BDNF is essential to promote persistence of long-term memory storage

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Persistence is a characteristic attribute of long-term memories (LTMs). However, little is known about the molecular mechanisms that mediate this process. We recently showed that persistence of LTM requires a late protein synthesis- and BDNF-dependent phase in the hippocampus. Here, we show that intrahippocampal delivery of BDNF reverses the deficit in memory persistence caused by inhibition of hippocampal protein synthesis. Importantly, we demonstrate that BDNF induces memory persistence by itself, transforming a nonlasting LTM trace into a persistent one in an ERK-dependent manner. Thus, BDNF is not only necessary, but sufficient to induce a late postacquisition phase in the hippocampus essential for persistence of LTM storage.

consolidation | hippocampus | forgetting | ERK1/2

Long-term storage of information is a hallmark feature of the brain. Memories may last for hours (short-term memory, STM) or for days, weeks ,and even a lifetime (long-term memory, LTM). LTM requires, whereas STM does not, a gene expression and protein synthesis-dependent stabilization process named consolidation that takes place in restricted areas of the brain, particularly in the hippocampus (1–3). Depending on the strength and/or saliency of the information to be remembered, consolidated LTMs can persist for just 24–48 h or for many days or weeks (4–6). For LTM to become long-lasting, changes must persist after acquisition to render the trace immune to molecular turnover (1, 2, 7). However, little is known about the molecular mechanisms that make some LTMs persist more than others.

BDNF regulates neuronal structure and function (8, 9). In particular, it is critical for synaptic plasticity and memory-processing in the adult brain (9–12). In fact, BDNF induces and is sufficient for long-term potentiation (LTP) in the hippocampus (13–16), a form of synaptic plasticity thought to underlie LTM formation (17–19). Recently, we described the requirement of a late phase of protein synthesis and BDNF expression in the hippocampus, 12 h after training, for persistence of consolidated LTM (12). We found that blocking BDNF expression and function in the hippocampus during a critical time period caused a deficit in memory persistence without affecting LTM formation, indicating that synthesis of BDNF during this late protein synthesis-dependent phase is crucial for persistence of memory storage. Here, we investigated whether BDNF is sufficient for memory persistence and whether it can, by itself, promote persistence of LTM storage. Given that BDNF activates several signaling effectors, including ERK (20, 21), we examined the mechanisms for BDNF-induced LTM persistence.

Results

To determine the effect of BDNF on LTM persistence, we used a one-trial inhibitory avoidance (IA) paradigm, a hippocampus-dependent task extensively used for studying posttraining memory processing and recall (2, 19, 22, 23). To test whether BDNF action during the late posttraining critical time period is not only required but sufficient for persistence of LTM storage, we infused the protein synthesis inhibitor anisomycin (Ani; 80 μg/0.8 μl per side) in the dorsal hippocampus [supporting information (SI) Fig. 4] 12 h after IA training, which causes a selective deficit in memory retention 7 days, but not 2 days, after training (12). Fifteen minutes after Ani infusion, we delivered human recombinant BDNF (hrBDNF, 0.25 μg/0.8 μl per side) or vehicle (Veh) into the hippocampus and tested the animals for retention 7 days thereafter. The impairment in LTM at 7 days after training caused by Ani given at 12 h after training was completely rescued by hrBDNF (Fig. 1*A*), indicating that BDNF is a key factor synthesized during this late protein synthesis-dependent phase necessary for persistence of LTM storage. We also confirmed that BDNF is required for persistence of LTM storage (12). Infusion of BDNF antisense oligonucleotides [BDNF ASO; (24)] into the dorsal hippocampus 10 h after training, a treatment that blocks expression of BDNF 12 h after training, impaired persistence but not formation of IA LTM (Fig. 1*B*). We next explored whether BDNF can induce persistence of memory storage by itself. Differences in LTM duration can be achieved by modifying the amount or the strength of training (Fig. 2*A*) (5). Thus, IA training using a strong foot shock, which generates a persistent LTM, resulted in increased BDNF expression in the dorsal hippocampus 12 h after training, whereas training with a mild foot shock, which produces a rapidly decaying LTM, did not change BDNF levels at 12 h after training [Fig. 2 *A* and *B* (12)]. Therefore, to determine whether BDNF infusion in the hippocampus 12 h after training induces persistence of IA LTM storage, we used a weak training protocol. In animals thus trained, infusion of hrBDNF (0.25 μg/0.8 μl) 12 h after training induced persistence of memory retention measured at 7 days (Fig. 2*C*). Consistent with the hypothesis that, late after training, BDNF expression induces LTM persistence but not LTM formation, rats infused with hrBDNF or Veh 12 h after training showed no differences in IA memory retention 2 days later (Figs. 1*A* and 2*C*; see also ref.12). When given 24 h after training, BDNF did not affect 7-day-old IA LTM (Fig. 2*D*).

