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# Transcriptional Regulation of Brain-Derived Neurotrophic Factor in the Amygdala during Consolidation of Fear Memory

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## ABSTRACT

We have demonstrated previously that brain-derived neurotrophic factor (BDNF) signaling in the amygdala is required for the consolidation of fear memory. This study is designed to characterize the signal cascades by which fear conditioning modulates transcriptional and translational expression of BDNF. Real-time reverse transcription-coupled polymerase chain reaction showed a significant increase in BDNF exon I- and III-containing mRNA in the amygdala of fear-conditioned rats, indicating that fear conditioning was capable of up-regulating BDNF mRNA. Bilateral administration of actinomycin D or anisomycin to the amygdala attenuated conditioning-induced increase in BDNF protein. Inhibitors for *N*-methyl-D-aspartate (NMDA) receptor, L-type voltage-dependent calcium channel (L-VDCC), adenylyl cyclase, cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent kinase IV (CaMKIV) significantly reduced the increase. Moreover, DNA affinity precipitation and chromatin immunoprecipitation assays showed that phosphorylated cAMP response element-binding protein (p-CREB) binding activity in the proximal region of *BDNF* promoter I and III was significantly increased after fear conditioning. Intra-amygdala administration of cAMP response element decoy DNA before training impaired fear learning. Taken together, these results suggest that calcium influx through NMDA receptors and L-VDCCs during fear conditioning activates PKA and CaMKIV resulting in CREB phosphorylation. The phosphorylated CREB binds to *BDNF* promoter and up-regulates the expression of BDNF in the amygdala, which helps the consolidation of fear memory.

Neuronal adaptive responses involving long-term synaptic plasticity and structural modifications require alterations in neuronal gene expression (Davis and Squire, 1984). It is suggested that neuronal activity enhances expression of a modulator and that this modulator could reinforce the efficacy of synaptic transmission and the growth of dendrites and axons (Bekinschtein et al., 2007). One class of the molecules that may serve as activity-dependent synaptic modulation is the neurotrophins, BDNF in particular (Poo, 2001; Lu, 2003). BDNF plays an important role in regulating the survival and differentiation of selective populations of neurons (Hetman et al., 1999), drug-seeking behavior (Lu et al., 2004), and synaptic plasticity such as long-term potentiation (LTP) (Korte et al., 1995; Pang et al., 2004) and learning and memory (Mizuno et al., 2000; Tokuyama et al., 2000). Contextual fear conditioning increased BDNF mRNA in the hippocampus (Hall et al., 2000). In parallel, antisense oligodeoxynucleotide that inhibited protein expression locally in the dorsal hippocampus blocked consolidation of contextual fear memory (Lee et al., 2004).

Neuronal plasticity in the lateral (LA) and basolateral (BLA) amygdala nuclei is believed to underlie the acquisition and expression of Pavlovian fear conditioning. Cued fear

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**ABBREVIATIONS:** BDNF, brain-derived neurotrophic factor; BLA, basolateral nucleus of amygdala; CaMK, calcium/calmodulin-dependent kinase; ChIP, chromatin immunoprecipitation assay; CRE, cAMP response element; CREB, cAMP response element-binding protein; DAPA, DNA affinity precipitation assay; D-APV, D-2-amino-5-phosphonovalerate; ELISA, enzyme-linked immunosorbent assay; LA, lateral nucleus of amygdala; LTP, long-term potentiation; *m*-AIP, myristoylated-autocamtide-2-related inhibitory peptide; NMDA, *N*-methyl-D-aspartate; PKA, cAMP-dependent protein kinase; L-VDCC, L-type voltage-dependent calcium channel; ACSF, artificial cerebrospinal fluid; PMSF, phenylmethylsulfonyl fluoride; HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Rp-cAMPS, *R*p-diastereomer of cyclic adenosine 3′,5′-phosphorothioate; NF-κB, nuclear factor κB; ANOVA, analysis of variance; ITI, intertrial interval; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; KN62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; CS, conditioned stimulus; KT-5720, (9α,10β,12α)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-*fg*:3′,2′,1′-*kl*)pyrrolo (3,4-*i*)(1,6)benzodiazocine-10-carboxylic acid, hexyl ester; KN93, *N*-[2-[*N*-(4-chlorocinnamyl)-*N*-methylaminomethyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt.

conditioning resulted in an increase in BDNF mRNA and protein levels in the amygdala (Rattiner et al., 2004a; Ou and Gean, 2006). Moreover, expression of a dominant-negative TrkB receptor to antagonize BDNF signaling in the amygdala impaired consolidation of both fear learning and extinction retention, indicating essential roles of BDNF in the excitatory and inhibitory amygdala-dependent memory (Rattiner et al., 2004a; Chhatwal et al., 2006). Although it is now generally recognized that synaptic activity can have a profound effect on BDNF expression, the signal cascades that underlie activity-dependent BDNF expression have not yet been identified. In the present study, we aim to investigate transcriptional regulation of BDNF expression during consolidation of fear memory.

## Materials and Methods

**Fear Conditioning.** Male Sprague-Dawley rats (8–9 weeks old) were trained and tested in a stabilimeter device. A piezoelectric device mounted below stabilimeter detects and transduces the motion of the cylinder produced by the whole body startle response of the rat (San Diego Instrument, San Diego, CA). The whole set-up was enclosed in a ventilated, sound-attenuating cabinet (length, 38 cm; width, 38 cm; and height, 55 cm). The acoustic startle stimulus was a 50-ms burst of white noise at the intensity of 95 dB. The visual CS was a 3.7-s light produced by an 8-W fluorescent bulb attached to the back of stabilimeter. The unconditioned stimulus was a 0.6-mA foot shock with a duration of 0.5 s.

**Acclimation.** On 3 consecutive days, rats were placed in the startle test boxes for 10 min and then returned to their home cages.

**Matching.** On 2 consecutive days, rats were placed in the startle box and 3 min later were presented with 10 startle stimuli at a 2-min intertrial interval (ITI). On the basis of their mean startle amplitudes in the second of these two sessions, rats were matched into groups with similar response levels.

**Training.** Rats were placed in the startle boxes and received 10 light-foot shock pairings with an ITI of 2 min. Unpaired controls received the same number of light and foot shock presentations but in a pseudorandom fashion in which the US could occur at anytime except at the 3.2 s after the CS.

**Test.** Twenty-four hours after training, rats were tested for fear-potentiated startle. This involved 30 startle-eliciting noise bursts presented alone (noise-alone trial) and 30 noise bursts presented 3.2 s after the onset of the 3.7-s light (light-noise trials). The two trial types were presented in a balanced mixed order (ITI, 30 s). The percentage of fear-potentiated startle was computed as follows: [(startle amplitude on CS-noise − noise-alone trials)/(noise-alone trials)] × 100.

