Behavioral/Systems/Cognitive

Region-Specific Involvement of BDNF Secretion and Synthesis in Conditioned Taste Aversion Memory Formation

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Brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase receptor B (TrKB), play a critical role in activity-dependent plasticity processes such as long-term potentiation, learning, and memory. It has been shown that BDNF exerts different or even opposite effects on behavior depending on the neural circuit. However, the detailed role of BDNF in memory process on the basis of its location has not been fully understood. Here, we aim to investigate the regional specific involvement of BDNF/TrkB in hippocampal-independent conditioned taste aversion (CTA) memory processes. We found region-specific changes in BDNF expression during CTA learning. CTA conditioning induced increased BDNF levels in the central nuclei of amygdala (CeA) and insular cortex, but not in the basolateral amygdala (BLA) and ventromedial prefrontal cortex. Interestingly, we found that the enhanced TrkB phosphorylation occurred at the time point before the increased BDNF expression, suggesting rapid induction of activity-dependent BDNF secretion by CTA learning. Moreover, targeted infusion of BDNF antibodies or BDNF antisense oligonucleotides revealed that activity-dependent BDNF secretion and synthesis in the CeA, but not the BLA, was respectively involved in the short- and long-term memory formation of CTA. Finally, we found that infusion of exogenous BDNF into the CeA could enhance CTA learning. These data suggest that region-specific BDNF release and synthesis temporally regulate different CTA memory phases through activation of TrkB receptors.

Introduction

Brain-derived neurotrophic factor (BDNF), a molecule known to regulate neuronal survival and differentiation, plays a critical role in synaptic plasticity, long-term potentiation, learning, and memory (Barde et al., 1987; Leibrock et al., 1989; Huang and Reichardt, 2001; Chao, 2003). Increasing evidence indicates that inhibition of BDNF signaling via the tropomyosin-related kinase receptor B (TrkB) in the hippocampus or amygdala leads to impaired memory acquisition, retention, and/or recall in the water maze, fear-potentiated startle, and passive avoidance tests (Lin-narsson et al., 1997; Minichiello et al., 1999; Mu et al., 1999; Alonso et al., 2002; Rattiner et al., 2004). Despite the importance of BDNF/TrkB signaling in learning and memory, most studies are currently focused on the hippocampal- or amygdala-dependent memory processes, while reports focusing on the role of BDNF/TrkB in hippocampal-independent cortical learning are limited. Moreover, there is indeed considerable evidence showing that BDNF exerts different or even opposite effects on depression-like behaviors depending on the neural circuit (Eisch et al., 2003; Krystal and Duman, 2004; Berton and Nestler, 2006; Berton et al., 2006). However, the detailed role of BDNF in memory processes on the basis of its regional location has not been fully understood.

Studies have demonstrated that blocking BDNF function by delivering BDNF antisense oligonucleotide (ASO) or BDNF antibodies into hippocampus could impair long-term memory (LTM) in contextual fear conditioning or inhibitory avoidance tests (Alonso et al., 2002; Lee et al., 2004). However, there are conflicting reports about whether BDNF is involved in short-term memory (STM) formation (Alonso et al., 2002; Lee et al., 2004). Recently, the human BDNF Val66Met polymorphism, which leads to decreased activity-dependent BDNF secretion, was found to be associated with hippocampal-dependent episodic STM deficit (Egan et al., 2003; Chen et al., 2004, 2006). Together, it remains of interest to investigate the contribution of BDNF and its activity-dependent secretion in distinct memory process.

Conditioned taste aversion (CTA) is a form of learning where the subject associates a novel taste [termed the conditioned stimulus (CS)] with a subsequent transient visceral illness [termed the unconditioned stimulus (US)], and is an established model for studying the molecular mechanisms of nondeclarative memory in different brain regions. CTA is formed by single-trial training and is a long-lasting memory that provides a useful model for studying the different phases of memory, such as acquisition, consolidation, and retrieval. On the basis of the typical CTA paradigm, the brain regions involved in CTA formation should be
those where taste and general visceral information associate such as the insular cortex (IC), the amygdala, the parabrachial nucleus (PBN), and the nucleus of the solitary tract (Gallo et al., 1998; Berman et al., 2000). In addition, recent studies have reported that ventromedial prefrontal cortex (vmPFC) plays an important role in CTA memory (Mickley et al., 2005; Yu et al., 2009). In the present study, the regional specific involvement of BDNF secretion and synthesis in the CTA memory process is investigated.

Materials and Methods

Animals. Wistar rats (2-month-old males, 250–300 g) were caged individually at 22°C under 12 h light/dark cycles. Water and food were available ad libitum except when experimental requirements dictated otherwise. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee of Shandong University.

