{Dyn}), blocked depotentiation. ***D***, Representative EPSC traces for GluA2$_{3Y}$ and inactive GluA2$_{3A}$ (Ctrl ^{3Y}) at different time points (5, 20, and 60 min, which represent EPSC levels before, after LTP, and after depotentiation induction, respectively). ***E***, Normalized EPSC amplitude at 60 min (32 min after induction of depotentiation) for LTP ($n = 6$) and depotentiation (DP, $n = 6$) and in the presence of GluA2$_{3Y}$ (3Y, $n = 5$), inactive control GluA2$_{3A}$ (Ctrl ^{3Y}, $n = 5$), dynamin-derived peptide (Dyn, $n = 9$), and inactive dynamin-control peptide (Ctrl ^{Dyn}, $n = 6$).

results suggest that depotentiation requires the activity-dependent synaptic removal of GluA2/AMPARs. The rats used for slice electrophysiology were younger than those used in the behavioral experiments. Age-dependent changes, however, have been shown to be absent in DP in rats (Kumar et al., 2007). Therefore, LTD and depotentiation are candidate mechanisms that could be involved in the forgetting of memories over time observed in the studies described above.

Discussion

We investigated whether interfering with the regulated synaptic removal of GluA2-containing AMPARs could prevent the forget-

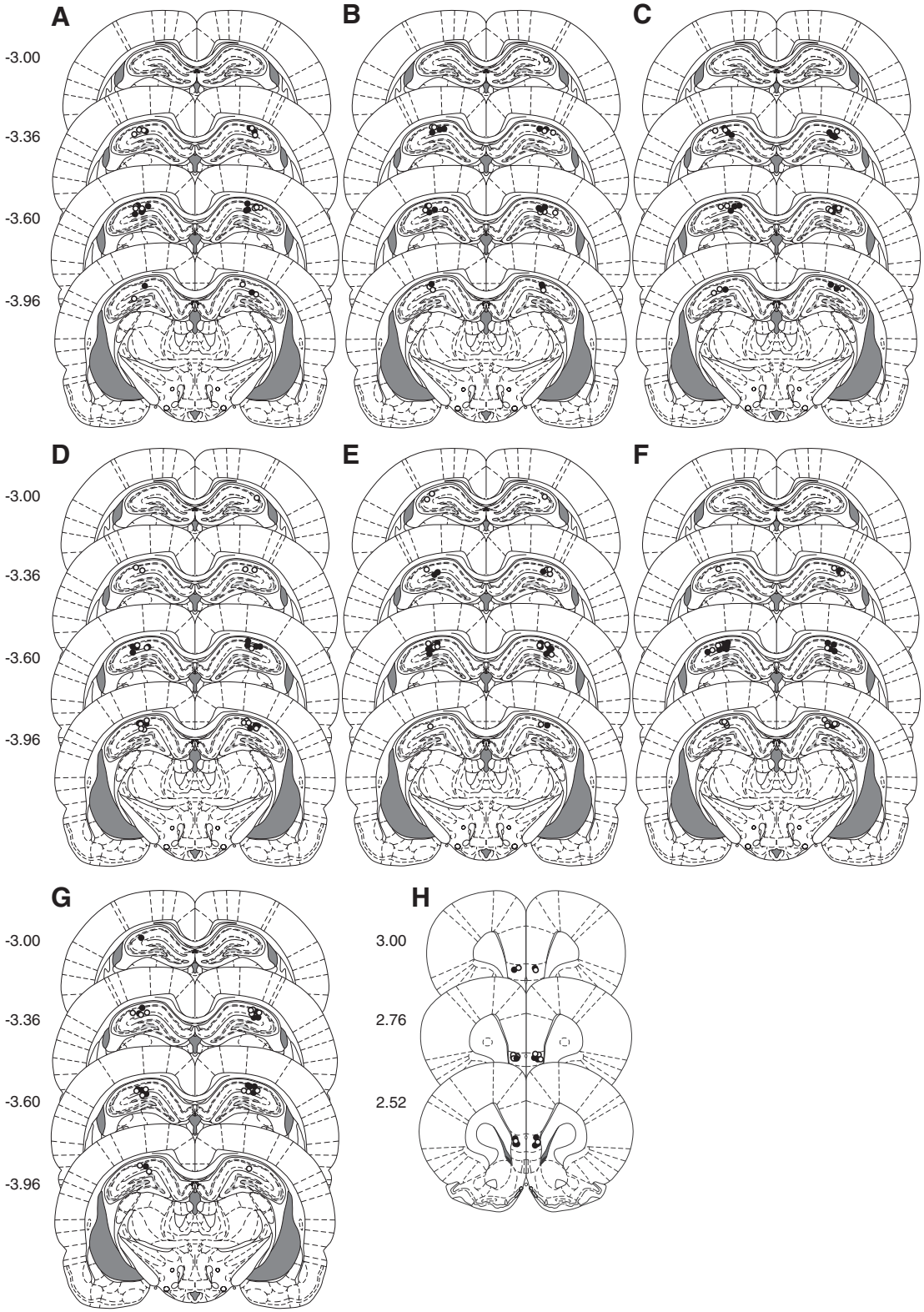

Figure 8. Cannula placements. ***A***, Figure 1*D–F* (white circles: Ctrl; black circles: $GluA2_{3Y}$). ***B***, Figure 2*A–C* (white circles: Ctrl; black circles: G2CT). ***C***, Figure 2*D–F* (white circles: Ctrl; black circles: $GluA2_{3Y}$). ***D***, Figure 3*A–C* (white circles: PI; black circles: RI). ***E***, Figure 3*D–F* (white circles: Ctrl; black circles $GluA2_{3Y}$). ***F***, Figure 4*D–F* (white circles: Ctrl; black circles $GluA2_{3Y}$). ***G***, Figure 5*D–F* (white circles: Ctrl; black circles $GluA2_{3Y}$). ***H***, Figure 6*A–C* (white circles: Ctrl; black circles $GluA2_{3Y}$).

ting of long-term memories. To this end, we infused the peptides $GluA2_{3Y}$ and G2CT into the dorsal hippocampus during the memory retention interval. This treatment preserved long-term object location memories and CPP, preventing their natural forgetting over time. Exploring possible functional roles of GluA2/AMPAR internalization, we found that infusing $GluA2_{3Y}$ into the dorsal hippocampus blocked the generalization of contextual fear, whereas infusing it into the infralimbic cortex after extinc-

tion training prevented spontaneous recovery of auditory fear. We identified depotentiation as a possible physiological correlate of this type of forgetting because $GluA2_{3Y}$ or a dynamin-derived peptide, which both disrupt AMPAR endocytosis, prevented the induction of depotentiation. These results suggest that forgetting of long-term memory involves the activity-dependent removal of GluA2/AMPARs.

Similar results were recently reported for shorter-lasting memories (Dong et al., 2015). In these experiments, rats received inhibitory avoidance training that resulted in memory lasting for at least 1 but not 24 h. Consistent with our results, infusions of $GluA2_{3Y}$ after training prevented synaptic loss of GluA2/AMPARs and preserved the conditioned response for at least 24 h. Similarly, systemic injections of $GluA2_{3Y}$ after weak stimulation of CA1 in dorsal hippocampus prolonged LTP for at least 24 h, which would otherwise have decayed within 4 