**Surgery.** Male Sprague-Dawley rats (8–9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and subsequently were mounted on a stereotaxic apparatus. Two cannula made of 22-gauge stainless steel tubing (C313G; Plastic Products, Roanoke, VA) were implanted bilaterally into the LA or BLA (anteroposterior, −2.7 mm; mediolateral, ± 5.5 mm; dorsoventral, −7.6 mm) (Paxinos and Watson, 1986). A 28-gauge dummy cannula was inserted into each cannula to prevent clogging. Three jewelry screws were implanted over the skull serving as anchors, and the whole assembly was affixed on the skull with dental cement. The rats were monitored and handled daily and were given 7 days to recover. Actinomycin D, anisomycin, TrkB/Fc chimera, D-2-amino-5-phosphonovalerate (D-APV) (Sigma, St. Louis, MO), verapamil, KT5720, KN62 (Tocris, Bristol, UK), Rp-cAMP, water-soluble myristoylated autocamtide-2-related inhibitory peptide (BIOMOL Research Laboratories, Plymouth Meeting, PA), water-soluble KN93, helenalin, and α2-antiplasmin (Calbiochem, La Jolla, CA) were administered bilaterally to the amygdala in a volume of 0.5 to 0.8 μl at a rate of 0.1 μl/min. cAMP response element (CRE) decoy DNA of *BDNF* promoters I and III (2 mM, 1 μl) were infused into the amygdala at a rate of 0.1 μl/min (two injections at 24 and 2 h before conditioning). The sequences of the nonbiotinylated promoter I and III CRE decoy DNA were the same as the probes used in the DNA affinity precipitation assay. The mutant CRE decoy DNA of *BDNF* promoter I and III were 5′-AGTTGGTCACGGACCTGGCTCAGAGAGG-3′ (Tabuchi et al., 2002) and 5′-TGACAGCCAGCTGCAAGGCAGC-3′ (Tao et al., 1998).

**Slice Preparation.** Rats were decapitated, and their brains were rapidly removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) solution. Thereafter, the brain was hemisected and cut transversely posterior to the first branch and anterior to the last branch of the superior cerebral vein. The resulting section was glued to the chuck of a Vibroslice tissue slicer. Transverse 400-μm slices were cut, and the appropriate slices were placed in a beaker of ice-cold oxygenated ACSF. The LA and BLA subregions were rapidly dissected out under a dissecting microscope at ice-cold oxygenated ACSF. ACSF solution had the following composition: 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM $NaH_2PO_4$, and 11 mM glucose. The ACSF was bubbled continuously with 95% $O_2$/5% $CO_2$ and had a pH of 7.4. To dissect out the amygdala in fresh tissues obtained from rats that received intra-amygdala infusions of drugs, brain slices were analyzed under a light microscope to identify the location of cannula placement. Tissues around the tip of the cannula were cut with a small surgical knife. Only brains with needle tracks in the LA or BLA were used for analysis.

**Amygdala Homogenates Preparation.** The LA and BLA subregions of the amygdala were sonicated briefly in ice-cold buffer (10 mM Tris-base, pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, 4 μg/ml aprotinin, and 1% Triton X-100). After sonication, the samples were centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant was obtained. Protein concentration in the soluble fraction was then measured using a Bradford assay, with bovine serum albumin as the standard.

**BDNF Immunoassay.** BDNF protein was quantified using an enzyme-linked immunosorbent assay (ELISA, ChemiKine BDNF Sandwich ELISA kit; Chemicon International Inc., Temecula, CA) according to the manufacturer's protocol. Microplate precoated with antibody against human BDNF is to capture BDNF proteins from the LA and BLA homogenates prepared as described above. Samples (100 μg) and serial dilutions of BDNF standard for generating a standard curve were added in duplicate wells in each plate and were shaken gently at 4°C overnight. After washing the plate five times, biotinylated mouse anti-BDNF monoclonal antibody (1:1000) was added in each well and incubated at room temperature for 3 h to detect the captured BDNF. The wells were washed, and streptavidin-HRP conjugate solution was added in each well and incubated at room temperature for 1 h. After washing the plate to remove nonbound streptavidin-HRP conjugate solution, the HRP activity was revealed with TMB/E substrate at room temperature for 15 min. After that, the reaction was stopped by adding stop solution to each well, and the amount of BDNF was determined by reading the absorbance at 450 nm. Unknown BDNF concentrations were compared with known BDNF concentrations using a calibration curve. The assay has a detection limit of 7.8 pg/ml, and no cross-reactivity with other related neurotrophic factors. The intra- and interassay coefficients of variations were 3.7 and 8.5%, respectively. BDNF protein levels in paired and unpaired rats were expressed as a percentage of those in naive controls.

To examine whether fear conditioning-induced increase in BDNF protein requires transcription and translation, rats were given intra-amygdala injection of anisomycin (62.5 μg/side dissolved in 50% DMSO), actinomycin D (1 μg/side dissolved in 50% DMSO), TrkB-IgG (2 μg/side dissolved in PBS solution), 50% DMSO, or PBS solution 30 min before training. To determine the effects of NMDA receptor and L-VDCC blockers on conditioning-induced increase in BDNF protein, rats were infused with D-APV (12.5 nmol/side dis-

solved in distilled $H_2O$), verapamil (4 μg/side dissolved in distilled $H_2O$), D-APV plus verapamil, or distilled $H_2O$ bilaterally into the amygdala 30 min before training. To determine the effects of various inhibitors on conditioning-induced increase in BDNF protein, rats were infused with Rp-cAMPS (40 nmol/side dissolved in distilled $H_2O$), KT5720 (0.5 μg/side dissolved in 50% DMSO), *m*-AIP (250 pmol/side dissolved in distilled $H_2O$), KN93 (10 nmol/side dissolved in distilled $H_2O$), KN62 (340 ng/side dissolved in 50% DMSO), helenalin (40 pmol/side dissolved in 50% DMSO), U0126 (1 μg/side dissolved in 50% DMSO), or vehicles (50% DMSO or distilled $H_2O$) bilaterally into the amygdala 30 min before training. One hour after fear conditioning, tissues from the LA and BLA were dissected out. BDNF protein was detected and quantified using ELISA.

**Semiquantitative Reverse Transcription-Coupled Polymerase Chain Reaction.** RNeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA) was used to extract total RNA from amygdala slice and ImProm-II Reverse Transcription System (Promega, Madison, WI) was used to synthesize cDNA. To determine the relative amount of cDNA molecules per sample, we performed real-time PCR using protocols provided by Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) system. Because of four alternative exons of the rat *BDNF* gene, 5′-end primers were designed that are specific for BDNF exons I (5′-ACTCAAAGGGAAACGTGTCTCT-3′), II (5′-CGGTGTAGGCTGGAATAGACT-3′), III (5′-CTCCGCCATGCAATTTCCACT-3′), or IV (5′-GTGACAACAATGTGACTCCACT-3′), and a 3′-end primer (5′-GCCTTCATGCAACCGAAGTA-3′) was generated that is common to all alternative spliced transcripts (Tabuchi et al., 2002). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from each sample was also amplified to serve as an internal control. The primers for detection of GAPDH cDNA were 5′-TGACAACTTTGGCATCGTGGAAGG-3′ and 5′-CAACGGATACATTGGGGGTAGGAAC-3′. The annealing temperature of four BDNF transcripts and GAPDH was 59°C. The levels of BDNF transcripts were normalized to the respective GAPDH and expressed as a percentage of those in naive controls. The amplified products were confirmed by running 1.5% agarose gels.