Besides promoting LTM persistence, BDNF could also facilitate retention of a 7-day-old long-lasting memory trace. In a different set of experiments, we replicated our previous findings by observing that intrahippocampal infusion of hrBDNF 12 h after a weak IA training induced a fourfold increase in retention scores at 7 days after training [+435% vs. Veh-injected animals ($n = 8$), $P < 0.01$]. However, this same treatment did not affect retention when rats were trained by using a strong training protocol (Fig. 2*E*). This suggests that BDNF have no effect on

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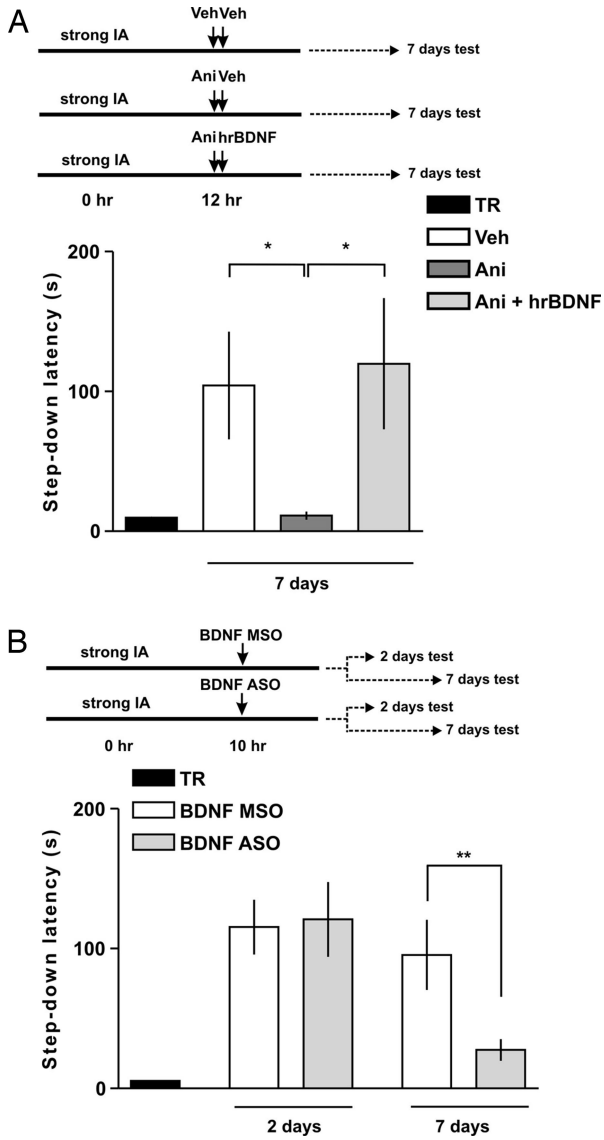

Fig. 1. BDNF is necessary and sufficient for memory persistence. (*A*) Human recombinant BDNF (hrBDNF) rescues the impairment in memory persistence caused by inhibition of protein synthesis. Anisomycin (Ani; 80 μg per side) caused amnesia 7 days after training when infused 12 h after learning (dark gray). This effect was reversed by infusion of hrBDNF (0.25 μg per side) 15 min later (light gray). *, $P < 0.05$, $n = 8$. (*B*) Intrahippocampal infusion of BDNF antisense oligonucleotide (BDNF ASO) late after IA training blocks memory retention at 7, but not at 2, days after training. BDNF ASO, but not BDNF missense oligonucleotide (BDNF MSO), infusion 10 h after training hinders memory persistence at 7 days but leaves memory intact 2 days after training. Animals were infused into the dorsal hippocampus with BDNF MSO (2 nmol per side) (white bars) or BDNF ASO (2 nmol per side) (gray bars) 10 h after training. Data are expressed as mean (±SEM) of training (TR, black bars) or test session step-down latency 2 or 7 days after IA training. **, $P < 0.01$; ASO vs. MSO at 7 days; Student's *t* test, $n = 8$–10.

memory persistence when infused 12 h after training if the training experience is strong enough to induce late BDNF expression by itself (see also Fig. 2*B*).