Behavioral procedures. The behavioral protocol of CTA was performed according to previously published protocols (Desmedt et al., 2003). For the CTA study, saccharin (0.1% w/v, sodium salt) was used as an unfamiliar taste (CS), and intraperitoneal injection of LiCl (0.15 M, 2% body weight) was the unconditioned stimulation (US). In brief, rats were deprived of water for 24 h and then given two pipettes containing 10 ml of water each for 10 min once a day. They were trained 3–4 d to get their daily water ration. On conditioning day, the rats were presented with two pipettes containing 5 ml of saccharin each for 10 min. Forty minutes later, they were injected with LiCl intraperitoneally. Four hours after conditioning, the rats were presented with a random array of six pipettes, three containing 5 ml of saccharin and three containing 3 ml of water for 10 min. The rats explored to consume or avoid the contents of the pipettes, and their liquid consumption was recorded to test CTA STM. On the next 2 d, rats were presented daily for 10 min with two pipettes containing 10 ml of water each. On the third day after conditioning, rats were presented with an array of six pipettes, three containing 5 ml of saccharin and three containing 3 ml of water, to test CTA LTM. Conditioned rats preferred a water/saccharin ratio of 9:1 on their STM and LTM tests in this multiple-choice situation, whereas unconditioned rats preferred saccharin to water. For the weak CTA conditioning, all rats were trained as above, except that on the conditioning day, rats received intraperitoneal injection of 0.05 ml LiCl (2% body weight) instead of 0.15 ml LiCl. The results were quantified by an aversion index (AI), defined as AI = ([milliliters of water]/[milliliters of water + milliliters of saccharin]) × 100% consumed in the test.

Quantitative real-time reverse transcriptase-PCR. After CTA conditioning, rats in the experimental group were killed at different time points after CS-US pairing (n = 5 per time point). Different from the CS-US paired group (CTA group), on the conditioning day the CS-alone group was given saccharin without LiCl injection before being killed, while the US-alone group received only an injection of LiCl. In addition, in accordance with a previous study showing that there was an absence of learning when the abdominal irritation (LiCl injection) was manipulated 24 h after taste (Gutiérrez et al., 2003), in the CS-US long (CS-US-L) group rats received LiCl injection 24 h after receiving saccharin. Last, a control group (n = 5) that underwent habituation was killed from their home cages in the animal housing facility. For the rats infused with BDNF antibodies or BDNF oligonucleotides (ODNs), at least five animals from each group were killed at 4 h after CTA conditioning.

Brains were removed after decapitation, and then coronal sections (1 mm thick) were obtained using a rat brain slicer (Braintrace Scientific). Five different brain regions from each rat [vmPFC, IC, basolateral amygdala (BLA), central nuclei of amygdala (CeA), and hippocampus] were punched according to the atlas (Paxinos and Watson, 1998) using a blunt-end 17 gauge syringe needle (1 mm inner diameter) and frozen in liquid nitrogen followed by RNA extraction. Total RNA was isolated using TRNzol-A RNA isolation reagent (catalog #DP4241–02, TIANGEN) according to the manufacturer’s instructions. A 0.5 µg aliquot of each sample was treated with DNase to avoid DNA contamination, and then was reversely transcribed using the RevertAid First Strand cDNA Synthesis Kit (catalog #K1621, Fermentas). Primer sequences used were as follows: BDNF forward primer: 5’ TAAATGAGGTTTATATAGCAG TAC ATG GGT TCT ACA 3’ and reverse primer: 5’ AGT TGTCGGGCAAATGCTT GTT T 3’ (Alfonso et al., 2006); nerve growth factor (NGF) forward primer: 5’ GCT CAT CCA CCC ACC GAT CAG TCT TCC 3’ and reverse primer: 5’ GCT GCACAAGTGGTG TCT CGCTAAC 3’, and reverse primer: 5’ GCG ATC CAG CGC CAG C 3 (Harada et al., 2005). Primers specific for β-actin were used as a control (forward: 5’ CAA CTT GAT GTA TGA AGG CTT G TG T 3’ and reverse: 5’ A CTT TTT ATT GTC CTA AAG TCA GTG TAC GAG 3’) (Alfonso et al., 2006). Quantitative real-time PCR was performed in a Cycler (Light Cycler 2.0, Roche) using SYBR Green (DRR041A, Takara). The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. Each sample was assayed in duplicate, and the mRNA levels were normalized for each well to the β-actin mRNA levels using the 2–ΔΔCT method.

ELISA. The vmPFC, IC, BLA, CeA, and hippocampus were dissected at various time points after CS-US pairing (n = 5–6 per time point) and were stored in microcentrifuge tubes at −80°C until analysis. Brain tissue samples were homogenized in ice-cold lysis buffer containing 137 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mM sodium vanadate. The tissue homogenate solutions were centrifuged at 14,000 × g for 5 min at 4°C. The supernatants were collected and used for quantification of total protein and BDNF levels. BDNF levels in each region were assessed using a commercially available assay kit from Promega. In brief, flat-bottom plates were coated with the captured BDNF antibodies. The captured BDNF was bound by a second specific antibody, which was detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. All unbound conjugates were removed by subsequent wash steps according to the Promega protocol. After incubation with chromogenic substrate, color change was measured in an ELISA plate reader at 450 nm.