**DNA Affinity Precipitation Assay.** A 300-μg sample of the LA and BLA homogenates was mixed with 2 μg of biotinylated double-strand DNA, and 50 μl of streptavidin-agarose beads (Pierce Chemical, Rockford, IL) in a final volume of 450 μl of binding buffer [1 μg of poly(dI-dC), 20 mM HEPES-KOH, pH 7.9, 0.1 mM KCl, 2 mM $MgCl_2$, 15 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM PMSF, 20 μg/ml leupeptin, and 4 μg/ml aprotinin]. Negative control was done without DNA probes. The mixture was incubated at room temperature for 1 h with rotating. Beads were pelleted and washed three times with ice-cold PBS. Proteins bound to the beads were eluted and separated by 8.5% SDS-polyacrylamide gel electrophoresis for Western blot analysis. The relative binding activities were expressed as a percentage of those in naive controls. The antibodies were p-CREB (Ser133) (1:1000, Upstate Biotechnology, Lake Placid, NY), nuclear factor κB (NF-κB) p65 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), and NF-κB p50 antibody (1:2000; Abcam Inc., Cambridge, UK). The biotinylated oligonucleotides to assay p-CREB binding activity were 5′-AGTTGGTCACGTAACTGGCTCAGAGAGG-3′ (*BDNF* promoter I) and 5′-TGACAGCTCACGTCAAGGCAGC-3′ (*BDNF* promoter III) (Tabuchi et al., 2002). The probe of NF-κB binding sequence located on *BDNF* promoter III is 5′-TCGTGGACTCCCACCCACTTTCCCAT-3′ (Lipsky et al., 2001).

**Chromatin Immunoprecipitation Assay.** ChIP assay kit was used for ChIP assay (Upstate Biotechnology), and the protocols were modified slightly for brain tissues. The LA and BLA slices were cross-linked in 1% formaldehyde at 37°C for 15 min, and 0.125 M glycine was incubated for 5 min to stop the fixation. After several washes using ice-cold PBS, SDS lysis buffer containing 1 mM sodium orthovanadate, 1 mM PMSF, 20 μg/ml leupeptin, and 4 μg/ml aprotinin was used to homogenize the tissues. The homogenates were sonicated to shear DNA to lengths between 200 and 800 base pairs. Sonicated DNA (400 μg) taken from each sample was incubated with anti-p-CREB (Ser133) antibody (20 μl; Upstate Biotechnology) at 4°C overnight and then with 60 μl of salmon sperm DNA/protein A agarose-50% slurry for 1 h to form the antibody/DNA/agarose complex. Negative control was done by using rabbit-IgG instead of anti-p-CREB (Ser133) antibody. The precipitated DNA was eluted by 250 μl of elution buffer. After adding 10 μl of 5 M NaCl, histone-DNA cross-links were reversed at 65°C overnight followed by the addition of 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris-HCl, and 2 μl of 10 mg/ml proteinase K (Sigma) incubated for 1 h at 45°C. DNA fragments were recovered by using the QIAquick PCR purification kit (Qiagen) for subsequent real-time PCR. ChIP data were normalized to input DNA from each sample. The amounts of p-CREB binding to respective *BDNF* promoters were expressed as a percentage of those in naive controls. Primers for real-time PCR were designed to amplify the proven calcium-sensitive CRE regions of *BDNF* promoter I and III (Tabuchi et al., 2002). The primer sequences used were the following: promoter I forward, 5′-GCACGAACTTTTCTAAGAAGTTT-3′; promoter I reverse, 5′-GGAACTTGTTGCTTTCCTG-3′; promoter III forward, 5′- ATGCAATGCCCTGGAAC-3′; promoter III reverse, 5′-GTGAATGGGAAAGTGGGTG-3′. The annealing temperature of both cases was 59°C. The amplified products were confirmed by running 1.5% agarose gels.

**Western Blot Analysis.** Equivalent amounts of protein for each sample taken from the LA and BLA homogenates were resolved in 12.5% SDS-polyacrylamide gels, blotted electrophoretically to polyvinylidene difluoride membrane, and blocked overnight in Tris-buffered saline (20 mM Tris-base, pH 7.5, and 50 mM NaCl) containing 5% nonfatty milk. For detection of mature BDNF, blots were incubated with anti-BDNF antibody (0.1 μg/ml; Leinco Technology, St. Louis, MO). To control the content of proteins per lane, membranes were stripped with 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl, pH 6.8, for 15 min at 65°C and reprobed with goat anti-β-actin antibody (1:10,000, Santa Cruz Biotechnology). An enhanced chemiluminescence kit (PerkinElmer Life and Analytical Sciences, Waltham, MA) was used for detection. Western blots were developed in the linear range used for densitometry. The density of the immunoblots was determined by an image analysis system installed with the software BIO-ID (Vilber Lourmat, Marne-La-Vallee, France). Mature BDNF levels in each treatment were expressed as a percentage of those in naive controls.

**Histology.** To identify cannula placements, animals received an overdose of pentobarbital (100 mg/kg) at the end of behavioral experiments. The brains were removed from the skull and fixed in buffered 4% paraformaldehyde, pH 7.4, for 48 h. Brains were sectioned with a sliding MicroSlicer (DTK-1000; Ted Pella Inc., Redding, CA), and sections (40 μm thickness) were stained for Nissl bodies.

**Data Analysis.** Differences among the groups were evaluated with one-way ANOVA followed by the Newman-Keuls post hoc tests. The level of significance was $p < 0.05$. All values in the text and figure legends are mean ± S.E.M.

## Results

We investigated whether conditioning-induced increase in BDNF protein required new mRNA and/or protein synthesis by pharmacologically blocking transcription and translation with actinomycin D and anisomycin, respectively. Rats were given intra-amygdala injection of anisomycin (62.5 μg/side, $n = 10$), actinomycin D (1 μg/side, $n = 11$), or vehicle ($n = 5$) 30 min before receiving 10 pairings of light and foot shock. The dose of anisomycin used in the present study has been shown to be effective in blocking fear conditioning (Schafe and LeDoux, 2000). Fear conditioning elicited an increase in BDNF expression compared with the unpaired control. Figure 1A shows that pretraining application of anisomycin or

actinomycin D inhibited conditioning-induced increase in BDNF. ANOVA showed a main effect for group ($F_{3,28}$ = 15.84, $p < 0.001$), and Newman-Keuls post hoc tests revealed that the levels of BDNF were significantly reduced in the anisomycin and actinomycin D groups compared with either paired controls without receiving injection or vehicle (50% DMSO) groups ($p < 0.01$). There was no difference between paired control and vehicle group ($p > 0.05$). Cannula tip placements from rats infused with anisomycin, actinomycin D, or vehicle are shown in Fig. 1D. These data suggest a requirement for gene transcription and protein synthesis in conditioning-induced increase in BDNF protein.

