HIPPOCAMPUS 12:551–560 (2002)

BDNF–Triggered Events in the Rat Hippocampus Are Required for Both Short- and Long-Term Memory Formation

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ABSTRACT: Information storage in the brain is a temporally graded process involving different memory types or phases. It has been assumed for over a century that one or more short-term memory (STM) processes are involved in processing new information while long-term memory (LTM) is being formed. Because brain-derived neutrophic factor (BDNF) modulates both short-term synaptic function and activity-dependent synaptic plasticity in the adult hippocampus, we examined the role of BDNF in STM and LTM formation of a hippocampal-dependent one-trial fear-motivated learning task in rats. Using a competitive RT-PCR quantitation method, we found that inhibitory avoidance training is associated with a rapid and transient increase in BDNF mRNA expression in the hippocampus. Bilateral infusions of function-blocking anti-BDNF antibody into the CA_1 region of the dorsal hippocampus decreased extracellular signal–regulated kinase 2 (ERK2) activation and impaired STM retention scores. Inhibition of ERK1/2 activation by PD098059 produced similar effects. In contrast, intrahippocampal administration of recombinant human BDNF increased ERK1/2 activation and facilitated STM. The infusion of anti-BDNF antibody impaired LTM when given 15 min before or 1 and 4 hr after training, but not at 0 or 6 hr posttraining, indicating that two hippocampal BDNF-sensitive time windows are critical for LTM formation. At the same time points, PD098059 produced no LTM deficits. Thus, our results indicate that endogenous BDNF is required for both STM and LTM formation of an inhibitory avoidance learning. Additionally, they suggest that this requirement involves ERK1/2-dependent and -independent mechanisms. ***Hippocampus 2002;12:551–560.*** **© 2002 Wiley-Liss, Inc.**

KEY WORDS: endogenous BDNF; anti-BDNF antibody; memory consolidation; inhibitory avoidance learning; ERK1/2

INTRODUCTION

Brain-derived neutrophic factor (BDNF) is a small dimeric protein, structurally related to nerve growth factor, which is widely expressed in the adult mammalian brain (Murer et al., 2001). Classically, BDNF regulates the long-term survival and differentiation of specific populations of neurons in the developing and adult brain, and it is involved in activity-dependent modulation of dendritic and axonal growth (Lu and Chow, 1999; McAllister et al., 1999; Poo, 2001).

A growing body of evidence indicates that BDNF regulates both short-term synaptic function and long-term activity-dependent synaptic plasticity (Korte et al., 1998; Li et al., 1998; Rutherford et al., 1998; Kafitz et al., 1999; McAllister et al., 1999; Poo, 2001). It has been shown that BDNF acutely modulates both pre- and postsynatic aspects of synaptic transmission (Poo, 2001) and it is crucially involved in long-term potentation (LTP) (Figurov et al., 1996; Kang et al., 1997; Korte et al., 1998; Chen et al., 1999; Poo, 2001).

The finding that BDNF plays an important role in the induction and/or maintenance of hippocampal LTP and that CREB, a transcription factor involved in long-term memory (LTM) formation, is a major mediator of neu-

Grant sponsors: the National Research Council of Brazil (CNPq) through the National Program for Nuclei of Excellence (PRONEX) Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and the Ministry of Health of Argentina.
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Accepted for publication 23 October 2001
DOI 10.1002/hipo.10035

ronal BDNF responses (Finkbeiner et al., 1997), fueled the study of the role of this neurotrophin in learning and memory.

Memory is not acquired in its definitive form. It is a temporally graded process during which new information becomes consolidated and stored (McGaugh, 1966, 2000; Izquierdo and Medina, 1997; Milner et al., 1998). From mollusks to mammals, memory can be divided into at least two phases: a protein and RNA synthesis-independent phase that lasts minutes to 1–3 hr (short-term memory, or STM), and a protein and RNA synthesis-dependent phase that lasts several hours to days, weeks, or even longer periods (LTM) (McGaugh, 1966, 2000; Davis and Squire, 1984; Emptage and Carew, 1993; Izquierdo et al., 1998).

In this regard, intracerebral pretraining administration of antisense BDNF oligonucleotides impaired LTM formation in spatial (Mizuno et al., 2000) and avoidance learning tasks (Ma et al., 1998; Johnston and Rose, 2001). Pretraining infusion of BDNF antibodies caused amnesia for a spatial task (Mu et al., 1999) in rats and for an inhibitory avoidance in day-old chicks (Johnston et al., 1999). Transgenic mice overexpressing BDNF showed deficits in LTM (Croll et al., 1999). In contrast, BDNF mutant mice exhibited learning, but not memory retention, deficits of a multitrial spatial task (Linnarsson et al., 1997). In addition, acute or chronic intracerebral administration of BDNF produced no effects on memory retention (Fischer et al., 1994; Pelleymounter et al., 1996; Johnston et al., 1999; Cirulli et al., 2000). In spite of these inconsistencies, there is a general consensus that BDNF participates in learning and memory processes. However, the requirement of endogenous BDNF and its underlying signaling pathways in both STM and LTM formation remains largely unknown.

Therefore, the aim of the present study was to examine whether endogenous BDNF in the hippocampus is required for STM and LTM processing of a one-trial inhibitory avoidance training in rats. In addition, we determined whether avoidance training is associated with changes in the level of BDNF mRNA and protein in the hippocampus.

MATERIALS AND METHODS

Subjects

Male Wistar rats (2–3 months; weight, 160–250 g) from our own breeding stock were used. The animals were housed in plastic cages with water and food available ad libitum, under a 12-hr light/dark cycle (light on at 7:00 A.M.) at a constant temperature of 23°C.