h. As was the case in our studies, $GluA2_{3Y}$ did not increase the strength of inhibitory avoidance memory or synaptic potentiation. Together, these findings suggest that $GluA2_{3Y}$ did not enhance memory consolidation of weak memory, thereby transforming it into long-term memory. Rather, consistent with our findings showing that infusing $GluA2_{3Y}$ after or during initial training did not increase novelty preference, these results suggest that $GluA2_{3Y}$ blocked a physiological forgetting mechanism that erases memories constitutively.

Our findings indicate that this form of forgetting resembles memory decay, not interference arising from ongoing new learning after original memory formation (Lewis, 1979; Wixted, 2004; Roediger et al., 2010). First, the long-term object location memories acquired with our protocol were relatively resistant to interference (Fig. 3*B*). Rats that were exposed to new objects at different locations in a different context did not show impaired memory for the locations of objects learned previously or subsequently. Therefore, highly similar learning experiences led to neither proactive nor retroactive interference. Second, infusing $GluA2_{3Y}$ before and after each training session did not block formation of long-term object location memories (Fig. 3*E*). This suggests that infusing $GluA2_{3Y}$ during the retention interval did not prevent new learning and memory acquisition. Therefore, it is unlikely that interference caused forgetting.

Studies exploring memory interference usually focus on a time point shortly after learning or memory reactivation to interfere with the processes of cellular consolidation or reconsolidation, respectively (Wixted, 2005; Hupbach et al., 2007; Dewar et al., 2012). Given our results, it indeed appears that outside of these processing phases, long-term memories that are supported by the hippocampus are relatively immune to interference, even during the learning of very similar material (Fig. 3*A–C*). This might indicate that pattern separation in dentate gyrus reduces the amount of overlap in neuronal populations recruited during different epochs of memory formation, thus decreasing the probability of interference. It is not a contradiction that new learning can interfere with memories during consolidation and reconsolidation. In other areas of the hippocampus, such as CA3, most neurons are connected to each other directly, which could facilitate interference during these processing phases (Hardt et al., 2013). In such densely connected networks, a Hebbian learning mechanism could link previously acquired and new memories even if their neural populations overlap marginally or not at all, provided they are processed concurrently. Conversely, interference could arise from limited processing resources for which coactive memories compete (Wixted, 2004, 2005). Inactive established memories, such as the location memories in our interference study (Fig. 3*A–C*), will not use resources during the acquisition of other memories, which can thus form uncompromised so that we neither observed proactive nor retroactive interference (Fig. 3*B*). Notwithstanding the actual neurobiological processes underpinning interference, our findings strongly suggest that a form of memory decay removes long-term memories in the hippocampus.