The increase in LTM persistence after BDNF infusion 12 h after weak IA training cannot be explained by a protracted effect on attention, motivation, perception, and/or motor coordination, because rats given BDNF 12 h after IA acquired and retained an object-recognition LTM 7 days later as unfailingly as control animals did (SI Fig. 5). Moreover, delayed infusion of BDNF 24 h after training did not affect IA LTM retention at 7 days, indicating that hrBDNF injected several hours after the critical time period does not produce any effect on performance (Fig. 2*D*) and ruling out the possibility that late posttraining administration of BDNF caused a protracted action on memory retrieval. Furthermore, the fact that the effect of BDNF on LTM persistence was only observed after weak, but not strong, IA training demonstrates that it results from a specific action that induces persistence of the memory trace in a time-dependent manner and not from a nonspecific enhancement of the animal's performance.

BDNF promotes ERK activation and BDNF-induced LTP requires ERK activity (10, 20, 25). A strong, but not a weak, training protocol that increases BDNF levels 12 h after training (Fig. 2*B*) induced ERK phosphorylation at 12 h after training (Fig. 3*A*). So we next examined whether blocking ERK activation in the hippocampus 12 h after training also produced a selective impairment in memory persistence (12). Intrahippocampal infusion of the MEK inhibitor, U0126 (0.38 μg/0.8 μl) 12 h after training impaired IA LTM retention 7, but not 2, days after training (Fig. 3*B*). In contrast, pretraining infusion of U0126 resulted in a clear-cut deficit in LTM retention at 2 and 7 days after training, thus confirming that the ERK cascade is required at the moment of training for LTM formation (25, 26). U0126 decreased hippocampal ERK phosphorylation *in vivo* by 34% (SI Fig. 6). Thus, ERK activation is required during training for LTM formation and again 12 h thereafter for persistence of LTM storage. In addition to ERK, BDNF can also activate the phosphoinositide-3 kinase/mammalian target of rapamycin (PI3K/mTOR) cascade (20, 21). In fact, when given in the hippocampus 15 min before training, both Ly294002 (10 mM/0.8 μl), a PI3K inhibitor, and rapamycin (60 nM/0.8 μl), a mTOR inhibitor (27), blocked IA LTM formation (SI Fig. 7 *A* and *C*). However, Ly294002 and rapamycin had no effect on LTM persistence when infused 12 h after IA training (SI Fig. 7 *A* and *C*). Moreover, there was no increase in Akt or mTOR phosphorylation levels 12 h after training (SI Fig. 7 *B* and *D*). Furthermore, intrahippocampal infusion of the phospholipase C (PLC) inhibitor U-73122 (0.05 μg/1 μl) 12 h after training did not affect IA LTM retention tested at 2 or 7 days after training (SI Fig. 8). This suggests that, although PLCγ has been implicated in BDNF signaling, its activation is not required for LTM persistence.

Given that BDNF-induced ERK-activation can be blocked by selective MEK inhibitors [SI Fig. 6 (10)] and that intrahippocampal infusion of BDNF antisense oligonucleotides hinders the IA training-induced activation of ERK at 12 h (Fig. 3*C*), we examined whether ERK is indeed required for BDNF-induced persistence of memory storage. As shown in Fig. 3*D*, intrahipocampal infusion of U0126 at 5 min before BDNF application 12 h after training, blocked the effect of BDNF on memory persistence.

Discussion

In this report, we show that infusion of recombinant BDNF in the CA1 region of the dorsal hippocampus 12 h after IA training reverses the deficit in memory persistence caused by local inhibition of protein synthesis. Importantly, we also demonstrate that BDNF is able to induce memory persistence by itself, transforming a nonlasting long-term memory trace into a persistent one through an ERK-dependent mechanism. Our results suggest that BDNF is not only necessary, but sufficient to induce a late postacquisition memory-processing phase in the hippocampus essential for persistence of LTM storage.