Behavioral Procedure

Inhibitory avoidance was as follows (Bernabeu et al., 1997; Izquierdo et al., 1998). Rats were placed on a 2.5 cm high, 8.0 cm wide platform at left of a 50.0 × 25.0 × 25.0 cm yellow acrylic training apparatus, whose floor was a series of parallel 0.2 cm caliber bronze bars spaced 1.0 cm apart. Latency to step down onto the grid with all four paws was measured. In the training trial, immediately after this, the animals received a 0.4 mA, 4.0 sec scrambled foot shock. In the test session performed 1.5 hr (STM) and 24 hr (LTM) after training, the procedures were similar except that foot shock was omitted.

Surgery and Infusion Procedures

Male Wistar rats were implanted under deep thionembutal anesthesia with 30 gauge guide canulas in the dorsal CA_1 region of the hippocampus at the coordinates of the atlas Paxinos and Watson (1986): anterior, −4.3; lateral, ±4.0; ventral, −2.4. The cannulae were fixed to the skull with dental acrylic (Bernabeu et al., 1997; Izquierdo et al., 1998). After recovery from the surgery, these animals were trained in inhibitory avoidance and tested 1.5 hr and 24 hr later. Cannulated rats received, 15 min prior to training or immediately, 1, 4, or 6 hr after training, a bilateral infusion of saline or recombinant human BDNF (0.25 μg/side; Promega, Madison, Wi) or function-blocking anti-BDNF antibody (0.5 μg/side; Chemicon, Temecula, CA; AB1513P) or PD098059 (50 μM; RBI, Natick, MA), a specific extracellular signal–regulated kinase (MEK1/2) inhibitor. Additional groups received, 15 min prior to training or 4 hr after training, a control IgG (2.5 μg/side; Sigma, St. Louis, MO). Infusions were in all cases bilateral and had a volume of 0.8 μl (except for experiment with PD098059, where it had 0.5 μl). The entire infusion procedure took around 2 min, including 45 sec for the infusions themselves, first on one side and then on the other, and the handling. Histological examination of cannulae placements was performed as described previously (Bernabeu et al., 1997; Izquierdo et al., 1998). Briefly, 24 hr after the end of the behavioral procedures, 0.8 μl of a solution of 4% methylene blue in saline was infused as indicated above into each implanted site. Animals were killed by decapitation 15 min later and the brains were stored in formalin for histological localization of the infusion sites. Infusions spread with a radius of less than 1.0 mm^3, as described before (Bernabeu et al., 1997; Vianna et al., 1999; Walz et al., 1999), and were found to be correct (i.e., within 1.5 mm^3 of the intended site) in 95% of the animals. Only the behavioral data from animals with the cannula located in the intended site were included in the final analysis.

Biochemical Procedures

The rest of the animals were divided in three experimental groups: animals withdrawn from their home cages at the same time points used to sacrifice the other two groups and killed immediately (naive group, N); animals trained in inhibitory avoidance task and killed at different times after training (trained group, T); and animals submitted to a identical foot shock used for avoidance training and killed at different times after the procedure (shocked group, S). After animals were killed, the brain were immediately removed, hippocampi were dissected out, frozen on dry ice, and stored at −70°C until used.

Enzyme-Linked Immunosorbent Assay (ELISA)

Total BDNF-like immunoreactivity was measured by a two-site ELISA kit (Promega, G6981) according to the manufacturer's protocol. Samples were weighted and then homogenized in 20 vol of

lysis buffer (137 mM NaCl, 20 mM Tris-base, 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate, pH 7.4). Cell extract was centrifuged for 20 min at 15,000 g to remove particulates. Tissue samples were acidified for 15 min and neutralized because this has been reported to aid dissociation of bound trophic factors from their receptors (Okragly and Hakk-Frendscho, 1997). Protein was assayed by the Bradford method and BDNF values were expressed as pg of BDNF/mg of total protein. BDNF concentrations for hippocampal cell extracts were calculated from regression analysis of human recombinant BDNF standard curve (diluted in homogenization buffer) run in each assay.

Competitive RT-PCR

The RNA from hippocampus was extracted using a guanidine method modified from Chomczynski and Sacchi (1987). Briefly, tissues were homogenized in 1 ml of solution containing 453 μl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), 50 μl of 2 M sodium acetate (pH 4), and 500 μl of phenol saturated with water. One μg of poli-inosinic acid was added as carrier for the subsequent precipitation. Then, the mixture was extracted with 100 μl of chloroform-isoamylalcohol mixture (49:1). The aqueous phase was precipitated with 500 μl of isopropanol and resuspended in 40 μl of RNAse-free water; 5 μl of the total RNA were treated with RQ RNAse-free DNAse (Gibco-BRL, Rockville, MD) according to manufacturer's protocol. Finally, the total DNAse-treated RNA was reverse-transcribed according to the manufacturer's protocol (Superscript II RT kit, Gibco-BRL) using oligo-dT(12–18) (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) as primers.

The multicompetitor standard pRat6 (Pitossi and Besedovsky, 1996) was linearized with *Eco*RI and quantified. 100 fg of the competitor were coamplified with 8 ng (β2), 160 ng (BDNF), 640 ng (IL-1β), or 80 ng (NT3) of the total cDNA from hippocampus in a 50 μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each of the sense and antisense primers, 200 μM each dNTP, and 2.5 units Taq polymerase (Gibco-BRL). The primers used were β2 sense primer (5′-TCTTTCTGGTGCTTGTCTC-3′), β2 antisense primer (5′-AGTGTGAGCCAGGATGTAG-3′), BDNF sense primer (5′-TCACAGTCCTGGAGAAAGTC-3′), BDNF antisense primer (5′-ATGAACCGCCAGCCAATTCT-3′), IL-1β sense primer (5′-TCCATGAGCTTTGTACAAGG-3′), IL-1β antisense primer (5′-GGTGCTGATGTACCAGTTGG-3′), NT3 sense primer (5′-ACGAGGTGTAAAGAAGCCAG-3′), and NT3 antisense primer (5′-TGGGGACAGATGCCAATTCA-3′). β2-microglobulin and IL-1β PCRs were performed in a PTC-200 thermocycler (MJ Research, Watertown, MA) as follows: 2.5 min denaturation at 94°C, followed by 30 cycles of 45 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 3 min at 72°C. Similar PCR was performed for BDNF and NT3, during 42 cycles with an annealing temperature of 60°C. The expected sizes for target and standard amplification products are 241 and 480 bp for β2-microglobulin, 237 and 480 bp for IL-1β, 202 and 480 bp for BDNF, and 214 and 480 bp for NT3.