The rat *BDNF* gene consists of four distinct 5′ exons each driven by a specific promoter and each spliced to a common 3′ exon that encodes the BDNF protein (Timmusk et al., 1993). To investigate which exon-containing BDNF transcripts were responsive to fear conditioning, a semiquantitative reverse transcription-coupled polymerase chain reaction assay was used to measure the level of each BDNF mRNA. For this purpose, 5′-end primers were designed that are specific for BDNF exons I, II, III, or IV, and a 3′-end primer was generated that is complementary to sequences in exon V, the exon that is common to all eight BDNF transcripts. Figure 1B shows that fear conditioning significantly increased exon I-containing BDNF transcript at 30 min after training and lasted for at least 4 h ($F_{4,44}$ = 6.31, $n$ = 4, 20, 6, and 8, respectively, for each time point, $p < 0.001$). Likewise, exon III-containing BDNF transcript was significantly increased after conditioning at all 0.5, 1, 2, and 4 h time points ($F_{4,34}$ = 5.08, $n$ = 4, 15, 5, and 8, respectively, for each time point, $p < 0.005$). On the other hand, exon II- and IV-containing BDNF transcripts were not affected by fear conditioning at all 0.5-, 1-, 2-, and 4-h time points (exon II: $F_{4,22}$ = 0.07, $n$ = 3, 12, 3, and 4, respectively, for each time point, $p > 0.5$; exon IV: $F_{4,34}$ = 0.89, $n$ = 3, 13, 5, and 7 for each time point, $p > 0.1$). We have reported previously that BDNF protein level was increased 30 min after training, peaked at 1 h, and returned to control levels within 4 h (Ou and Gean, 2006). A comparison of the time course of BDNF transcripts showed that the exon I- and III-containing BDNF transcripts persist after BDNF translation has shut off, suggesting that these two BDNF transcripts are relatively stable. These results are consistent

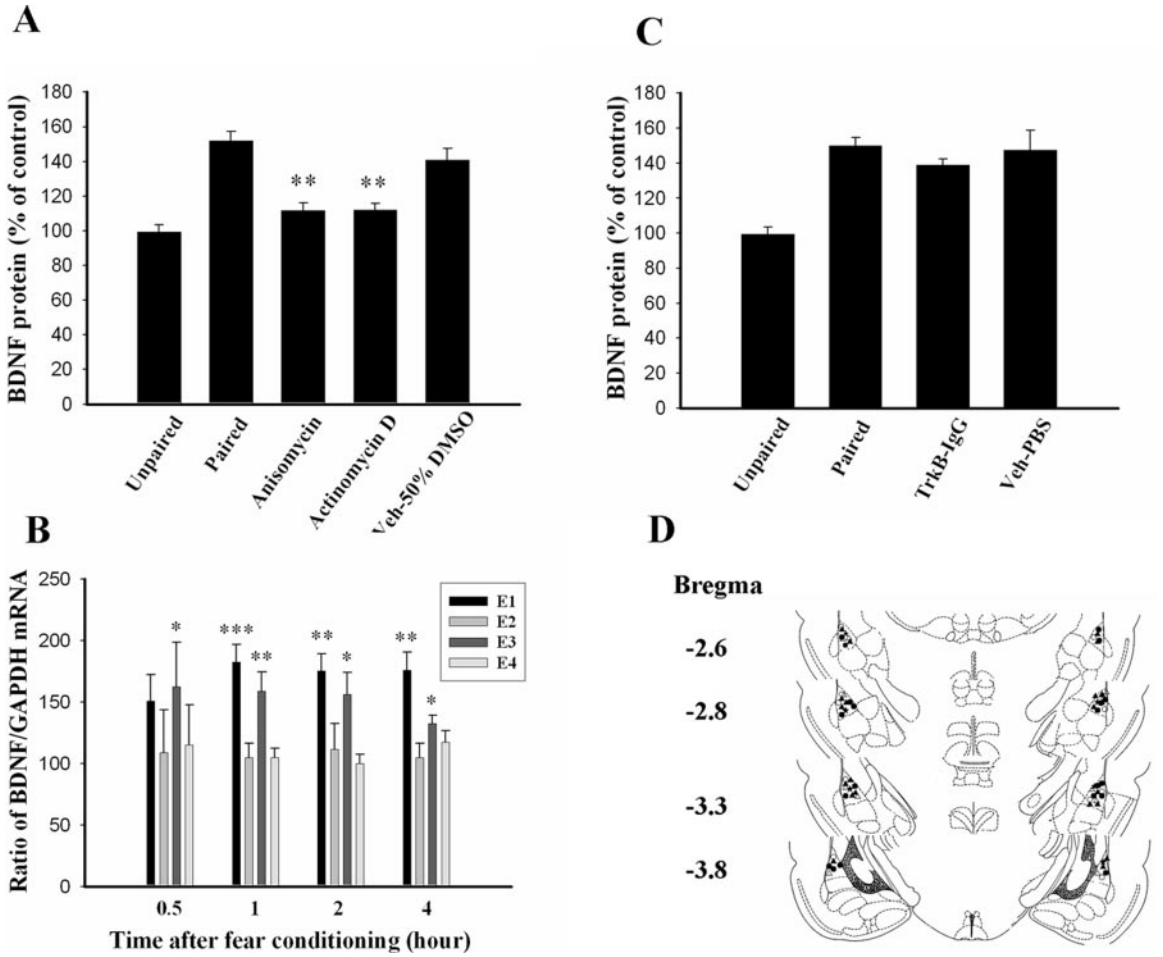


**Fig. 1.** Fear conditioning-induced increase in BDNF protein requires transcription and translation. A, rats were given intra-amygdala injection of anisomycin (62.5 μg/side, $n$ = 10), actinomycin D (1 μg/side, $n$ = 11), or vehicle ($n$ = 5) 30 min before training. One hour after fear conditioning, tissues from the LA and BLA were dissected out. BDNF protein was detected and quantified using ELISA. **, $p < 0.01$ versus vehicle control. B, conditioning increases the expression of exon-specific BDNF transcripts. One microgram of total RNA was reverse-transcribed into single-strand cDNA, and a portion of the cDNA was amplified by real-time PCR with exon-specific primers. GAPDH transcripts were also reverse transcription-coupled polymerase chain reaction-amplified and used as an internal control for RNA. The relative level of four BDNF transcripts was normalized against the GAPDH of the same sample and expressed as a percentage of those in naive controls. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus naive control. C, rats were given intra-amygdala injection of TrkB-IgG (2 μg/side, $n$ = 3) or vehicle ($n$ = 7) 30 min before training. D, cannula tip placements from rats infused with Anisomycin (●), actinomycin D (▲), TrkB-IgG (□), 50% DMSO (○), or PBS(△) in A and C.

with previous in situ hybridization experiments showing a selective increase in BDNF transcripts containing exons I and III in the amygdala after fear conditioning (Rattiner et al., 2004b).

In the hippocampus, BDNF mRNA level was increased after 3 days of voluntary exercise. The exercise-induced increase in mRNA was abolished by blocking TrkB receptor with a Trk-selective tyrosine kinase inhibitor K252a, suggesting that BDNF may regulate its own expression (Vaynman et al., 2004). We examined whether endogenous BDNF might regulate its protein level triggered by fear conditioning by using a TrkB-specific "receptor body" that contains the ligand-binding domain of TrkB receptor coupled to Fc fragment of human immunoglobulin. The compound acts as a false receptor and scavenges unbound TrkB ligands (Patterson et al., 2001). TrkB-IgG (2 $\mu$g/side, $n = 3$) was microinjected bilaterally into the amygdala 30 min before training. Figure 1C reveals that sequestration of BDNF during fear conditioning did not affect conditioning-induced increase in BDNF protein level ($F_{2,14} = 2.15$, $p > 0.2$). Newman-Keuls post hoc tests showed that the level of BDNF was not different from those paired controls without receiving injection or vehicle group ($p > 0.05$). These results suggest that conditional regulation of BDNF is not mediated by BDNF binding to TrkB receptors.