What are the mechanistic differences between a persistent LTM and another LTM that is rapidly forgotten? Our experiments indicate that BDNF is a key molecule involved in memory persistence, because the deleterious effect of general protein synthesis

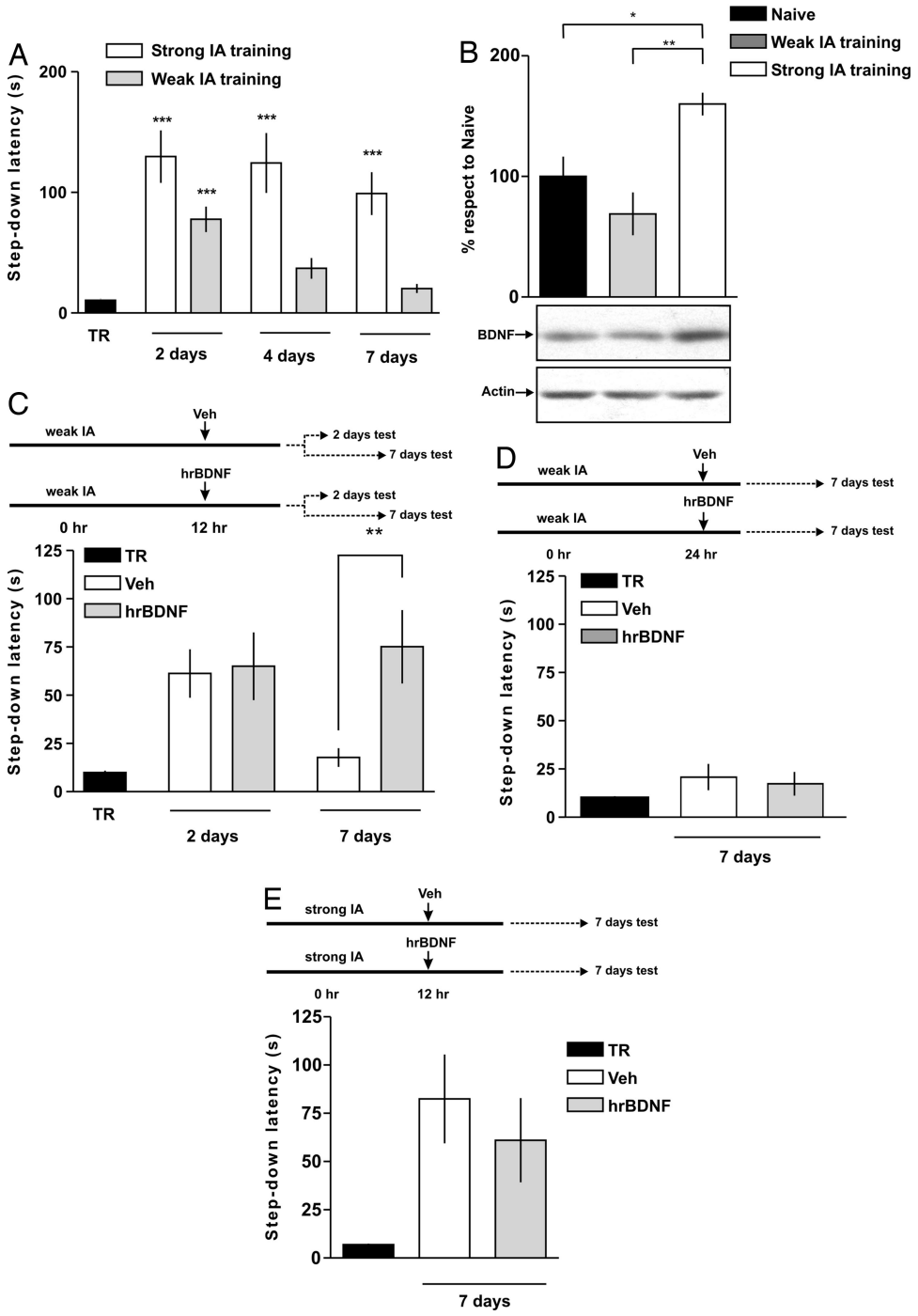

Fig. 2. BDNF promotes persistence of LTM storage. (*A*) A strong (0.7 mA), but not a weak (0.4 mA), foot shock during IA training creates a persistent LTM, lasting for at least 7 days. Data are expressed as mean (±SEM) of training (TR, black bars) or test session step-down latency at 2, 4, or 7 days after training. ***, $P < 0.001$; *, $P < 0.05$ vs. TR; $n = 10$. (*B*) (*Upper*) A strong, but not a weak, training is associated with an increase in BDNF in the dorsal hippocampus. Bars show normalized mean percentage level of BDNF with respect to the naïve group. Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ vs. naïve in Newman–Keuls test after ANOVA, $n = 6$. (*Lower*) Representative blots showing BDNF and actin levels. (*C* and *D*) Infusion of hrBDNF 12 h, but not 24 h, after training prevents memory decay. Rats were infused into the dorsal hippocampus with vehicle (Veh) or hrBDNF (0.25 μg per side) after a weak training and tested for retention 2 or 7 days afterward. Data are expressed as mean ± SEM of training (TR, black bars), Veh (white bars), or hrBDNF (gray bars) test session step-down latency at 2 or 7 days after training. **, $P < 0.01$ vs. Veh group; Student's *t* test, $n = 10$. (*E*) Infusion of hrBDNF 12 h after strong IA training does not enhance memory retention at 7 days. Rats were bilaterally infused into the dorsal hippocampus with vehicle (Veh) or hrBDNF (0.25 μg per side) after strong IA training and tested for retention 7 days afterward. Data are expressed as mean ± SEM of training (TR, black bars) or test session step-down latency 7 days after training.

inhibition can be overcome by exogenous BDNF infusion closer in time. Interestingly, it has been recently shown that the blocking effect of Ani on LTP maintenance can also be reversed by BDNF administration (13). We have also demonstrated that persistent LTMs (i.e., those lasting at least 7 days) are associated with increased BDNF protein levels and ERK activation in the hippocampus 12 h after training, whereas rapidly decaying LTMs are not (see Figs. 2*B* and 3*B* and SI Fig. 4). This BDNF-induced