Amplicons were separated by 1.5% agarose gel electrophoresis containing 5 μg/μl ethidium bromide for 1 hr at 7.5 V/cm. Bands were visualized by excitation at 316 nm and digitalized with a frame grabber (White/UV transilluminator; UVP, Cambridge, U.K.). The bands were quantified with the software Gelworks 1D Intermediate version 3.01 (UVP).

Because of differences in the amplification efficiencies of cDNA and competitor, reference curves were constructed using 100 fg of competitor and sequential dilutions of either rat spleen cDNA (β2 or IL-1β) or hippocampus cDNA (BDNF or NT3) (Alvarez et al., 2000). The logarithm of the cDNA/competitor ratio was lineally related to the logarithm of the cDNA dilution amplified, with a slope of 0.6351 for β2, 0.8006 for IL-1β, 0.6435 for BDNF, and 0.9511 for NT3. These slopes were used to correct the cDNA/competitor ratio, so that the values reported represent relative cDNA dilutions and permit a precise semiquantitative analysis (Alvarez et al., 2000). To sum up, for each measured molecule, the UV quantitation of the cDNA amplicon is normalized by the cf amplicon of the same tube and corrected using the reference curve. The result for BDNF, IL-1β, and NT3 measures are then divided by the quantitation of β2-microglobulin (which represents the amount of cDNA in the sample). For easy visualization, the values are expressed as a percentage in respect to the media of the naive animals.

Immunoblot Assays

To investigate whether intrahippocampal infusion of recombinant BDNF, anti-BDNF antibody, or PD098059 affects ERK1/2, AKT, and CAMKII activation, 3 mm thick slices were taken 5–7 min after the infusions from the area in which the infusion cannulae were placed. 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, 50 mM NaF, and 1 mM sodium orthovanadate). Samples of homogenates (16–30 μg of protein) were subjected to SDS-PAGE (10% gels), and immunoblots were performed as described previously (Cammarota et al., 2000; Viola et al., 2000). Membranes were incubated with the following antibodies: anti-ERK1 and -ERK2 (1:3,000; New England Biolabs, Beverly, MA), anti–activated ERK1 and ERK2 (1:4,000; New England Biolabs), anti–activated AKT (1/2,000; New England Biolabs), and anti–activated CAMKII (1:5,000; Promega). Densitometric analysis of the films was performed by using an MCID Image Analysis System (version 5.02; Imaging Research, St. Catharines, Ontario, Canada). Western blots were developed to be linear in the range used for densitometry.

Data Analysis

Statistical analysis was performed by one-way ANOVA using the Dunnet's or Newman-Keuls tests. Mann-Whitney U-test was used for nonparametric analysis after Kruskal-Wallis test.

RESULTS

Rapid and Transient Induction of BDNF mRNA in Hippocampus During Avoidance Training

To investigate the role of hippocampal BDNF in memory formation, we first determined if learning of a one-trial inhibitory avoidance task is associated with an induction of BDNF mRNA expression. This fear-motivated learning is an hippocampal-dependent task that is acquired in a single and brief training session (Winocur and Bindra, 1976; Thompson, 1977; Gold, 1986; Lorenzini et al., 1996; Taubenfeld et al., 1999), which makes it ideal for investigating the role of signaling pathways initiated during memory processing without the interference from retrieval of the learned behavior that might occur in multitrial tasks (Izquierdo and Medina, 1997).

By using a competitive RT-PCR quantitation method, we found that BDNF mRNA levels in the hippocampus increased by 90% 1 hr after avoidance training ($P < 0.05$; n = 5; Fig. 1A). No changes were seen in shocked animals, indicating that BDNF mRNA upregulation is associated with the acquisition of the stepping down–shock association and could not be explained by the effect of shock alone. To evaluate whether this change is selective for BDNF mRNA, hippocampal IL-1β and NT3 mRNA levels were also measured. No significant changes were observed in IL-1β or NT3 mRNA expression in trained rats as compared to control animals (IL1β: trained, 100% ± 25%; shocked, 156% ± 38%; NT3: trained, 127% ± 18%; shocked, 143% ± 42% with respect to control values; $P > 0.05$; n = 4–8).

To determine the time course of the training-associated increase in hippocampal BDNF mRNA expression, we next assayed BDNF mRNA levels at different time points after avoidance training. No changes between groups were found in hippocampal BDNF mRNA levels at 2–9 hr after training (Fig. 1B). Only a nonsignificant decrease (−60%) was found in both the trained and shocked groups at the 6 hr time point. The amount of IL-1β mRNA was not altered between 1 and 6 hr after training (data not shown). Therefore, these findings indicate that avoidance learning resulted in a rapid, selective, and transient induction of BDNF mRNA expression in the hippocampus.

Despite some previous works showing induction of BDNF mRNA expression following different learning tasks (Ma et al., 1998; Hall et al., 2000; Mizuro et al., 2000; Tokuyama et al., 2000), so far no information is available concerning induction of BDNF protein. To test whether avoidance training–associated induction of BDNF mRNA is accompanied by changes in BDNF protein, we determined hippocampal BDNF endogenous levels at different time points after training. As shown in Figure 1C, a slight decrease in the amount of BDNF protein was found 6 hr after training (−30%; $P < 0.05$; n = 10) with respect to control values. However, we also observed a nonsignificant decrease in shocked group (−20%; $P > 0.05$; n = 10) at the same time point. These results suggest that the reduction in BDNF protein and mRNA levels is probably associated with the foot shock–aversive experience.