In situ hybridization. Rats were deeply anesthetized with 10% chloral hydrate anesthesia (4 ml/kg, i.p.) and transcardially perfused with 4% paraformaldehyde. Brains were removed and postfixied overnight, then equilibrated at 4°C in 30% sucrose/PBS (PO4 7–7.4, NaCl 0.15 M, pH 7.4) for at least 3 d before sectioning. Brains were sectioned at 40 µm thickness on a Microm cryostat (HM 500) at −20°C onto gelatin-coated slides. Every sixth serially obtained section was processed and analyzed for in situ hybridization.

Riboprobes were prepared from an open reading frame of BDNF coding sequence (387 bp) subcloned into a pBluescriptII SK vector. The primers for BDNF were as follows: forward primer: 5’ CGT CCT TCT GC 3’, reverse primer: 5’ G TAT ACG ACC CAG CCA AT 3’, Digoxigenin (DIG)–labeled probes were synthesized by T7 or T3 RNA polymerase with the DIG-RNA labeling mix (Roche Diagnostics) using linearized plasmid as template, according to the manufacturer’s instructions. In situ hybridization was performed with sense and antisense riboprobes.

The in situ hybridization experiments were performed as described previously (Tongiorgi et al., 1998). Prehybridization was performed at 55°C for 60–90 min in the prehybridization solution. In situ hybridization was carried overnight (at least 16 h) at 55°C. After hybridization, the sections were washed twice in 2× saline sodium citrate containing 50% deionized formamide (Roche Diagnostics) at 55°C for 30 min, 20 min in 2× SSC at 55°C, and twice in 0.2× SSC at 60°C for 30 min. Slices hybridized with DIG-labeled riboprobes were processed for detection with anti-DIG antibodies conjugated with alkaline phosphatase (Roche Diagnostics) for 2 h at room temperature. Finally, sections were incubated in the developing buffer containing NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Roche Diagnostics). To obtain reproducible and comparable results and to avoid saturation of the reaction, alkaline phosphatase development was always performed for exactly 5 h at room temperature.

Microphotographs of BDNF mRNA were captured by using a Nikon 80i light microscope equipped with a CCD camera interfaced to a per-
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Figure 1. Temporal and spatial changes in neurotrophin expression after CTA conditioning. A, Temporal and spatial changes of BDNF mRNA expression after CTA conditioning using real-time PCR. Rats were killed at 0, 1, 2, 4, 6, or 8 h after conditioning, and the relative levels of BDNF mRNA expression normalized to levels at 0 h in the vmPFC, IC, BLA, CeA, and hippocampus (HIP) are shown (n = 5 per time point; *p < 0.05, **p < 0.01 vs the values at 0 h time point). B, C, Levels of NGF mRNA (B) (n = 5–8 per group) and NT4/5 mRNA (C) (n = 6–7 per group) do not significantly change at 4 h after CTA conditioning in different brain regions. D, Temporal and spatial changes of BDNF protein levels after CTA conditioning using ELISA. Rats were killed at 0, 1, 4, 8, 12, or 16 h after conditioning and BDNF protein levels in the vmPFC, IC, BLA, CeA, and HIP are measured (n = 5–6 per time point; *p < 0.05, **p < 0.01 vs the amount at 0 h time point). All values are presented as mean ± SEM.

Surgical procedures. Rats were anesthetized with 10% chloral hydrate, restrained in a stereotaxic apparatus (8001, RWD Life Science), and implanted bilaterally with guide cannula (stainless steel, 23 gauge) aimed at the coordinates in reference to bregma: infralimbic cortex (IL) region of the vmPFC: anteroposterior (AP), +3 mm; lateral (L), ±5.5 mm; dorsoventral (V), −4 mm; IC: AP, +1.2 mm; L, ±5.3 mm; V, −5.5 mm; BLA: AP, −2.38 mm; L, ±5 mm; V, −7 mm; and hippocampus: AP, −3.3 mm; L, ±2 mm; V, −3.4 mm (Paxinos and Watson, 1996). The cannulas were positioned in place with acrylic dental cement and secured with skull screws. A stylos was placed in the guide cannula to prevent clogging. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations.

Microinjection. For microinjection, the stylus was removed from the guide cannula and a 28 gauge infusion cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The infusion cannula was connected via PE20 tubing to a micro-syringe driven by a micro-infusion pump (KD5310, KD Scientific), K252a (dissolved in artificial CSF/0.05% DMSO, 25 µM, 1 µl/side, Calbiochem) (Jiang et al., 2001), BDNF antibodies (dissolved in 0.1 M PBS, 1 µg/µl, 0.5 µl/side), BDNF ODNs (dissolved in sterile saline, 2 nM, 0.5 µl/side; (Alonso et al., 2002), BDNF ODNs (dissolved in sterile saline, 0.25 µg/µl, 1 µl/side, PeproTech) (Bekinschtein et al., 2007), ibotenic acid (dissolved in 0.1 M PBS, 10 µg/µl, 0.5 µl/side, Sigma) (Larrard, 1989), or their respective vehicles were microinjected into different brain regions. The infusions were performed at a rate of 0.5 µl/min, and injection cannulas were left in the position for an additional 2 min after the infusion was completed to minimize dripping of the injected liquid along the injection tract. BDNF ODNs were HPLC-purified phosphorothioate end-capped 18-mer sequences. The ODN sequences used were as follows: BDNF antisense ODN (ASO): 5′-TCT TCC CCT TTT AAT GGT 3′; BDNF sense ODN (MSO): 5′-ATA CTT GTT CTT GTC 3′ (Lee et al., 2004). Both ODNs were phosphorothioated on the three terminal bases of both the 5′ and 3′ ends, which results in increased stability and less toxicity. Compared with the specific matches of BDNF ASO for the rat BDNF mRNA sequence, BDNF MSO, which included the same 18 nt as the ASO but in a scrambled order, did not generate any full matches to identified gene sequences in the database.