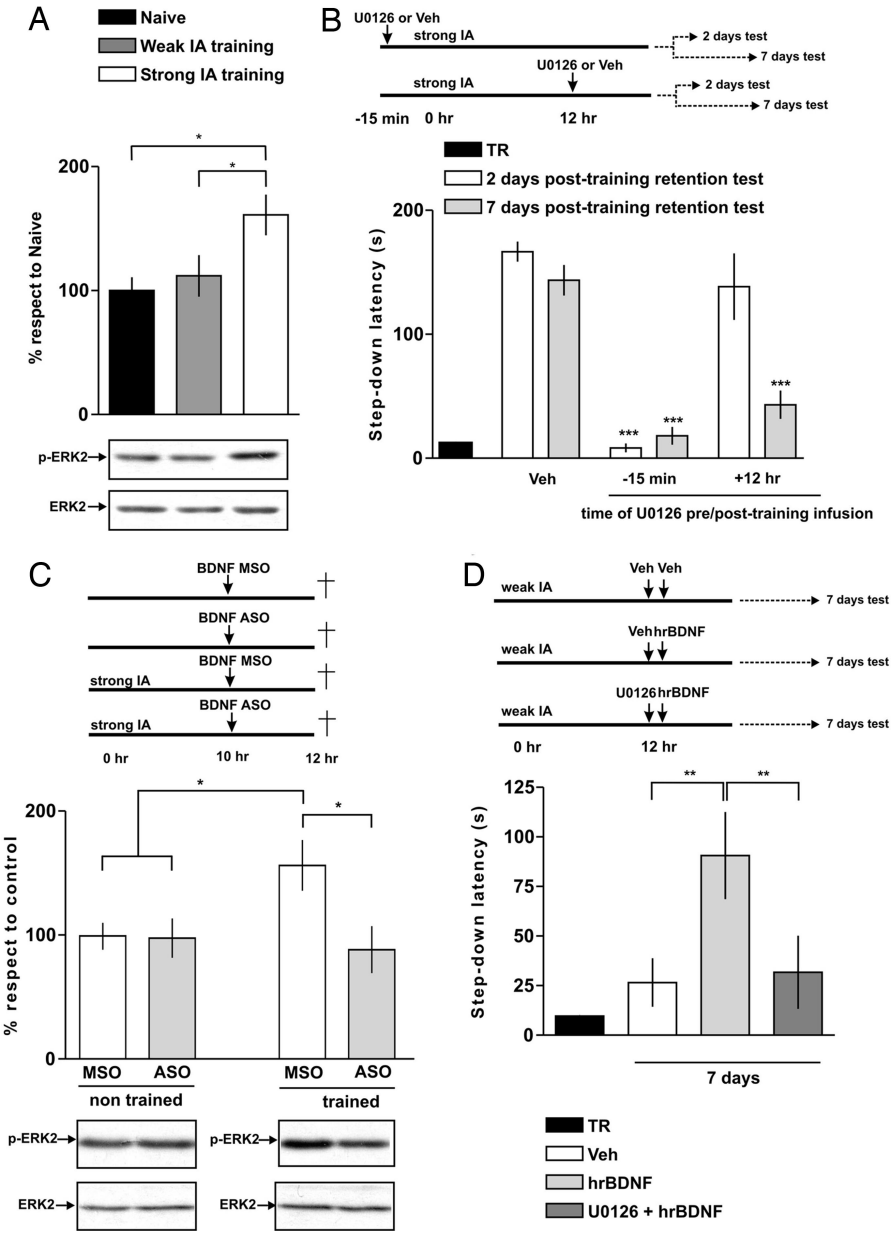

Fig. 3. ERK activation is necessary for BDNF induction of persistence of LTM storage. (*A*) (*Upper*) Strong, but not weak, IA training is associated with an increase in phospho-ERK2 (*p*-ERK2) in the dorsal hippocampus 12 h after training. Bars show normalized mean percentage level (±SEM) of *p*-ERK2 with respect to the naïve group. ***, $P < 0.05$ in Newman–Keuls test after ANOVA, $n = 6$. (*Lower*) Representative blots showing *p*-ERK2 and ERK2 levels. (*B*) U0126, but not Veh, infusion 12 h after strong IA training hindered memory at 7 days but left memory intact 2 days after training. Animals were infused into the dorsal hippocampus with U0126 (0.38 μg per side) or Veh 15 min before or 12 h after training. Data are expressed as mean ± SEM of training (TR, black bars) or test step-down latency, 2 (white bars) or 7 days (gray bars) after training. ***, $P < 0.001$ in Student's *t* test, $n = 8$–10. (*C*) (*Upper*) Intrahippocampal BDNF ASO infusion 10 h after training prevented the learning-associated increase in *p*-ERK2 12 h after training. Naïve or trained rats received bilateral infusions of BDNF MSO (white bars) or BDNF ASO (gray bars). Two hours later, the dorsal hippocampus was dissected out and used for Western blot analysis of *p*-ERK. Bars show the normalized mean percentage levels (±SEM) with respect to the naïve animals injected with MSO. *, $P < 0.05$ in Newman–Keuls test after ANOVA, $n = 5$. (*Lower*) Representative blots showing *p*-ERK2 and ERK2 levels. (*D*) ERK activation is required for BDNF induction of