In cultured neurons, the expression of *BDNF* gene is enhanced by an increase in intracellular calcium concentrations (Zafra et al., 1990; West et al., 2001). We determined whether conditioning-induced increase in BDNF protein level required calcium influx through NMDA receptors and/or L-VDCCs. Rats were infused with NMDA receptor antagonist D-APV (12.5 nmol/side, $n = 4$), L-VDCC blocker verapamil (4 $\mu$g/side, $n = 7$), or vehicle ($n = 8$) bilaterally into the amygdala 30 min before training. Treatment with D-APV or verapamil reduced BDNF protein level from 150.0 ± 4.8% ($n = 13$) to 121.9 ± 5.7% ($p < 0.01$) and 118.4 ± 7.1% ($p < 0.01$), respectively (Fig. 2). Furthermore, concomitant administration of D-APV with verapamil completely abolished conditioning-induced increase in BDNF (103.9 ± 9.5%, $n = 7$, $p < 0.01$). These results suggest that the increase in BDNF protein depends on the activation of NMDA receptors and L-VDCCs.

We used selective inhibitors to determine the roles of various signal transduction pathways in conditioning-induced increase in BDNF protein. Rats were infused with PKA inhibitors Rp-cAMPS (40 nmol/side, $n = 7$) and KT5720 (0.5 $\mu$g/side, $n = 6$), CaMKII/IV inhibitors KN93 (10 nmol/side, $n = 13$) and KN62 (340 ng/side, $n = 4$), CaMKII inhibitor myristoylated autocamtide-2-related inhibitory peptide (*m*-AIP, 250 pmol/side, $n = 8$) (Ishida and Fujisawa, 1995), NF-$\kappa$B inhibitor helenalin (40 pmol/side, $n = 12$), mitogen-activated protein kinase kinase inhibitor U0126 (1 $\mu$g/side, $n = 8$), or vehicles (50% DMSO, $n = 5$; $H_2O$, $n = 8$) bilaterally into the amygdala 30 min before training. ANOVA showed a main effect for group ($F_{9,72} = 6.58$, $p < 0.001$), and post hoc tests revealed that the levels of BDNF were significantly reduced in the Rp-cAMPS ($p < 0.001$), KT5720 ($p < 0.001$), KN62 ($p < 0.05$), and KN93 ($p < 0.05$) groups compared with paired group (Fig. 3). By contrast, *m*-AIP, U0126, and helenalin were without effect ($p > 0.05$). These results suggest that PKA and CaMKIV pathways are involved in the conditional regulation of BDNF expression.

Previous studies have shown that calcium influx through L-VDCC induces CREB phosphorylation at Ser133 and that phosphorylation at Ser133 activates CREB to stimulate CRE-dependent transcription (Tao et al., 1998; West et al., 2001). To examine whether binding affinity of phosphorylated CREB to *BDNF* promoter was altered after fear conditioning, DAPA was used to quantify the DNA binding activity of p-CREB. The oligonucleotides corresponding to the sequence of CRE in the proximal region of *BDNF* promoter I and III were used as probes because these probes have been shown to bind to CREB or CREB family proteins in electrophoretic mobility shift assay (Tabuchi et al., 2002). As shown in Fig. 4A, there was a time-dependent increase of p-CREB binding to *BDNF* promoter I after fear conditioning. There were differences between control, 15-min ($n = 10$, $p < 0.001$),

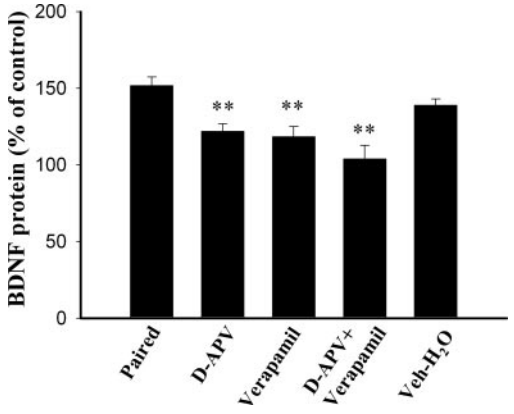


**Fig. 2.** Effects of NMDA receptor antagonist and L-VDCC blocker on the conditioning-induced increase in BDNF protein. Rats were infused with D-APV (12.5 nmol/side), verapamil (4 $\mu$g/side), D-APV plus verapamil, or vehicle bilaterally into the amygdala 30 min before training. One hour later, tissues from the LA and BLA were dissected out and BDNF protein was detected and quantified using ELISA. **, $p < 0.01$ versus paired control.

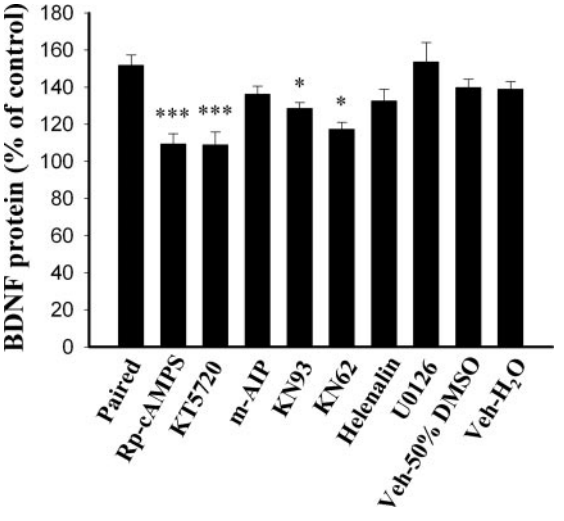


**Fig. 3.** Effects of various signal pathway inhibitors on the conditioning-induced increase in BDNF protein. Rats were infused with Rp-cAMPS (40 nmol/side), KT5720 (0.5 $\mu$g/side), *m*-AIP (250 pmol/side), KN93 (10 nmol/side), KN62 (340 ng/side), helenalin (40 pmol/side), U0126 (1 $\mu$g/side), or vehicles (50% DMSO and distilled $H_2O$) bilaterally into the amygdala 30 min before training. One hour later, tissues from the LA and BLA were dissected out and BDNF protein was detected and quantified using ELISA. *, $p < 0.05$, ***, $p < 0.001$ versus paired control.

and 30-min ($n = 11$, $p < 0.001$) time points. No significant difference was detected between control and 0 ($n = 10$) and 60-min ($n = 4$) time points ($p > 0.05$). Likewise, there was a significant increase of p-CREB binding to *BDNF* promoter III after fear conditioning (Fig. 4B). There were differences between control, 15-min ($n = 4$, $p < 0.05$), and 30-min ($n = 8$, $p < 0.001$) time points. No significant difference was detected between control, 0 ($n = 6$), and 60-min ($n = 3$) time points ($p > 0.05$).