Measurement of anxiety-like behavior. Stressed rats were systematically restrained 2 h per day for 14 consecutive days using cylindrical restrainers, secured at the head and tail ends with large binder clips. Twenty-four hours after the last restraint, anxiety-like behavior was measured using open field (OF) and elevated plus-maze (EPM) tests. The OF apparatus consists of a (100 × 100 × 50 cm) wood cube with a white floor divided into 5 × 5 identical squares. The arena was set up in a dim room under a digital camera, connected to a video recorder. A single rat was placed in the center of the OF arena and left free to explore for 10 min. After each trial, the apparatus was cleaned with a 50% ethanol solution to prevent olfactory cues. The total number of squares crossed and time spent in the inner 4 × 4 squares were recorded to test locomotor activity and anxiety-like behavior, respectively.

The EPM was constructed of black Plexiglas, consisting of two opposite open arms (50 × 10 cm), an open platform (10 × 10 cm) in the center, and two opposite closed arms (50 × 10 cm) with 40 cm high opaque walls. The apparatus was raised 50 cm above the floor and set up under an infrared-sensitive digital camera connected to a video recorder. The test animal was placed in the central platform facing one of the open arms and allowed to explore the apparatus for 5 min. The maze was cleaned with a 50% ethanol solution and dried after each trial to eliminate possible odor cues left by previous subjects. Anxiety levels were
measured by the percentage of both open-arm entries and time spent in open arms.

Histology. At the end of the experiments, rats were deeply anesthetized and 1 μl of methylene blue was microinfused. Rats were perfused with saline followed by 4% paraformaldehyde. Brain sections (60 μm) were analyzed to verify the microinfusion sites and the scope of solution diffusion.

Statistical analyses. Data were analyzed with Student’s t test or one-way ANOVA, followed by least significant difference post hoc comparisons. The significance level was set to 0.05 for all statistical analyses, and all values in the text and figures represent means ± SEM. Data analyses were performed using SPSS statistical program, version 10.0.

Results

Time course of BDNF changes after CTA conditioning

BDNF and its receptor TrkB have been shown to participate in hippocampal-dependent learning and memory. To investigate the role of BDNF in CTA acquisition, we initially examined BDNF gene expression at various time points in distinct brain regions (vmPFC, IC, BLA, CeA, and hippocampus) after CTA conditioning using real-time PCR. As shown in Figure 1A, among the five brain regions we analyzed significant changes in BDNF mRNA after CTA conditioning were only observed in the CeA (ANOVA: F(5,24) = 4.459, p = 0.005) and IC (ANOVA: F(5,24) = 6.788, p = 0.001). BDNF mRNA increased starting at 2 h (p < 0.05) and peaked at 4 h (p < 0.01) after CTA conditioning in the CeA. BDNF mRNA levels peaked at 6 h in the IC and began to return to baseline at 8 h after conditioning. Surprisingly, although the BLA region is believed to play an important role in memory formation, BDNF mRNA level in the BLA did not change significantly subsequent to CTA conditioning (ANOVA: F(5,24) = 0.884, p = 0.994). To test whether other neurotrophins such as NGF and NT4/5 were also increased, we further examined mRNA levels of these two neurotrophic factors in different brain regions at the 4 h post-CTA conditioning time point using real-time PCR. Post hoc analysis showed no statistical significance of NGF mRNA (Fig. 1B; ANOVA: F(5,47) = 0.413, p = 0.922) and NT4/5 mRNA (Fig. 1C; ANOVA: F(5,22) = 0.503, p = 0.866) compared with the control group, which indicates that only BDNF mRNA levels appeared to show activity-dependent changes after CTA conditioning in specific brain regions.

We next investigated whether the increased BDNF mRNA would lead to BDNF protein level elevation after CTA conditioning. BDNF protein level was examined using ELISA at 0, 1, 4, 8, 12, or 16 h after CTA conditioning. Consistent with the changes of BDNF mRNA, increased BDNF protein levels were detected in the CeA (Fig. 1D; ANOVA: F(5,30) = 7.016, p = 0.001) and IC (Fig. 1D; ANOVA: F(5,30) = 7.016, p < 0.001), but not in the BLA (Fig. 1D; ANOVA: F(5,30) = 0.584, p = 0.712). Compared with the time course of BDNF mRNA level change, BDNF protein showed a delayed increase, which started at 4 h (p < 0.05, vs 0 h) and peaked at 8 h (p < 0.01, vs 0 h) postconditioning in the CeA, suggesting that the altered protein synthesis occurred in concert with the increases in gene transcription. The increased BDNF protein levels began to return to baseline at 16 h after conditioning. The temporal change of BDNF expression in the CeA and IC after CTA conditioning suggests that BDNF in the CeA and IC may be involved in CTA memory formation.