No differences between groups were seen in BDNF protein at the other time points. It is interesting to note here that, with remarkable parallel to the present findings, long-term potentiation-induced increase in BDNF mRNA expression (Dragunow et al., 1993) is not accompanied by changes in BDNF protein levels in the rat hippocampus (Walton et al., 1999).

Hippocampal BNDF Is Required for Short-Term Memory Formation

Although these experiments demonstrated that hippocampal BDNF mRNA is induced specifically during inhibitory avoidance learning, they do not address the question of whether endogenous BDNF is necessary for the acquisition and consolidation of this behavioral training.

We therefore assessed whether neutralizing endogenous BDNF biological activity by delivering function-blocking anti-BDNF antibodies (Rasika et al., 1999) into the hippocampus (Fig. 2) blocks memory formation. Given that BDNF in the hippocampus has both short- and long-term synaptic plasticity actions (Chen et al., 1999; Kafitz et al., 1999; Lu and Chow 1999; Poo, 2001), we first examined the effects of BDNF antibodies on short-term memory of the avoidance training as determined by the retention performance in a test session carried out at 1.5 hr after training (Izquierdo et al., 1998).

As shown in Figure 3A, the infusion of neutralizing BDNF antibodies (0.5 μg/side) into the CA_1 region of the dorsal hippocampus 15 min prior to training blocks STM formation. No effects were seen when anti-BDNF antibody was given immediately or 1 hr after training, indicating that the amnesic effect is not attributable to a retrieval deficit, to a lingering effect on performance, or to more general effects on anxiety or motor activity. Furthermore, the intrahippocampal infusion of a control IgG (2.5 μg/side) produced no effect on retention performance [test session latency = 48.7 (21.1/180); n = 9; $P = 0.96$, Mann-Whitney U-test], indicating that the impairment of retention did not seem to be the result of a nonspecific protein-loading effect.

The infusion of recombinant human BDNF (0.25 μg/side) into the dorsal hippocampus 15 min before or immediately after training strongly facilitated STM retention (Fig. 3A). No effect was seen when BDNF was administered 1 hr posttraining. It is important to mention here that no differences between groups shown in Figure 3A were seen in training test session latencies ($P = 0.17$, Kruskal-Wallis test).

Given that BDNF stimulates, via activation of TrkB receptors, ERK1/2 cascade in hippocampal and cortical neurons (Marsh and Palfrey, 1996; Blanquet, 2000; Han and Holtzman, 2000; Cavanaugh et al., 2001), we next examined whether the infusion of recombinant BDNF or anti-BDNF antibody into the CA_1 region of the dorsal hippocampus alters ERK1/2 activation in microdissected samples of the dorsal hippocampus. Using immunoblot techniques to detect dually phosphorylated, activated ERK1/2, we found that the infusion of recombinant BDNF (0.25 μg/side) produced an increase in the phosphorylation state of ERK1/2 (ERK2: +90%, $P < 0.001$; ERK1: +190%, $P < 0.001$; n = 4) and that the administration of anti-BDNF antibody (0.5 μg/side)

decreased ERK2, but not ERK1, activation (−45%; $P < 0.05$; n = 5; Fig. 3B). When blots were stripped and reprobed using an antibody that recognizes ERK1/2 independently of their phosphorylation states (total ERK1/2), no changes were observed (Fig. 3B). These findings suggest that ERK1/2 is a downstream component of the signaling pathway mediating the effect of endogenous BDNF in STM formation.