memory persistence. Intrahippocampal hrBDNF infusion 12 h after weak IA training induced persistence of LTM (hrBDNF, light gray) that was abolished by U0126 injection (0.38 μg per side) 15 min before (U0126+hrBDNF, dark gray). Data are expressed as mean ± SEM of training (TR, black bars) or test session step-down latency 7 days after IA training. **, $P < 0.01$, Student's *t* test, $n = 8$.

memory phase is essential for creating persistent LTM traces that last longer than 1 or 2 days. Administration of BDNF protein 12 h, but not 24 h, after training transforms a rapidly decaying memory trace into a persistent one, suggesting that the increase in hippocampal BDNF that occurs 12 h after training is critical to prevent memory loss. In this sense, memory maintenance might be the result of recurrent waves of protein expression, such as BDNF, either in the hippocampus or in other brain structures. Our results also point to the existence of an information processing phase specifically implicated in the generation of a persistent form of LTM. Just as there is a protein synthesis-independent STM that maintains the accessibility of memory while protein synthesis-dependent LTM is being formed, there seems to be a BDNF-dependent mechanism that mediates persistence of long-lasting LTMs. The mechanisms that trigger this BDNF-dependent phase remain to be elucidated. One possibility is that, during training, a

protracted gene expression and protein synthesis process that may last for several hours or days is switched on (28–30), first to consolidate and then to ensure LTM persistence.