Selective induction of BDNF expression after associative CTA conditioning

The previous experiments suggest that there are temporal- and spatial-specific changes in BDNF gene expression after CTA conditioning. To further investigate whether BDNF mRNA changes are specific to associative CTA conditioning rather than only ex-
expression have been found in the BLA (Fig. 2). Previous observations, no significant changes in BDNF gene expression (Gu-

eles and ventral posteromedial thalamic nucleus (data not shown). Meanwhile, no significant changes were found in the BLA (t test, p = 0.790), vmPFC (t test, p = 0.78), or hippocampus (t test, p = 0.874). As a control, sections hybridized with the BDNF sense riboprobes yielded no signal (data not shown). Together, these findings further confirm our real-time PCR results that there are temporal- and spatial-specific changes in BDNF gene expression after CTA conditioning, which suggests that BDNF in different brain regions may play different roles in CTA memory formation.

**Figure 3.** In situ hybridization analysis of regional specific BDNF mRNA increases at 4 h after CTA conditioning. A. Representative photomicrographs of coronal rat brain sections from CS-US-L control group or CTA conditioning group depicting BDNF mRNA expression in the vmPFC, IC, BLA, CeA, and hippocampus (HIP). Scale bars, 500 μm. Magnification of sections containing the CeA from CS-US-L control and CTA groups are presented at the bottom. Scale bar, 20 μm in the magnified sections at the bottom. B. Quantitative analysis of BDNF mRNA from in situ hybridization in the vmPFC, IC, BLA, CeA, and HIP at 4 h after CTA conditioning normalized to CS-US-L control (n = 5 per group; *p < 0.05). All values are presented as mean ± SEM.

TrkB is activated after CTA conditioning with increased BDNF secretion and synthesis

The release of BDNF and its binding to TrkB receptor, leads to the dimerization and autophosphorylation of tyrosine residues in the intracellular domain of the receptor and subsequent activation of downstream signaling molecules (Kaplan and Miller, 2000). Though both BDNF and NT4/5 can bind to and induce TrkB receptor phosphorylation, we have previously found that NT4/5 expression levels did not show statistically significant changes after CTA conditioning, thus the phosphorylation of TrkB was likely triggered by the release of BDNF during the CTA process. To test whether TrkB activation was accompanied with BDNF protein increase, tissues were dissected at 8 h after CTA conditioning. p-TrkB levels normalized to total TrkB were measured by immunoprecipitation and immunoblotting. Quantitative densitometric analyses of p-TrkB receptors showed elevated levels of p-TrkB in the CeA (Fig. 4B; ANOVA: F(2,7) = 8.34, p < 0.05; post hoc: control vs 8 h, p < 0.05) and IC (Fig. 4B; ANOVA: F(2,9) = 4.771, p < 0.05; post hoc: control vs 8 h, p < 0.05), but not in the BLA (Fig. 4B; ANOVA: F(2,9) = 2.517, p = 0.135) after conditioning. To investigate whether TrkB was activated only subsequent to the increased BDNF expression, tissues were dissected at 1 h after CTA conditioning when both BDNF mRNA and protein levels showed no change. Interestingly, post hoc analysis showed significantly enhanced p-TrkB levels in the CeA (p < 0.01) and IC (p < 0.05) at 1 h postconditioning, which suggested induced rapid activity-dependent BDNF release in advance of the increased BDNF synthesis associated with CTA training. Collectively, the immediate activation of TrkB in the same brain regions with increased BDNF expression, suggests that BDNF secretion and synthesis may be spatially and temporally involved in CTA memory formation.
vated learning tasks like spatial maze, inhibitory avoidance, and CTA (Bermúdez-Rattoni and McGaugh, 1991; Bermúdez-Rattoni, 2004). Our results showed that microinfusion of K252a into the IC before conditioning impaired both the STM and LTM of CTA (Fig. 5B,C; t test; STM, p < 0.001; LTM, p < 0.001). In contrast, microinfusion of K252a into the IL of the vmPFC (Fig. 5B,C; t test; STM, p = 0.481; LTM, p = 0.51) or hippocampus (Fig. 5B,C; t test; STM, p = 0.558; LTM, p = 0.866) has no effect on CTA memory formation. Together with our previous findings, these results suggest that BDNF is not only selectively induced by CTA conditioning in specific brain regions, but also spatially involved in STM and LTM of CTA.