It has been reported that there was an NF-κB binding site within the 5′-flanking region *cis* to exon III of the *BDNF* gene that played an important role in NMDA-mediated neuroprotection (Lipsky et al., 2001). We examined whether NF-κB bound to the promoter region of BDNF exon III was altered after conditioning. Figure 4, C and D, shows that the transcription factors NF-κB p65 and p50 binding to promoter region of BDNF exon III were unaffected by fear conditioning ($p > 0.1$). Furthermore, conditioning-induced increases of p-CREB binding to *BDNF* promoter I and promoter III were significantly reduced by the treatment with D-APV, verapamil, Rp-cAMPS, KT5720, or KN93 (Fig. 5). ANOVA showed a main effect for group ($F_{5,34} = 6.65$, $p < 0.001$) in p-CREB binding to *BDNF* promoter I, and Newman-Keuls post hoc tests revealed that the levels of binding were significantly reduced in the D-APV ($n = 4$), verapamil ($n = 6$), KN93 ($n = 6$), Rp-cAMPS ($n = 6$), and KT5720 ($n = 7$) groups compared with the paired group ($p < 0.01$). Likewise, the levels of p-CREB binding to *BDNF* promoter III were significantly reduced in the D-APV ($n = 4$), verapamil ($n = 8$), KN93 ($n = 8$), Rp-cAMPS ($n = 8$), and KT5720 ($n = 7$) groups compared with paired group ($F_{5,37} = 8.03$, $p < 0.001$).

Because DAPA analyze p-CREB binding activity to *BDNF* promoter in an indirect way, we used ChIP assay to determine whether p-CREB bound to *BDNF* promoter region on the endogenous chromatin. ChIP assay using real-time PCR showed that there was a significant increase of p-CREB binding to *BDNF* promoters I (216.0 ± 33.9%, $n = 4$, $p < 0.05$) and III (235.4 ± 27.7%, $n = 4$, $p < 0.01$) 30 min after fear conditioning (Fig. 6).

To examine whether p-CREB binding to *BDNF* promoter region of exons I and III was important for fear learning, the same DNA probes as shown in Fig. 4, A and B, but without biotinylation were used as CRE decoy DNA to compete for endogenous p-CREB binding so as to inhibit CREB-dependent BDNF transcription. Bilaterally cannulated rats were infused 24 and 2 h before behavioral training with promoter I or III CRE decoy DNAs. Figure 7 shows that both *BDNF* promoter decoy DNAs reduced fear-potentiated startle from 174.7 ± 10.7% ($n = 16$) to 121.0 ± 22.3% ($n = 14$, $p < 0.05$) and 102.0 ± 27.3 ($n = 13$, $p < 0.05$), respectively. The decoy DNA with mutation of two bases in the CRE-like sequence of *BDNF* promoter I, which failed to affect DNA binding activity of CREB (Tabuchi et al., 2002), was without effect ($n = 12$, $P > 0.05$). Likewise, administration of probe with mutation of five bases in the CRE-like sequence of *BDNF* promoter III did not affect fear-potentiated startle ($n = 10$, $p > 0.05$) (Fig. 7). These results suggest that p-CREB binding to CRE sequence of *BDNF* promoter I and III is important for fear memory.

Like other secreted proteins, the 32-kDa pro-BDNF is the main form secreted from the neurons and subsequently is proteolytically cleaved to mature BDNF (Mowla et al., 1999), which is responsible for LTP formation (Pang et al., 2004).

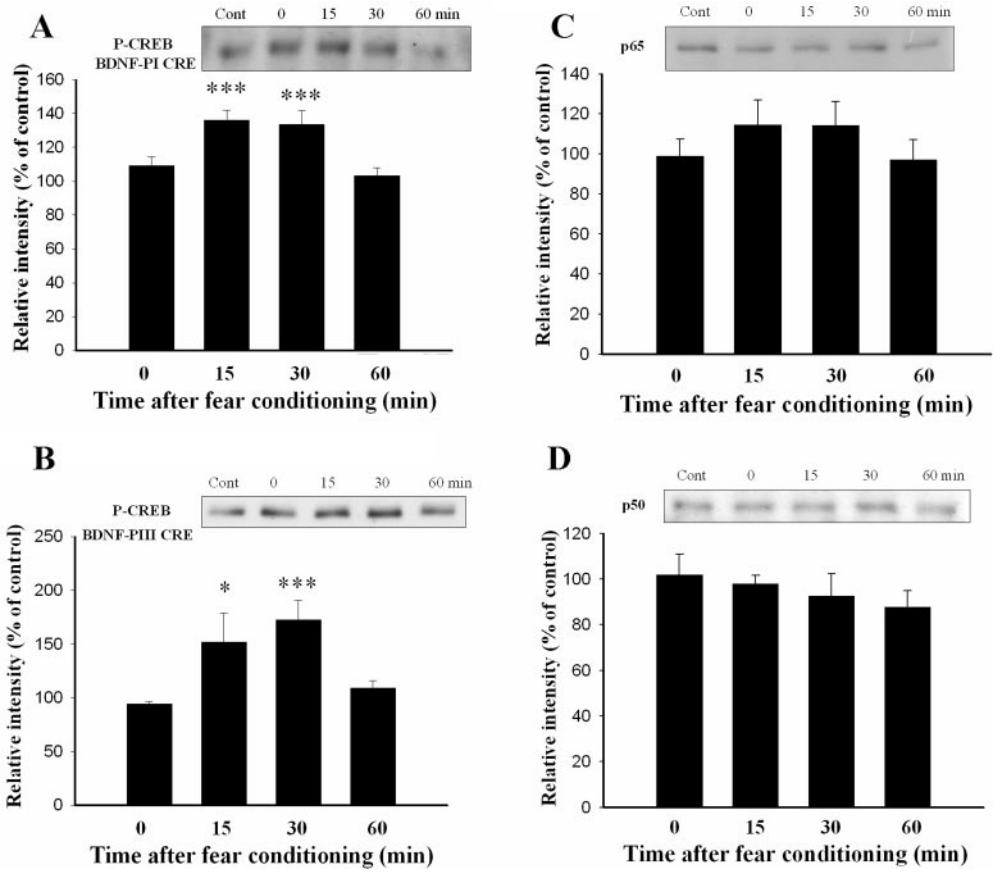


**Fig. 4.** Fear conditioning increases p-CREB binding to *BDNF* promoter regions of exon I and III. At various time points after fear conditioning, whole-cell lysate of the LA and BLA was prepared and then precipitated with biotinylated double-strand DNA of *BDNF* promoter I and III. Samples were then analyzed by immunoblotting with antibody against p-CREB (A and B). C and D, whole-cell lysate was precipitated with biotinylated double-strand DNA of NF-κB binding site of *BDNF* promoter III. Samples were then analyzed by immunoblotting with antibodies against p65 or p50. *, $p < 0.05$, ***, $p < 0.001$ versus control.