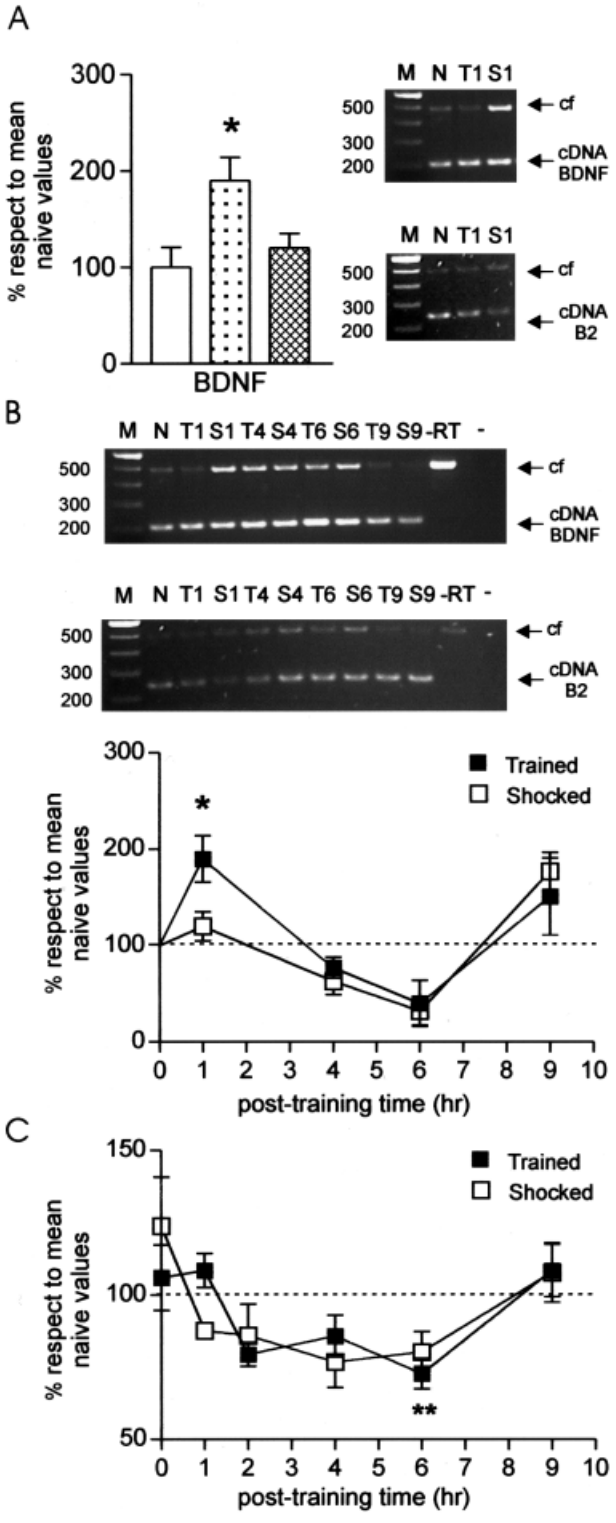

FIGURE 1. Rapid and transient induction of BDNF mRNA in the hippocampus during avoidance training. A: Representative competitive RT-PCR for BDNF and β2 cDNA and densitometric analysis, 1 hr after training, in trained (dotted bars) and shocked (hatched bars) groups with respect to naive animals (open bars). Data are expressed as mean ± SEM percentage of mean naive values (naive: 380 ± 81, arbitrary units; n = 5-8). Asterisk: $P < 0.05$ with respect to naive and shocked groups, Newman Keuls test. B: Representative competitive RT-PCR for BDNF and β2 cDNA and densitometric analysis of competitive RT-PCR time course of BDNF mRNA expression after training. Data are expressed as mean ± SEM percentage of mean naive values (n = 4-8). Asterisk: $P < 0.05$ with respect to naive and shocked groups, Newman Keuls test. M: molecular weight; N: naive; T1: trained 1 hr; S1: shocked 1 hr; T4: trained 4 hr; S4: shocked 4 hr; T6: trained 6 hr; S6: shocked 6 hr; T9: trained 9 hr; S9: shocked 9 hr; −RT: control without reverse transcription; −: negative control; cf: competition fragment. C: Time course of BDNF protein after training. Data are expressed as mean ± SEM percentage of mean naive values (naive: 96 ± 8 pg of BDNF protein/mg of total protein; n = 5-10). Double asterisk: $P < 0.01$ with respect to naive values, Dunnet's test.

FIGURE 2. Schematic drawing of plane AP: Bregma-4.3 mm of Paxinos and Watson (1986), showing (shaded) the extent of the area reached by the infusion in the dorsal hippocampus.

To examine this assumption further, we next determined whether inhibiting MEK1, the specific protein kinase that phosphorylates and activates ERK1/2, blocks STM formation. The bilateral infusion of the specific MEK1/2 inhibitor PD098059 (50 μM/side) into the CA_1 region of the dorsal hippocampus 15 min prior to or immediately after training caused amnesia in the test session performed 1.5 hr after training (Fig. 3C). Importantly, PD098059 has no effect on memory retention when given 1 hr after training, indicating that PD098059 infusions did not produce a nonspecific impairment of retrieval.

To confirm that the dose of PD098059 used in these experiments effectively inhibits ERK1/2 activation in vivo, the phosphorylation levels of ERK1/2 were measured in samples of the dorsal hippocampus of rats administered with saline or PD098059 5–7 min before. As shown in Figure 3B, PD098059 (50 μM/side) caused a significant reduction in ERK2 activation (−62%; $P < 0.01$; n = 5). No changes were observed in total ERK1/2 (Fig. 3B). It is important to note here that PD098059 had no effect on CAMKII or AKT/PKB activation (n = 4; data not shown). These findings imply that this drug delivered into the dorsal hippocampus modulate specifically the activation of ERK1/2 in vivo.