Our result showing unaffected spatial learning when animals were infused with $GluA2_{3Y}$ into the dorsal hippocampus seemingly stands in contrast to demonstrations that $GluA2_{3Y}$ impairs acquisition of place memory in the Morris water maze in rats (Dong et al., 2013, but see Dong et al., 2015, who report that $GluA2_{3Y}$ infusions enhance water maze place learning in the APP23/PS45 mouse model for Alzheimer's disease). These opposing outcomes likely reflect that different forms of learning recruit different forms of synaptic plasticity. For example, whereas the allocentric learning of a hidden platform location over several training trials may require increasing and reducing synaptic potentiation, the learning of object locations in an open field (Figs. 1, 2, 3) or the acquisition of appetitive CPP (Fig. 4) may predominantly depend on the former mechanism. It has been shown consistently that $GluA2_{3Y}$ does not impair the induction of LTP (see also Fig. 7) or other forms of hippocampal learning such as contextual fear conditioning (Ahmadian et al., 2004; Brebner et al., 2005; Dalton et al., 2008). Instead, $GluA2_{3Y}$ blocks LTD, depotentiation (Fig. 7), reversal learning, and the acquisition of fear extinction memory, all of which critically involve the synaptic removal of GluA2/AMPARs (Dalton et al., 2008; Dong et al., 2013). Future research will address which learning mechanisms these tasks indeed recruit.

We infused $GluA2_{3Y}$ twice daily into the hippocampus for up to 13 d, aiming to affect a significant part of the retention period because we had no hypotheses about when and for how long forgetting processes take place. Nevertheless, our data suggest that interfering with GluA2/AMPAR endocytosis may preserve memory for as long as it can be expressed because infusing animals with $GluA2_{3Y}$ shortly before forgetting would naturally occur (i.e., on day 8 after training) effectively blocked forgetting. At this late time point after learning, $GluA2_{3Y}$ did not prevent forgetting by enhancing memory consolidation. Therefore, it is unlikely that enhanced memory consolidation underpins preserved long-term memory in the experiments in which infusions began 24 h after the last training session. This conclusion is further supported by the fact that $GluA2_{3Y}$ did not affect memory formation (Fig. 3*E*). It remains unclear how these artificially prolonged long-term memories compare with naturally aged counterparts. For example, it will be important to address whether natural forgetting will set in as soon as $GluA2_{3Y}$ infusions terminate. Furthermore, it remains to be determined whether infusions of $GluA2_{3Y}$ into one area of the brain can affect memory processes in other connected brain regions, which may contribute to attenuated forgetting of the targeted memory.

Our data indicate that preventing activity-dependent removal of GluA2/AMPARs "locks" the current synaptic state in terms of preserving present synaptic potentiation. Depending on the brain system and memory type, this brake on remodeling may reveal different cognitive–behavioral phenomena. In the case of long-term memory retention for object locations or CPP, $GluA2_{3Y}$ infusions into the dorsal hippocampus can prevent decay-like forgetting; when infused into the infralimbic cortex after auditory fear extinction training (Fig. 6), $GluA2_{3Y}$ may preserve inhibitory memory, potentially counteracting the natural decay that would otherwise occur in this region. An alternative

explanation that could account for our observations may be that forgetting in the hippocampus does not remove actual memory content, but rather eliminates the network alterations that permit retrieval of content stored previously (Hardt et al., 2009; Ryan et al., 2015); forgetting in the infralimbic cortex, however, may erase components of the actual inhibitory memory. When we infused $GluA2_{3Y}$ into the dorsal hippocampus after contextual fear conditioning, it prevented generalization of fear to novel contexts (Fig. 5). This may reflect that $GluA2_{3Y}$ preserved spatial memory and the ability to discriminate the original conditioning context from new ones, but it could also suggest that GluA2-dependent AMPAR endocytosis is essential for fine-tuning hippocampal networks during abstraction processes; that is, the extrapolation of general patterns from specific experiences. These various possible contributions of GluA2/AMPAR trafficking to memory processes could explain why $GluA2_{3Y}$ blocks reversal learning (Dong et al., 2013), reconsolidation (Rao-Ruiz et al., 2011), and extinction learning (Dalton et al., 2008; Dias et al., 2012).

Decay might recruit processes akin to LTD and depotentiation (Fig. 7). Like the forgetting of spatial memories, these processes require NMDAR activation (Villarreal et al., 2002; Shinohara and Hata, 2014). Together with our results, this suggests a role for NMDAR in synaptic GluA2/AMPAR removal during memory decay (Hardt et al., 2013, 2014). Due to their interaction with cell-adhesion and cytoskeletal proteins, GluA2/AMPAR removal could contribute to dissolving synaptic infrastructure essential for memory persistence (Hadziselimovic et al., 2014). Therefore, whereas active decay likely provides qualitative mnemonic functions and participates in memory system maintenance, its dysregulation could give rise to cognitive deficits in neurodegenerative diseases. Indeed, after a century of exploring consolidation (McGaugh, 2000), examining how the brain forgets established memories may offer promisingly fresh perspectives on memory and its disorders.

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