BDNF secretion and synthesis in the CeA are respectively involved in CTA STM and LTM

Since K252a is a pharmacological inhibitor of all Trk receptors and also may have nonspecific effects, BDNF ASO and anti-BDNF blocking antibodies could respectively block de novo BDNF synthesis and neutralize endogenously released BDNF, which allow us to distinguish the roles of BDNF synthesis and secretion in memory formation. BDNF ASO or anti-BDNF antibodies were further used to specifically investigate the role of BDNF in CTA memory formation. More importantly, BDNF ASO and anti-BDNF blocking antibodies could respectively block the increased p-TrkB level at 1 h postconditioning in the CeA (Fig. 5D) showed deficits in both CTA STM and LTM (Fig. 5E; test, STM, p < 0.001; LTM, p = 0.012), which suggested that BLA participates in the neural networks related to CTA formation. Together, these data indicate that BDNF in the two subnuclei of amygdala have different roles in CTA memory formation.

The IC is a region in the temporal cortex that has been implicated in the acquisition and storage of different aversive motivated learning tasks like spatial maze, inhibitory avoidance, and CTA (Bermúdez-Rattoni and McGaugh, 1991; Bermúdez-Rattoni, 2004). Our results showed that microinfusion of K252a into the IC before conditioning impaired both the STM and LTM of CTA (Fig. 5B,C; t test; STM, p < 0.001; LTM, p < 0.001). In contrast, microinfusion of K252a into the IL of the vmPFC (Fig. 5B,C; t test; STM, p = 0.481; LTM, p = 0.51) or hippocampus (Fig. 5B,C; t test; STM, p = 0.558; LTM, p = 0.866) has no effect on CTA memory formation. Together with our previous findings, these results suggest that BDNF is not only selectively induced by CTA conditioning in specific brain regions, but also spatially involved in STM and LTM of CTA.
It has been shown previously that BDNF ASO infusion into the CeA induced anxiety-like behaviors (Pandey et al., 2006). To examine whether changes in anxiety levels would modify CTA memory formation, a chronic restraint stress (CRS) protocol (2 h/14 d), which could produce anxiety-like behavior (Kim and Han, 2006), was used, and anxiety-like behaviors were evaluated 24 h after the CRS (Fig. 7A). Compared with the unstrained rats, stressed animals had decreased exploratory behavior as demonstrated by a reduction in the time spent in the center compartment in the OF test (Fig. 7B; t test, p < 0.01), as well as a significant decrease in the percentage of open-arm entries and the percentage of time spent in open arms in the EPM test (Fig. 7C; t test, p < 0.01). These results suggested that the CRS procedure produced anxiety-like behaviors. Next, CTA conditioning was conducted and the STM and LTM were evaluated (Fig. 7A). In contrast to the elevated anxiety-like behaviors, there were no significant differences in CTA STM (Fig. 7D; t test, p = 0.283) and LTM (t test, p = 0.254) between the stressed and unstrained groups. These series of experiments suggest that stress-induced increased anxiety level has no effect on the CTA STM and LTM memory formation.

**BDNF synthesis in the CeA is required for CTA consolidation**

Since our previous results have shown that BDNF participated in CTA LTM formation, we next investigated the functional role of BDNF in different memory processes, such as acquisition, consolidation, or retrieval by varying the time point of BDNF antibody microinjection into the CeA. BDNF antibodies were infused into the CeA at 1 h post-CTA conditioning and both the STM and LTM were measured (Fig. 8A). We found the intact STM and impaired LTM (Fig. 8B; ANOVA: F(3,28) = 7.556, p = 0.001; post hoc: vehicle vs BDNF antibody, STM, p = 0.934; LTM, p < 0.01) after infusion of BDNF antibodies at 1 h post-conditioning, which suggests that the increased BDNF synthesis is required for the CTA memory consolidation and the rapid BDNF secretion within 1 h after CTA conditioning is involved in CTA memory acquisition. LTM impairment could also be due to memory retrieval deficit. In an additional experiment, we microinjected BDNF antibodies into the CeA 15 min before LTM test and detected similar levels of LTM compared with vehicle group (Fig. 8A, B; t test, p = 0.151). Thus, BDNF is not necessary for the retrieval of acquired CTA memory. Overall, these results suggest that BDNF secretion and synthesis in the CeA are respectively involved in CTA memory acquisition and consolidation, but not in memory retrieval.
protocol in which rats were injected with a lower concentration of LiCl (LiCl concentration 0.05 M) compared with normal CTA conditioning (LiCl concentration 0.15 M). Post hoc comparisons revealed that rats in the weak training group showed significantly attenuated STM and LTM memory, compared with the normal conditioning group (Fig. 9A; ANOVA: STM, $F_{(2,12)} = 72.267, p < 0.001$; LTM, $F_{(2,12)} = 48.662, p < 0.001$). Paralleling the attenuated memory, we found that weak training abolished the increased BDNF gene expression at 4 h postconditioning (Fig. 9B; ANOVA: $F_{(4,10)} = 25.817, p < 0.001$; post hoc: weak vs normal, $p < 0.05$) and led to significantly decreased TrkB phosphorylation at 1 and 8 h after CTA conditioning in the CeA (Fig. 9C; ANOVA: $F_{(4,10)} = 25.817, p < 0.001$; post hoc: weak vs normal, $p < 0.001$) compared with normal CTA group, which further confirms our previous conclusion that BDNF signaling in the CeA plays an important role in CTA memory formation. Next, exogenous BDNF or vehicle was microinfused into CeA immediately after weak CTA training, and the STM and LTM of CTA were evaluated (Fig. 9D). We found that exogenous BDNF infusion could rescue the attenuated STM (Fig. 9E; ANOVA: $F_{(2,21)} = 27.681, p < 0.001$; post hoc: weak + hrBDNF vs normal + vehicle, $p = 0.477$) and LTM (Fig. 9F; ANOVA: $F_{(2,21)} = 16.860, p < 0.001$; post hoc: weak + hrBDNF vs normal + vehicle, $p = 0.238$) caused by weak training to normal levels compared with the normal conditioning group. These results suggest that BDNF in the CeA is not only necessary but also sufficient for CTA memory formation.