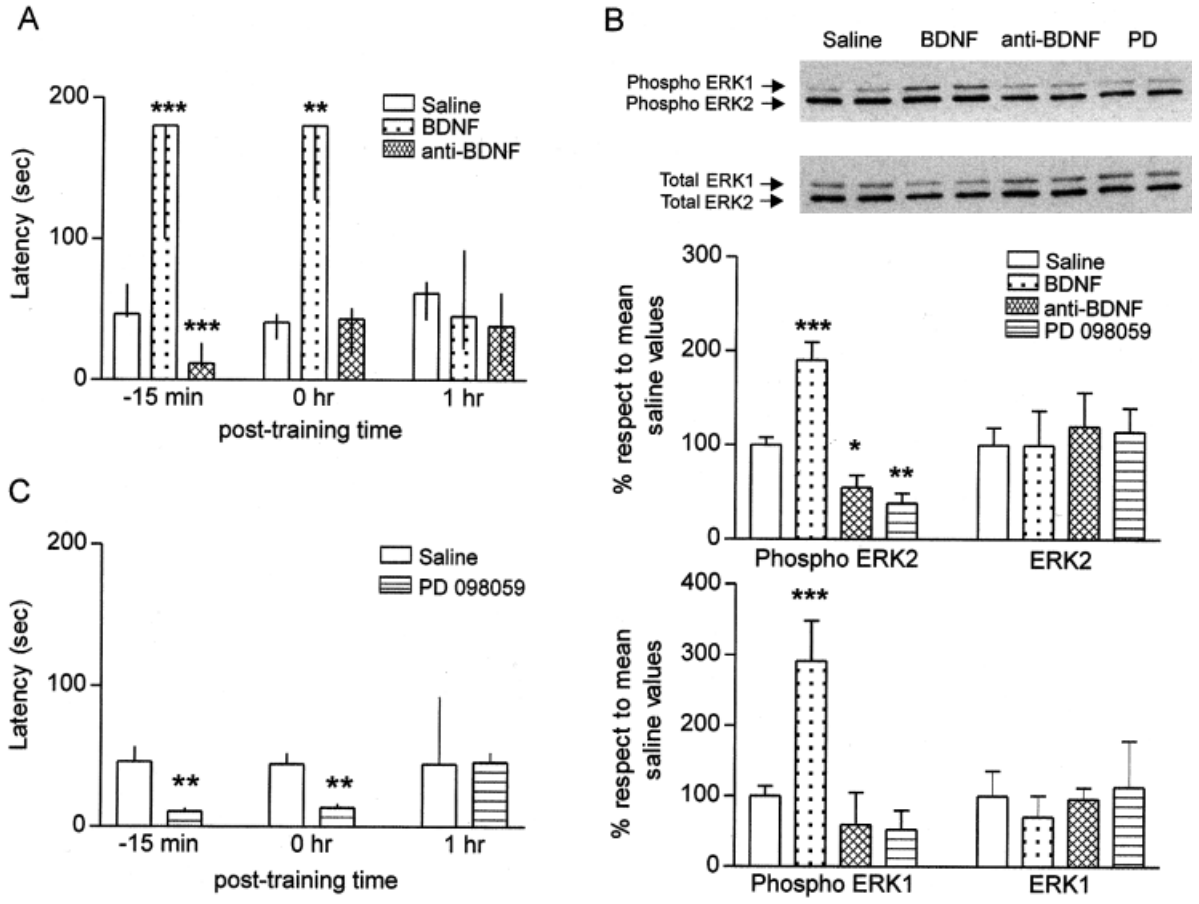

FIGURE 3. Hippocampal BNDF and ERK1/2 are required for short-term memory formation. A: Medians (interquartile range) of latencies to step down from the platform of the inhibitory avoidance box in the test session 1.5 hr after training. Animals were infused with saline or BDNF (0.25 μg/side) or anti-BDNF (0.5 μg/side), 15 min prior to or immediately or 1 hr after avoidance training. B: Representative Western blots and densitometric analysis of the data using anti-activated ERK1/2 (phospho ERK1/2) and anti-ERK1/2 (total ERK1/2) in the area of the dorsal hippocampus in which the infusion cannulae were placed, 5–7 min after animals were injected with saline or BDNF (0.25 μg/side) or anti-BDNF (0.5 μg/side) or PD098059 (50 μM). Data are expressed as mean ± SEM percentage of mean saline values (Phospho ERK2: 772.4 ± 60.2; total ERK2: 811.1 ± 151.3; Phospho ERK1: 41.3 ± 5.9; total ERK1: 73.1 ± 26.8 in arbitrary unit). Triple asterisk: $P < 0.001$; double-asterisk: $P < 0.01$; single asterisk: $P < 0.05$ with respect to mean saline values, Newman-Keuls test (n = 4–5). C: Same as in A but here we infused PD098059 (50 μM). In A and C, triple asterisk: $P < 0.001$; double asterisk: $P < 0.01$ vs. saline group, Mann-Whitney U-test (n = 8–10 per group).

Taken together, the present results demonstrate that the BDNF/ERK1/2 signaling pathway in the hippocampus is required for STM formation during a narrow time window around the time of training.

Hippocampal BDNF Is Also Required for Long-Term Memory Formation

Given that BDNF elicits long-term synaptic plasticity changes (Cohen-Cory, 1999; Lu and Chow, 1999; McAllister et al., 1999; Poo, 2001) and regulates gene expression required for neuronal survival, differentiation, and connectivity (Murer et al., 2001; Poo, 2001), and that cAMP response element-binding protein (CREB), which is crucially involved in the formation of LTM (Yin and Tully, 1996, Silva et al., 1998; Viola et al., 2000), is a major mediator of BDNF responses (Finkbeiner et al., 1997), we next determined the role of hippocampal BDNF on LTM formation. Function-blocking anti-BDNF antibody was administered into the CA_1 region of the dorsal hippocampus before, or after, avoidance training and rats were tested for retention 24 hr later.

As shown in Figure 4A, infusion of anti-BDNF antibody (0.5 μg/side) 15 min prior to, or 1 and 4 hr after training caused a clear-cut LTM retention deficit. On the other hand, when administered immediately or 6 hr after, anti-BDNF antibody had no effect on retention test performance. These findings indicate that two hippocampal BDNF-sensitive time periods are critical for inhibitory avoidance LTM retention. It is noteworthy that these two time windows closely parallel those found for the amnesic action of protein synthesis inhibitors in several one-trial learning tasks, including inhibitory avoidance (Freeman et al., 1995; Bourtchuladze et al., 1998; Quevedo et al., 1999).

No effect on LTM retention scores was observed if a control IgG (2.5 μg/side) was infused 15 min prior to training [test session