Memory storage is thought to rely on structural modification of synaptic connections and neuronal growth (1–3). In agreement with this idea, BDNF increases the number of dendritic spines of CA1 pyramidal neurons (31). Consistently with findings showing that ERK 1/2 pathway regulates dendritic and spine morphology (21), both the effects of BDNF on spine morphogenesis (32) and LTM persistence are ERK-dependent (see Fig. 3*D*). It has also been shown that BDNF released by θ activity promotes actin polymerization in dendritic spines in the hippocampal CA1 region (33). Interestingly, a recent report shows that, after their initial appearance, dendritic spines form synapses within 15–19 h, close to the 12-h time point critical for persistence of LTM storage (34). In addition, deficits in BDNF and in ERK signaling are associated to memory impairment in the aging brain (35, 36). Age-related memory impairments have often been characterized in terms of rapid forgetting, in which aged animals have relatively comparable learning and memory on tests performed 24–48 h after training, but poor LTM retention at later times (6). Unraveling the molecular mechanisms involved in memory persistence may help to understand and treat the deficits in memory storage associated with normal and pathological aging.

Importantly, the present findings may bridge the concepts of cellular consolidation developed by McGaugh (2) and extensively studied over the years by his group and by many others (18, 22, 23), including ours (6, 17, 19, 29, 39), and that of systems consolidation, originally posited by Squire and his collaborators (40–44). Squire and his associates produced substantial evidence that there is a consolidation process that may last for weeks in rodents and for years in humans (40). The brain systems involved in these prolonged processes are different from those involved in early posttraining cellular consolidation and include, to a large extent, the neocortex (41–45). The findings here and elsewhere (12) that defined cellular and molecular mechanisms in the hippocampus may define whether a given memory will persist over just a few days or over at least a week in rats clearly suggests a link between consolidation processes lasting a few hours and consolidation processes that take more than that (45).

Materials and Methods

Subjects. Male Wistar rats (2.5 months old/220–250 g) from our own breeding colony were used. Animals were housed five to a cage at 23°C, with water and food ad libitum, under a 12-h light/dark cycle (lights on at 7:00 a.m.). The procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of the University of Buenos Aires and the Pontifical Catholic University of Rio Grande do Sul.

Surgery. Rats were implanted under thionembutal anesthesia with 22-g guide cannulae in the dorsal CA1 region of the hippocampus at coordinates A −4.3, L ± 3.0, V 1.4 of the atlas of Paxinos and Watson (37). Cannulae were fixed to the skull with dental acrylic.

Inhibitory Avoidance. After recovery from surgery, animals were handled once a day for 2 days and then trained in inhibitory avoidance (between 7:00 and 9:00 a.m.) as described (29). Briefly, the training apparatus was a 50 × 25 × 25-cm Plexiglas box with a 5-cm-high, 7-cm-wide, and 25-cm-long platform on the left end of a series of bronze bars that made up the floor of the box. For training, animals were placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, they received either a 3-s, 0.7-mA scrambled foot shock (strong training) or a 3-s, 0.35-mA scrambled foot shock (weak training). Memory retention was evaluated in a nonreinforced test session carried out 2, 4, or 7 days after training. All animals were tested only once.

Object Recognition. The task was conducted in a 60 × 40 × 50-cm box built of white and transparent acrylic. Before training, animals were habituated to the experimental arena by allowing them to freely explore it 20 min per day for 2 days in the absence of stimulus objects. The stimulus objects were made of metal, glass, or plastic. The role (familiar or novel) and the relative position of the objects were counterbalanced and randomly permuted for each experimental animal. In the training session, each animal was introduced for 5 min in the arena containing two identical objects. Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. The time spent exploring each object was registered by an observer blind to the treatment and expressed as percentage of the total exploration time. Twenty-four hours after training, animals were tested by reintroducing them in the arena for 5 minutes. One of the objects was familiar (meaning it had been presented the day before) and the other was novel. Exploration was registered as in the training session.

