Research Paper

Brain-derived neurotrophic factor in the anterior cingulate cortex is involved in the formation of fear memory

LI Qing-Qing¹, LI Bao-Ming¹,²*

¹Institute of Neurobiology, State Key Laboratory of Medical Neurobiology, and Innovation Center for Brain Science, Fudan University, Shanghai 200032, China; ²Center for Neuropsychiatric Disorders, Institute of Life Science, Nanchang University, Nanchang 330031, China

Abstract: Brain-derived neurotrophic factor (BDNF), a small dimeric secretory protein, plays a vital role in activity-dependent synaptic plasticity, learning and memory. It has been shown that BDNF in the hippocampus and amygdala participates in the formation of fear memory. However, little is known about the functional role of BDNF in the anterior cingulate cortex (ACC). To address this question, we examined the mRNA and protein levels of BDNF in the ACC of rats at various time points after fear conditioning, using quantitative real-time PCR and enzyme-linked immunosorbent assay (ELISA). The results showed that BDNF exhibited a temporally specific increase in both mRNA and protein levels after CS (tone) and US (foot shock) was paired. Such increase did not occur after the animals were exposed to CS or US alone. When BDNF antibody was locally infused into the ACC prior to CS-US pairing, both contextual and auditory fear memories were severely impaired. Taken together, these results suggest that BDNF in the ACC is required for the formation of fear memory.

Key words: BDNF; anterior cingulate cortex; fear conditioning; rats

Brain-derived neurotrophic factor (BDNF), the most abundant member of neurotrophins, is widely expressed in the central nervous system, especially in those regions related with higher cognitive functions, such as...
the cerebral cortex, hippocampus, amygdala, striatum, and cerebellum. A great body of evidence indicates that BDNF not only regulates neuronal survival and differentiation, but also exerts vital effects on synaptic plasticity, long-term potentiation, learning and memory.

Increasing evidence demonstrates that blocking BDNF function via delivering anti-BDNF antibody or BDNF antisense oligonucleotide into the hippocampus or amygdala leads to impaired memory formation in contextual fear conditioning, inhibitory avoidance tests, or fear-potentiated startle. Despite the importance of BDNF in learning and memory function, most studies are centered on the hippocampus and amygdala, while reports focusing on the role of BDNF in the anterior cingulate cortex (ACC) in learning and memory are limited.

The ACC, a very important part of the forebrain, plays diverse roles in many higher brain functions, such as anticipation, pain, attention, error monitoring, and effortful recall. Moreover, activity-dependent gene imaging and regional inactivation studies have shown that the ACC is involved in fear and spatial memory. For example, muscimol-induced inactivation of the ACC blocked the formation of both contextual and auditory fear memories. Expression of zif268 and c-fos in the ACC was greatly elevated following the retrieval of 36-day contextual fear memory. Considering the crucial role of the ACC in higher brain functions, it would be of great interest to investigate whether BDNF in the ACC is involved in learning and memory. In the present study, we used real-time quantitative PCR and ELISA techniques to investigate the post-conditioning expression of BDNF in the ACC, and locally infused BDNF antibody into the ACC to see if fear memory formation was affected.

1 MATERIALS AND METHODS

1.1 Subjects
Male Sprague-Dawley rats (200–250 g) were housed 2–3 per cage under constant temperature (23 °C ± 1 °C) with a 12-h light/dark cycle. Water and food were available ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals issued by National Institutes of Health (NIH), and were approved and monitored by the Ethical Committee of Animal Experiments at the Fudan University Institute of Neurobiology (Shanghai, China).

1.2 Apparatus
The training chamber was constructed of stainless-steel bars (height/width/length: 33 × 26 × 31 cm³) and was equipped with metal grid floor (Coulbourn Instruments, USA). The chamber was enclosed in a ventilated and sound-attenuating cabinet, and a diffuse single light was turned on during the experiment. The photo beam of the chamber, which was linked to a computer, and FreezeFrame software (Coulbourn Instruments, USA) automatically monitored freezing responses of the animals. The sampling rate was four times per second. Freezing behaviour was defined as the absence of any visible movement except respiration and quantified as freezing score by FreezeFrame.

1.3 Behavioral procedures
On the training day, rats were subjected to a conditioning session, which consisted of several procedures. For starters, rats were placed in the training chamber for 2 min. Then, a tone conditioned stimulus (CS; 2 200 Hz and 96 dB for 20 s) was given and co-terminated with a foot shock unconditioned stimulus (US; 0.5 mA, 0.5 s). Such CS-US paring was repeated for five times, with inter-pairing interval of 90–120 s. Rats were returned to their home cages after conditioning.

Four control groups were included: 1) rats in the naïve group were handled for 7 d, but never exposed to the training chamber; 2) rats in the context control group were exposed to the training chamber for 10 min without receiving any tone and foot shock; 3) rats in the CS-alone group experienced the same training procedures as the CS-US pairing group except that no US was given; and 4) rats in the US-alone group were exposed to one foot shock (0.5 mA, 2.5 s) immediately after placed in the chamber and remained in the chamber for 10 min.

Contextual and auditory fear memory tests were performed 24 h post-conditioning. For the contextual fear memory test, rats were exposed to the training chamber for 3 min. The freezing score during this period was used as a behavioral measure for the contextual fear memory. For the auditory fear memory test, rats were placed into a novel chamber for 90 s and were then given three CS presentations, each lasting 30 s with inter-CS interval of 20 s. The freezing score during the CS presentations was used as a behavioral measure of the auditory fear memory.
1.4 Real-time quantitative PCR

After conditioning, rats in the experimental group were sacrificed at different time points ($n = 4$ per time point). Brains were removed after decapitation and placed into Aryl Brain Matrix (Large rat, coronal). The ACC was dissected according to the coordinates from Paxinos and Watsons [26], and frozen in liquid nitrogen followed by RNA extraction.