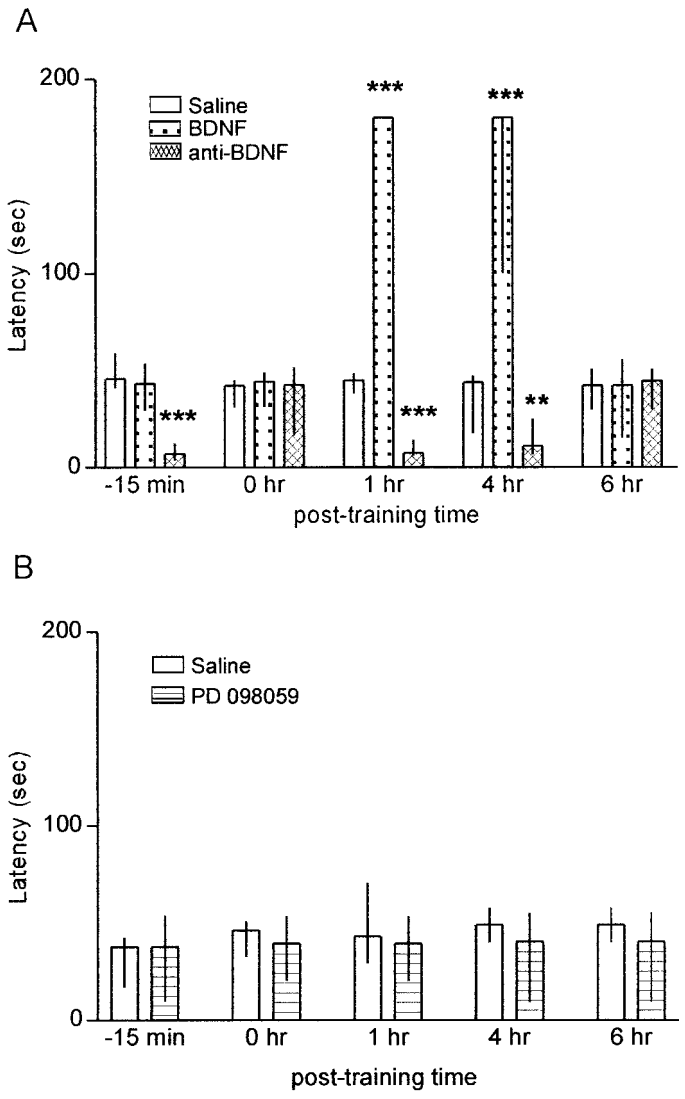

FIGURE 4. Hippocampal BDNF is also required for long-term memory formation. A: Medians (interquartile range) of latencies to step down from the platform of the inhibitory avoidance box in the test session 24 hr after training. Animals were infused with saline, BDNF (0.25 μg/side), or anti-BDNF (0.5 μg/side), 15 min prior to or immediately or 1, 4, or 6 hr after avoidance training. B: Same as in A but here PD098059 (50 μM) was infused. In A and B, triple asterisk: $P < 0.001$; double asterisk: $P < 0.01$ vs. saline group, Mann-Whitney U-test (n = 8–10 per group).

latency: 45.3 (15.4/117.3); n = 9; $P = 0.89$, Mann-Whitney U-test] or 4 hr after training [test session latency: 48.6 (18.3/85.5); n = 8; $P = 0.697$, Mann-Whitney U-test]. Also, no differences between groups shown in Figure 4A were seen in training test session latencies ($P = 0.338$, Kruskal-Wallis test).

Infusion of the recombinant BDNF (0.25 μg/side) into the CA_1 region of the dorsal hippocampus at 1 or 4 hr, but not immediately or 6 hr, after training facilitated LTM retention (Fig. 4A). BDNF has no effect when given 15 min prior to training, supporting the idea that BDNF effects are specific on LTM processing but not on acquisition.

To examine whether the activation of hippocampal ERK1/2 mediates endogenous BDNF-triggered events for LTM formation, we studied the effect of PD098059 (50 μM/side) infused into the CA_1 region at the same time points used for anti-BDNF antibody (Fig. 4A). Unexpectedly, the inhibition of ERK1/2 activation produced no LTM retention deficits (Fig. 4B), indicating that BDNF-triggered events critical for LTM formation appears not to involve the ERK1/2 cascade.

DISCUSSION

The main finding of the present study is that endogenous BDNF in the hippocampus is required for both STM and LTM formation of a one-trial fear-motivated learning task. This requirement involves ERK1/2-dependent and independent mechanisms.

We also found a facilitatory effect of recombinant BDNF on both short- and long-term memory retention scores. To our knowledge, this is the first report showing that a single infusion of exogenous BDNF into a selected brain region is able to improve memory performance in mammals. In addition, avoidance training results in a rapid, selective, and transient induction of BDNF mRNA in the hippocampus.

Our present results demonstrated that BDNF/ERK1/2 signaling pathway is required for STM formation of a hippocampal-dependent one-trial avoidance task. This is based on three series of data. First, the bilateral infusion of anti-BDNF antibody into the CA_1 region of the dorsal hippocampus prior to but not immediately or 1 hr after training impaired STM retention scores (Fig. 3A), indicating that the acquisition and/or consolidation, but not the retrieval, of STM of avoidance learning is affected. Interestingly, it has been previously reported that anti-BDNF antibody reduced hippocampal LTP when administered before but not 10, 30, or 60 min after theta-burst stimulation, indicating that endogenous BDNF is required for a limited time period only shortly before or around LTP induction (Chen et al., 1999). Second, infusions of recombinant BDNF, which facilitated STM retention performance (Fig. 3A), activated ERK1/2 (Fig. 3B) while infusions of anti-BDNF antibody decreased ERK2 activation (Fig. 3B). Third, PD098059-induced inhibition of ERK1/2 cascade (Fig. 3B) shortly before or immediately after training caused amnesia in the test session performed 1.5 hr after training (Fig. 3C). In this context, we recently demonstrated that ERK1/2 cascade is transiently activated immediately after inhibitory avoidance training (Alonso et al., 2001). Further studies will be needed to elucidate the involvement of several ERK1/2 substrates, such as the Kv4.2 potassium channel (Adams et al., 2000; Sweatt 2001), on STM formation.

Confirming and extending recent findings in day-old chicks (Johnston et al., 1999; Johnston and Rose, 2001), we found that endogenous BDNF is also required for LTM formation for a one-trial fear-motivated learning in rats (Fig. 4A). There are two time windows of anti-BDNF antibody-induced amnesia for avoidance

training: an early critical period around the time of training and a late critical period at 1–4 hr after training.