Because BDNF ELISA measured both pro-BDNF and mature BDNF, we used an antibody specific for the mature BDNF to determine whether conditioning induced increase in mature BDNF. Figure 8A shows that mature BDNF level was significantly increased after fear conditioning compared with naive controls ($p < 0.01$). The most significant protease that cleaves proneurotrophins is the serine protease plasmin (Lee et al., 2001). We tested whether proteolytical cleavage of pro-BDNF was required for fear learning by infusion of $\alpha_2$-antiplasmin (1 $\mu$g/side, $n = 7$), a 452-amino acid glycoprotein that forms a covalent complex with plasmin and inactivates it (Levi et al., 1993) into the amygdala 30 min before fear conditioning. Figure 8B shows that fear-potentiated startle was significantly reduced in $\alpha_2$-antiplasmin-treated rats compared with vehicle controls ($n = 8$, $p < 0.05$). In parallel, conditioning-induced increase in mature BDNF was blocked by $\alpha_2$-antiplasmin (Fig. 8A).

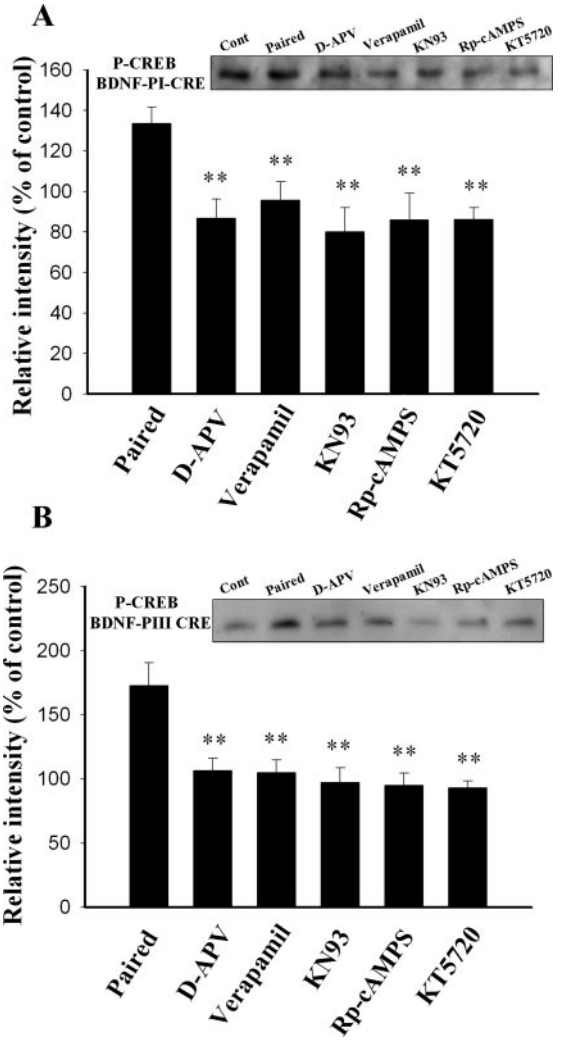


**Fig. 5.** p-CREB binds to exon I and III promoter regions of *BDNF* and its sensitivity to various signal pathway inhibitors. Rats were infused with D-APV (12.5 nmol/side), verapamil (4 $\mu$g/side), KN93 (10 nmol/side), Rp-cAMPS (40 nmol/side), KT5720 (0.5 $\mu$g/side), or vehicle bilaterally into the amygdala 30 min before training. Thirty min later, whole-cell lysate of the LA and BLA was prepared and then precipitated with biotinylated promoter I (A) and III (B) CRE DNA of *BDNF*. Samples were then analyzed by immunoblotting with antibody against p-CREB. **, $p < 0.01$ versus paired control.

## Discussion

It has been shown that conditioning trials of light paired with foot shock elicited an increase in BDNF expression in the amygdala. On the other hand, BDNF expression was not observed in the unpaired controls, suggesting that the increase depends on the learned association between light and foot shock (Rattiner et al., 2004a; Ou and Gean, 2006). In this report, we show a signal cascade of conditioning-induced *BDNF* gene transcription. Three steps in response to fear conditioning are proposed. First, calcium influx through NMDA receptors and L-VDCCs during fear conditioning activates adenylyl cyclase and protein kinase A. Second, activated PKA translocates to nucleus and subsequently induces CREB phosphorylation. Increase in intracellular calcium also activates CaMKIV resulting in the phosphorylation of CREB at Ser133. Third, within the nucleus, activated CREB binds to *BDNF* promoter to stimulate the transcriptional machinery and up-regulate the expression of BDNF in the amygdala.

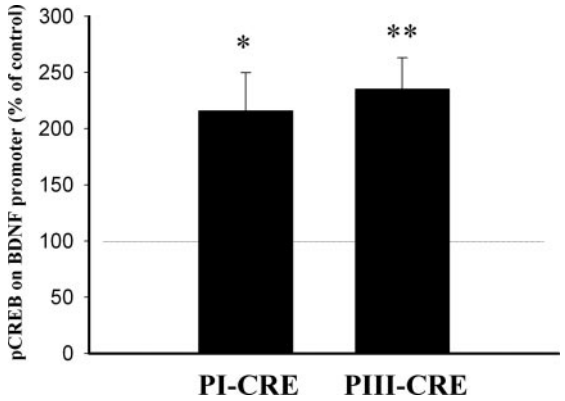


**Fig. 6.** ChIP analysis of p-CREB binding to *BDNF* promoter I and III after fear conditioning. ChIP assay was performed to measure the levels of DNA binding activity of p-CREB at CRE on *BDNF* promoter I and III 30 min after fear conditioning. ChIP data obtained from real-time PCR was normalized to input DNA from each sample. The amounts of p-CREB binding to respective *BDNF* promoters were expressed as a percentage of those in naive controls. *, $p < 0.05$, **, $p < 0.01$ versus naive control.

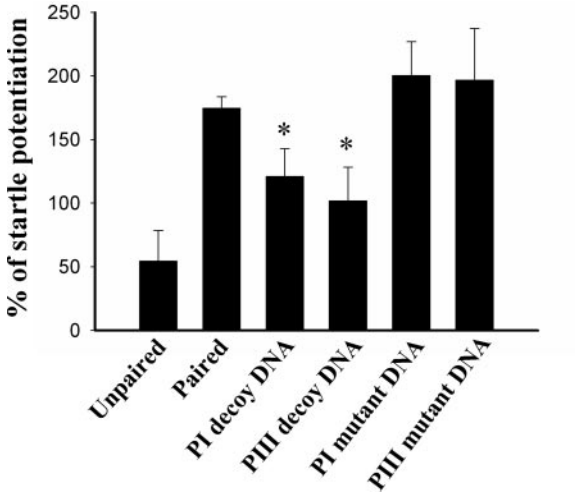


**Fig. 7.** Blockade of fear-potentiated startle by CRE decoy DNA. Rats were given *BDNF* promoter I CRE decoy DNA or promoter III CRE decoy DNA (2 mM, 1 $\mu$l/side) 24 and 2 h before fear training and fear-potentiated startle was tested 24 h later. *, $p < 0.05$ versus paired control.