**Discussion**

The purpose of this study is to investigate the spatial involvement of BDNF secretion and synthesis in CTA memory formation. We provided evidence that increased BDNF secretion and synthesis were temporally induced in specific brain regions after associative CTA conditioning. Inhibition of BDNF signaling in selective brain regions could block CTA acquisition. Moreover, we found that activity-dependent BDNF secretion and synthesis are respectively involved in STM and LTM of CTA. Finally, we demonstrated that the infusion of exogenous BDNF into CeA could enhance CTA memory formation.

Anatomical and pharmacological studies have highlighted the involvement of several brain structures (e.g., PBN, amygdala, IC, vmPFC) and cellular processes [e.g., expression of immediate early genes, Ras-mitogen-activated protein kinase (MAPK) signaling pathway, protein synthesis] in CTA learning (Welzl et al., 2001). Our data provide several new insights into the spatial-specific involvement of BDNF secretion and synthesis in the CTA memory process. First, we found that BDNF expression was temporally induced in selective brain regions after CTA conditioning, whereas levels of other neurotrophins such as NGF and NT4/5 did not change. BDNF has been extensively studied in hippocampal- and amygdala-dependent learning and memory (Kesslak et al., 1998; Hall et al., 2000; Rattiner et al., 2004; Chhatwal et al., 2006). However, little is known about the role of BDNF in hippocampal-independent cortical memory process. Elevated BDNF expression levels in the CeA and IC during CTA acquisition have been found after associative CS-US pairings, suggesting that an interaction of the representations of the CS and US is obligatory for BDNF involvement in novel taste learning. The differential changes of BDNF expression in the two subnuclei of amygdala during CTA acquisition imply that BDNF may play distinct roles in CTA memory formation in different brain regions. In addition, we found increased phosphorylation of BDNF receptor, TrkB, at the time point before and following increased BDNF synthesis in the CeA and IC, which suggests that associative CTA learning could first induce the rapid re-
lease of preexisting BDNF, followed by increased BDNF synthesis.

Second, we found that BDNF in selective brain regions was differentially involved in CTA memory formation. Notably, we found that BDNF/TrkB signaling in the CeA and BLA, two subnuclei of amygdala, play distinct roles in CTA memory formation. Intra-CeA infusion of K252a, BDNF ASO, or BDNF antibodies significantly impaired CTA memory formation; however, inhibition of BDNF/TrkB signaling in the BLA had no effect. Previous studies have shown diverse molecular mechanisms within the CeA and BLA to be implicated in mediating CTA acquisition. Local microinfusion of the protein synthesis inhibitor anisomycin or the H9252-adrenergic antagonist propranolol into the CeA but not BLA during the training session impaired CTA memory (Bahar et al., 2003); whereas, intra-BLA infusions of NMDA antagonist MK-801 or D-AP5 [D-(2)-2-amino-5-phoshonovaleric acid], AMPA antagonist CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione), or metabotropic glutamate receptors antagonist (S)-MCPG [(S)-H9251-methyl-4-carboxyphenylglycine] affected CTA memory formation (Yamamoto and Fujimoto, 1991; Tucci et al., 1998; Yasoshima et al., 2000). Our study demonstrates that BDNF in the CeA, but not BLA, is necessary for CTA memory formation. Our data are also consistent with previous findings that microinjection of antisense oligodeoxynucleotides against cAMP response element-binding protein (CREB) into CeA-impaired CTA formation (Lamprecht et al., 1997), as BDNF is known to activate CREB and CREB plays a central role in mediating BDNF responses in neurons (Finkbeiner et al., 1997). Although blocking BDNF signaling in the BLA has been shown to impair amygdala-dependent tasks such as fear-potentiated startle (Rattiner et al., 2004), BDNF in the BLA does not appear to affect memory formation in hippocampal-independent nondeclarative CTA memory tests, suggestive of selective roles for BDNF in different learning tasks. In addition, we found that BDNF/TrkB signaling in the IC, but not hippocampus, is essential for CTA memory formation. The role of the IC in CTA memory formation has been demonstrated in many studies by microinfusion of exogenous BDNF into the anesthetized adult rats induces a lasting potentiation of synaptic efficacy and reverses the CTA memory deficits produced by the inhibition of protein synthesis (Escobar et al., 2003; Moguel-González et al., 2008). In addition, BDNF microinfusion into the IC can enhance the CTA retention (Castillo et al., 2006), which is dependent on the activation of both MAPK and phos-