At least two different time windows for the amnesic effect of protein synthesis inhibitor anisomycin have been reported for fear-motivated learning in chicks (Freeman et al., 1995) and rats (Bourtchuladze et al., 1998; Quevedo et al., 1999), one around the time of training and the other 3–4 hr posttraining. Two similar periods were found for the amnesic action of PKA inhibitors in mice (Bourtchuladze et al., 1998) and rats (Vianna et al., 1999). In addition, we showed that following avoidance training, there are two periods of increased PKA activity and phosphoCREB immunoreactivity in CA_1 region, one immediately and another 3–6 hr after training (Bernabeu et al., 1997).

What are the BDNF-triggered signaling events required for LTM formation? Since BDNF activates ERK1/2 cascade and phosphorylates CREB in hippocampal and cortical neurons (Marsh and Palfrey, 1996; Xing et al., 1996; Finkbeiner et al., 1997; Han and Holtzman, 2000; Cavanaugh et al., 2001), a likely candidate is ERK1/2. Unexpectedly, our results demonstrated that the inhibition of hippocampal ERK1/2 at the same time points that are sensitive to anti-BDNF antibody did not impair LTM formation (Fig. 4B). Therefore, the ERK1/2 signaling cascade is not a major mediator of hippocampal BDNF in the formation of LTM for an avoidance learning. However, our findings do not rule out that the ERK1/2 cascade may participate in LTM formation at other time points (Atkins et al., 1998; Sweatt, 2001). Indeed, we have previously found that an NMDA glutamate receptor-dependent activation of ERK1/2 is required 3 hr after training for LTM consolidation (Walz et al., 1999; Cammarota et al., 2000). In this context, it is important to stress here that activation of ERK1/2 returned to control levels by 1 hr after the intrahippocampal infusion of PD098059 (Blum et al., 1999).

Given that BDNF rapidly enhances NMDA glutamate receptor responsiveness via postsynaptic TrkB receptors (Levine and Kolb, 2000; Poo, 2001), that BDNF-induced potentiation in hippocampal slices requires a transient increase in intracellular Ca^{2+} concentration (Kang and Schuman, 2000), and that blockade of hippocampal NMDA glutamate receptors at the time of training impaired LTM for inhibitory avoidance (Izquierdo and Medina, 1997), the possibility exists that NMDA glutamate receptors may be involved in the early critical period of anti-BDNF antibody-induced amnesia (Fig. 4A).

In this context, CAMKII/IV signaling pathways have also been reported to be associated with neuronal BDNF responses in the hippocampus (Blanquet and Lamour, 1997; Finkbeiner et al., 1997; Liu et al., 1999; Blanquet, 2000). A learning-specific increase in CAMKII activity was observed immediately after avoidance training (Cammarota et al., 1998) and we and others have previously shown that inhibition of CAMKII/IV at the time of training, but not later on, impaired LTM for inhibitory avoidance training (Wolfman et al., 1994; Tan and Liang, 1997). Taken together, these findings suggest that there is an early critical time period during which endogenous BDNF, probably via activation of NMDA glutamate receptor/CAMKII/IV signaling pathways, is required for LTM formation.

Given that BDNF stimulates CREB phosphorylation in CA_1 region via a CAMK-dependent mechanism (Finkbeiner et al., 1997) and that CREB-regulated transcription is considered a crucial step in LTM formation (Bourtchuladze et al., 1994; Yin and Tully, 1996; Guzowski and McGaugh, 1997; Izquierdo and Medina, 1997; Impey et al., 1998; Taubenfeld et al., 1999; McGaugh, 2000), our present results may suggest that endogenous BDNF in the hippocampus exerts its critical role in LTM formation, at least in part, via CREB activation.

In accordance with recent findings obtained in a contextual conditioning (Hall et al., 2000), one-trial inhibitory avoidance training resulted in a rapid and transient induction of BDNF mRNA expression in the hippocampus. Given that CREB activates BDNF transcription (Tao et al., 1998) and that avoidance training resulted in increased phosphorylation of CREB immediately after training in CA_1 region and dentate gyrus of the hippocampal formation (Bernabeu et al., 1997; Taubenfeld et al., 1999), it is tempting to suggest that CREB activation may participate in avoidance learning-induced increase in BDNF mRNA observed 1 hr after training. This induction of BDNF expression was not accompanied by rapid changes in the level of BDNF protein (Fig. 1C). Several works have shown that changes in BDNF mRNA and protein are not correlated (Nanda and Mack, 2000; Pollock et al., 2001). The lack of observed BDNF protein changes may reflect an increased turnover of BDNF protein or may be the lack of translation of the newly synthesized BDNF mRNA, suggesting that the BDNF gene is regulated at the level of translation as well as transcription. Alternatively, experience-dependent changes in protein BDNF trafficking may account for this discrepancy (Pollock et al., 2001). Evidence of anterograde/retrograde transport of BDNF protein in the CNS supports this hypothesis (Altar and DiStefano, 1998). We cannot rule out, however, the possibility that small BDNF protein changes occurred below the detectability level of the ELISA technique.

In contrast, we did observe a transient decrease in BDNF protein 6 hr after training in trained group and also similar nonsignificant decrease in shocked animals. At this time point, a marginal decrease in BDNF mRNA levels in both trained and shocked animals was observed (Fig. 1B). Previous work showed a decreased BDNF expression after corticosterone administration or exposure to stress (Smith et al., 1995; Schaaf et al., 2000). Therefore, the delayed training-associated reduction in BDNF protein may be due to the aversive component of the training.

In conclusion, our results suggest that endogenous BDNF in the hippocampus is required for STM formation via activation of ERK1/2 cascade and also plays a crucial role in long-lasting memories that is mainly independent of ERK1/2 signaling pathways.

Hippocampal BDNF appears to be necessary for LTM in two discrete time periods, the first around the time of training and the second 1–4 hr later, paralleling the periods sensitive to protein synthesis inhibitors found in different learning tasks. In addition, a rapid and transient BDNF mRNA upregulation in the hippocampus was found during memory formation.

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