Drugs and Infusion Procedures. Oligonucleotides (ODN) (Genbiotech) were HPLC-purified phosphorothioate end-capped 18-mer sequences, resuspended in sterile saline to a concentration of 2 nmol/μl. BDNF antisense ODN (BDNF ASO), 5′-TCT TCC CCT TTT AAT GGT-3′; BDNF missense ODN (BDNF MSO), 5′-ATA CTT TCT GTT CTT GCC-3′. Both ODN sequences were subjected to a BLAST search on the National Center for Biotechnology Information BLAST server using the GenBank database. The ASO sequence had positive matches only for their target mRNA sequences and no other rat or human coding sequences. The control MSO sequence, which included the same 18 nt as the ASO but in a scrambled order, did not generate any full match to identified gene sequences in the database. Anisomycin (Ani) was purchased from Sigma. It was first dissolved in 3 M HCl, the pH adjusted to ≈7 with 3 M NaOH, further diluted to working concentration with saline and bilaterally infused at a dose of 80 μg per side. Human recombinant BDNF (Alomone) was dissolved in sterile saline and injected at a dose of 0.25 μg per side. U0126 was purchased form Cell Signaling Technology and injected at a final concentration of 0.38 μg per side in 10% DMSO in saline. Rapamycin was purchased from Sigma, dissolved in 0.1% DMSO, and infused at a dose of 60 nM per side. Ly294002 was purchased from Cell Signaling Technology, dissolved in 10% DMSO, and infused at a dose of 5 mM per side. U-73122 was purchased from Sigma; it was first dissolved in DMSO and then diluted in saline to working concentration (final concentration of DMSO, 8%). U-73122 was injected at a dose of 0.05 μg per side.

Histological Analysis. After the behavioral procedures, rats received an overdose of thionembutal and were perfused transcardially with 0.9% saline and 4% paraformaldehyde. Brains were removed and placed in 10% buffered formalin containing 30% sucrose. For analysis of ODN spread, rats were injected with 2 nmol of biotinylated BDNF antisense ODN (BDNF ASO) and 2 h later were anesthetized and perfused with 4% paraformaldehyde. The brains were isolated and sliced, and ASO was detected by avidin–biotin staining. By 2 h, ASO diffused throughout the dorsal hippocampus and slightly into the overlying cortex. For analysis of BDNF delivery and spread, rats were injected with 0.25 μg of hrBDNF and 15 min later were anesthetized and perfused with 4% paraformaldehyde. The brains were isolated and sliced, and BDNF was detected by immunohistochemistry using highly diluted primary BDNF antibody (1:20,000; Santa Cruz Biotechnology) to minimize endogenous BDNF staining. BDNF did not diffuse >1.7 mm^3 beyond the injection site (data not shown). Examination of cannulae placement was performed as described (29). Briefly, 24 h after the end of the behavioral procedures, 0.8 μl of 4% methylene blue in saline was infused as indicated above. Animals were killed by decapitation 15 min later and the brains stored in formalin for histological localization of the infusion sites. Infusions spread with a radius of <1.7 mm^3 and were found to be correct (i.e., within 1.5 mm^3 of the intended site) in 93% of the animals. Only data from animals with cannulae located in the intended sites were included in the final analysis.

Immunoblot Assays. Tissue was homogenized in ice-chilled buffer (20 mM Tris·HCl (pH 7.4), 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 10 μg/ml bacitracin, 10 μg/ml pepstatin, 15 μg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate). Aliquots were subjected to SDS/PAGE under reducing conditions. Proteins were electrotransferred onto PDVF membranes for 2 h at 100 V at 4°C for BDNF analysis. For ERK1/2, Akt, and mTOR analysis, proteins were transferred onto PVDF membranes overnight at 4°C. Immunoblots were performed by incubating membranes with anti-BDNF (1:1,000), anti-phospho-ERK1/2 antibody (1:3,000), anti-ERK 1/2 (1:4,000), anti-phospho-Akt (1:2,000), anti-Akt (1:2,000), anti-phospho-mTOR (1:2,000), or anti-mTOR (1:2,000). All antibod-

ies were from Santa Cruz Biotechnology or from Cell Signaling Technology. Densitometry analysis was performed with an MCID Image Analysis System (version 5.02; Imaging Research).

Data Analysis. Behavioral data were analyzed by unpaired Student's *t* test or ANOVA, followed by Newman–Keuls or Bonferroni post hoc tests. Immunoblot data were analyzed by unpaired two-tailed Student's *t* test or ANOVA, followed by Newman–Keuls multiple comparison test.

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