**NMDA Receptors and L-VDCCs Contribute to BDNF Expression.** LTP at thalamic-LA synapses induced by pairing presynaptic stimulation with postsynaptic depolarization requires calcium influx through L-VDCCs (Weisskopf et al., 1999). On the other hand, calcium entry through NMDA receptors is necessary for LTP induced by tetanic stimulation of cortical-LA pathway (Huang and Kandel, 1998). Block of NMDA receptor in the amygdala impairs both short-term and long-term fear memory, whereas block of L-VDCC selectively disrupts long-term memory (Miserendino et al., 1990; Maren et al., 1996; Bauer et al., 2002). Thus, it is believed that combined activation of NMDA receptor and L-VDCC contributes to fear memory formation (Bauer et al., 2002). In cortical and hippocampal neurons, activation of L-VDCCs or NMDA receptors leads to an enhancement of BDNF mRNA levels and stimulates release of BDNF protein (Zafra et al., 1990; West et al., 2001). BDNF exon I responded predominantly to calcium signals evoked via activation of L-VDCC, whereas BDNF exon III responded to calcium influxes through L-VDCCs and NMDA receptors. Direct stimulation of NMDA receptor supported the activation of *BDNF* promoter III but not that of *BDNF* promoter I (Tabuchi et al., 2000). In the present study, we found that NMDA receptor antagonist and L-VDCC blocker inhibited conditioning-induced increase in BDNF protein to almost the same degree. A combination of both blockers completely abolished the increase. The requirement of L-VDCC and NMDA receptor for conditioning-induced BDNF protein increase resembled that of BDNF exon III-containing transcript in cortical neurons.

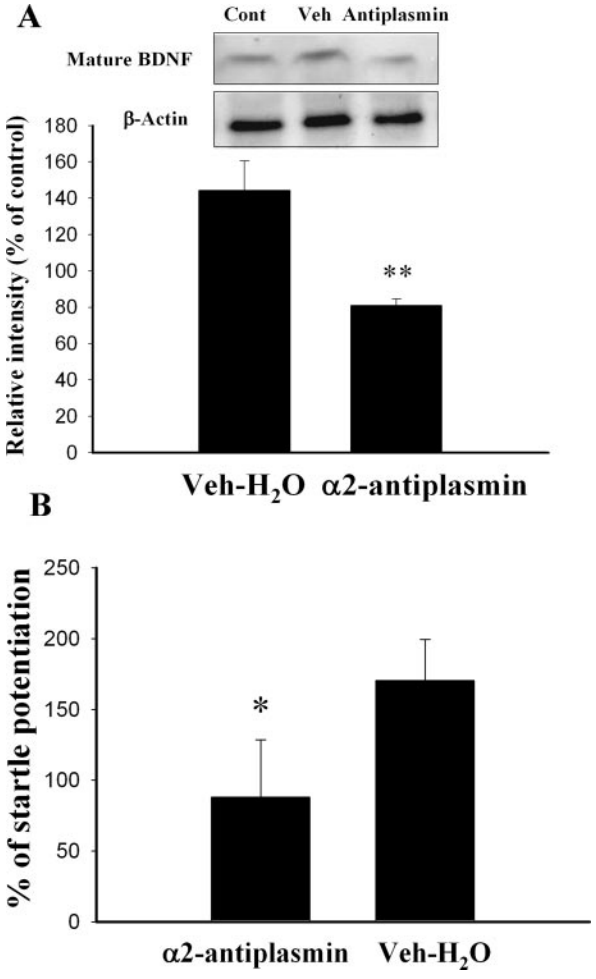


**Fig. 8.** Proteolytic cleavage of pro-BDNF is required for fear memory formation. A, 1 h after fear conditioning, protein level of mature BDNF was detected by Western blotting using an antibody specific for the mature BDNF. Conditioning-induced increase in mature BDNF was significantly reduced in $\alpha_2$-antiplasmin (1 $\mu$g/side)-treated rats. B, pretraining administration of $\alpha_2$-antiplasmin (1 $\mu$g/side) significantly reduced fear-potentiated startle. *, $p < 0.05$, **, $p < 0.01$ versus vehicle control.

**Signal Cascades.** We have applied pharmacological inhibitors to delineate the signal pathway downstream of calcium influx. The results showed that infusion of Rp-cAMPS, KT5720, KN62, and KN93 but not *m*-AIP or helenalin into the amygdala before training significantly reduced conditioning-induced increase in BDNF protein. Rp-cAMPS is an antagonist of cAMP binding to regulatory subunit of PKA, whereas KT5720 is an ATP analog that competitively inhibits its catalytic subunit of PKA binding to ATP. Reduction of conditioning-induced increase in BDNF protein by both Rp-cAMPS and KT5720 strongly suggests the involvement of PKA. Thus, calcium influx activated adenylyl cyclase resulting in the increase of cAMP. Gene transcription induced by cAMP is mediated through the activation of PKA and phosphorylation of CREB. Phosphorylated CREB binds to the conserved cAMP response element TGACGTCA typically found in the promoter of many cAMP-responsive genes. The DAPA and ChIP experiments further revealed a significant increase of p-CREB binding activity to *BDNF* promoter region of exons I and III during the consolidation of fear memory.

KN62 and KN93 are CaMK II and IV inhibitors, whereas *m*-AIP specifically inhibits CaMKII. We found that KN62 and KN93 but not *m*-AIP reduced conditioning-induced BDNF protein levels, suggesting that CaMKIV is involved in BDNF expression. Taken together, these results suggest that activation of PKA and CaMKIV pathways in response to fear conditioning occurs in concert to regulate the expression of BDNF.

It is noteworthy that that CREB phosphorylation and BDNF expression were increased 30 to 60 min after conditioning, suggesting that BDNF is involved in the consolidation. However, all injections of inhibitors occurred before training. The possibility that drugs' effects on consolidation were compounded with effects on initial encoding could not be excluded.

**Mature BDNF and Fear Conditioning.** BDNF arises from precursor pro-BDNF, which is proteolytically cleaved to mature BDNF (Mowla et al., 1999). Pro-BDNF binds with high affinity to $p75^{NTR}$, whereas mature BDNF binds preferentially to TrkB receptors. Both pro-BDNF and BDNF have been implicated in neuronal plasticity and apoptosis (Poo, 2001; Lu et al., 2005; Teng et al., 2005). However, pro- and mature BDNF in general work in opposition. Mature BDNF is growth-promoting and is a key protein-synthesis product that is required for long-term synaptic modifications underlying late-phase LTP (Pang et al., 2004). On the other hand, pro-BDNF is proapoptotic and promotes NMDA-dependent long-term depression probably via the regulation of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor expression (Lee et al., 2001; Rosch et al., 2005). We showed that fear conditioning significantly increased the protein level of mature BDNF. It is noteworthy that pretraining administration of plasmin inhibitor significantly reduced

fear-potentiated startle, suggesting that mature BDNF is the active form that mediates fear conditioning.

In summary, this study not only characterizes the signal pathway of transcriptional regulation of BDNF expression during consolidation of fear memory but also demonstrates that mature BDNF is the active form that mediates fear conditioning. The 32-kDa pro-BDNF is the main form secreted from neurons (Mowla et al., 1999), and mature BDNF is derived primarily from the cleavage of pro-BDNF by extracellular proteases. Thus, extracellular proteases such as plasmin may become a potential target of therapeutic intervention for the treatment of post-traumatic stress disorders.

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