Figure 7. Effect of chronic restraint stress-induced elevated anxiety on CTA memory formation. A, Schematic representation of the behavior protocol. Rats were restrained for 14 consecutive days (2 h/d). B–D, Anxiety-like behaviors (B, C) and CTA conditioning (D) were respectively performed at 24 and 48 h after the last stress. CTA STM and LTM tests were performed at 4 and 72 h after conditioning. B, Number of squares crossed and time spent in the center in the open field test in the stressed and unstressed rats. C, Percentage of time spent in the open arm and percentage of open-arm entries in the plus-maze test in the stressed and unstressed rats. D, Aversion index in the CTA STM and LTM tests in the stressed and unstressed rats. All results are presented as the mean ± SEM determined from analysis of 7–10 rats per group. (**p < 0.01).

Figure 8. Effects of BDNF antibodies on CTA memory consolidation and retrieval in the CeA. A, Schematic representation of the behavior protocol to evaluate the effect of BDNF on CTA consolidation and retrieval. B, To investigate the role of BDNF in CTA memory consolidation, rats received bilateral CeA microinfusion of BDNF antibodies or vehicle at 1 h post-CTA conditioning, and the STM and LTM tests were performed at 4 and 72 h after conditioning, respectively (**p < 0.01). To investigate the effect of BDNF on CTA memory retrieval, BDNF antibodies or vehicle were administrated into the CeA 15 min before the LTM test. The values represent the mean ± SEM determined from analysis of 8 rats per group.
phatidylinositol-3-kinase (Castillo and Escobar, 2011). However, the role of endogenous BDNF in the IC in CTA memory has not been addressed in these studies. Here, we found that BDNF in the IC was required for CTA STM and LTM formation. In contrast, inhibition of BDNF/TrkB signaling in the hippocampus had no effect on CTA memory formation, which is consistent with a previous report showing that hippocampal integrity is not required for the CTA memory process (Gallo and Cândido, 1995).

Third, this is the first study demonstrating that the activity-dependent rapid BDNF secretion and the subsequent increased BDNF expression in CeA after CTA conditioning are differentially involved in STM and LTM. Although BDNF has been shown to play an important role in LTM, it has not been established whether BDNF is involved in STM formation. Infusion of BDNF ASO in the hippocampus before contextual fear conditioning had no effect on STM; whereas, functional blocking of BDNF availability by delivering BDNF antibodies in the hippocampus did impair STM of inhibitory avoidance training (Alonso et al., 2002; Lee et al., 2004). These conflicting results may be attributable to the different approaches used to inhibit BDNF function. In our study, we found that BDNF ASO microinfusion could block de novo BDNF synthesis but had no effect on the rapid BDNF secretion induced by CTA conditioning; however, BDNF antibody administration could neutralize the secreted BDNF and block the following TrkB phosphorylation. Our study indicates that the increased BDNF secretion and synthesis in the CeA were respectively required for CTA STM and LTM, and therefore explained the discrepancy in previous studies. Furthermore, we found that BDNF was required for the acquisition and consolidation, but not the retrieval, of CTA memory in the CeA by varying the delivering time of BDNF antibodies. Interestingly, our results suggest that the elevation of p-TrkB is necessary for the subsequent increased BDNF gene transcription, implying that the initial release of BDNF accompanied by TrkB activation triggers a cascade of BDNF synthesis resulting in subsequent BDNF-mediated consolidation.

Finally, we showed that exogenous BDNF administration in the CeA could enhance CTA memory, which suggests that BDNF in the CeA is not only necessary but also sufficient for CTA learning. It has been reported that the transgenic mouse overexpressing TrkB receptor in the postnatal neurons could lead to increased TrkB phosphorylation and improved CTA learning (Koponen et al., 2004). In addition, previous reports have shown that microinfusion of BDNF into IC before CTA training could enhance the CTA learning (Castillo et al., 2006) and reverse the CTA memory deficits induced by protein synthesis inhibition (Moguel-González et al., 2008). Our findings further demonstrate the importance of BDNF signaling in the CeA in CTA memory formation.

In conclusion, using the CTA memory paradigm, we determined the spatial-specific roles of BDNF in CTA memory formation. We provided evidence, for the first time to our knowledge, that dynamic regulation of BDNF in the CeA, but not BLA, played important roles in CTA acquisition. In addition, activity-dependent secretion and synthesis of BDNF in the CeA were respectively required for CTA acquisition and consolidation through activation of TrkB. Finally, we found that microinfusion of BDNF in the CeA could enhance CTA memory. Our study will help further the understanding of the precise regulation of BDNF in different memory phases depending on its location in the neural circuit.
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