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. A 1-μg aliquot of each sample was reversely transcribed using the SuperScript™ III First Strand kit (Invitrogen, Carlsbad, CA, USA). To determine the relative amount of cDNA per sample, we performed real-time quantitative PCR in the Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using protocols provided by ReverTra Ace qPCR RT Kit (TOYOBO). Primer sequences were as follows: BDNF forward primer: 5’-TCACAGTCTCTG-GAGAAAGTC-3’, and reverse primer: 5’-ATGAC-CGCCAGCCAATCTC-3’ [8]; and β2 from each sample was also amplified to serve as an internal control (forward: 5’-TCTTTCTGGTGCTTGTCTC-3’, and reverse: 5’-AGTGTGAGCCAGGATGTAG-3’) [8]. Each sample was assayed in triplicate, and the mRNA levels were normalized for each well to the β2 mRNA levels using the $2^{-\Delta\Delta CT}$ method.

1.5 ELISA

The ACC was dissected at various time points after conditioning ($n = 4$ per time point) and frozen in liquid nitrogen. Brain tissue samples were homogenized in ice-cold lysis buffer NP-40 (Beyotime, China). The tissue homogenate solutions were centrifuged at 12 000 g for 20 min at 4 ºC. The supernatants were collected and used for quantification of total protein and BDNF levels. The total protein of each sample was assayed using the Micro BCA™ Protein Assay Kit (Thermo), and the color change was measured in a plate reader at 562 nm. BDNF levels were assessed using the BDNF E max ImmunoAssay System (Promega, USA) according to the manufacturer’s instructions. Each BDNF standard and sample (300 μg) was assayed in duplicate.

1.6 Surgery

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige SN-2, Japan). They were implanted bilaterally with stainless steel guide cannulae (outer diameter: 0.80 mm; inner diameter: 0.65 mm; length: 6 mm) aimed at the ACC (bregma, +2.7 mm; lateral, ±0.7 mm; depth, 1.0 mm). Dummy cannulae, 0.5 mm longer than the guide cannulae, were inserted into the guide cannulae to prevent clogging and reduce the risk of infection. Rats were given at least 5 days to recover before behavioral training.

1.7 Drug administration

Rats were held gently before drug infusion. The dummy cannulae were replaced with infusion cannulae (outer diameter: 0.40 mm). The tip of the injection needle was 1.5 mm beyond that of the guide cannulae, i.e., 2.5 mm beneath the skull surface. The infusion cannula was connected via polyurethane tubing to a 5-μL micro-syringe. BDNF antibodies (dissolved in 0.01 mol/L PBS, 1 μg/μL, Millipore Bioscience Research Reagents) were infused into the ACC bilaterally (0.5 μL/side) at a rate of 0.25 μL/min. The injection needles were left in the position for an additional 2 min after the infusion was completed. Infusions were performed 15 min pre-conditioning.

1.8 Histology

To verify location of infusions, rats were anesthetized with an overdose of sodium pentobarbital (80 mg/kg, i.p.). Rats were transcardially perfused with 0.9% saline solution, followed by 4% (v/v) formalin. After decapitation, brains were removed and submerged into 30% (w/v) sucrose solution until they sank. Then, the brains were cut into sections of 40 μm with a freezing cryostat (Leica, Germany). Brain sections were mounted on gelatin-subbed glass slides and stained with neural red (1% in ddH2O). The locations of the injection needle were examined under a light microscope.

1.9 Data analysis

Data were expressed as means ± SEM. Differences among the groups were evaluated with ANOVA followed by the Dunnett’s test, ANOVA with a post hoc quadratic trend analysis, Fisher’s least significant difference (LSD) test, or unpaired t test. A probability level of less than 0.05 was accepted as statistically significant. Data analysis was performed using the SigmaPlot software (Version 12.1; StatSoft, USA) or the SPSS (Version 22.0; IBM Corp.) statistics package.

2 RESULTS

2.1 BDNF shows a temporal change in expression after conditioning

To investigate the role of BDNF in the ACC, we initially
examined BDNF gene expression at various time points after fear conditioning using real-time quantitative PCR. As shown in Fig. 1A, BDNF mRNA significantly increased at 2 h, peaked at 4 h, and returned to control levels at 6 h after fear conditioning (ANOVA: quadratic trend analysis; \( F_{1, 18} = 30.35; P < 0.01 \)). Post hoc LSD multiple comparison revealed that BDNF mRNA levels at 2 \((P < 0.01)\) and 4 h \((P < 0.01)\) post-conditioning increased significantly, compared with that in the naïve control group. At other time points BDNF mRNA showed no significant difference with the naïve control \((P > 0.05)\). Thus, the mRNA level of BDNF demonstrated a temporarily specific increase after fear conditioning.

To test whether the increased BDNF mRNA would be accompanied by BDNF protein elevation, we determined BDNF protein levels in the ACC at different time points after conditioning. As shown in Fig. 1B, BDNF protein showed an increase at 2 h post-conditioning, peaked at 4 h, and returned to baseline at 6 h (ANOVA: quadratic trend analysis; \( F_{1, 18} = 52.55; P < 0.01 \)) post-conditioning in the ACC, which was consistent with the change of BDNF mRNA. Similarly, the LSD’s post hoc comparison found BDNF protein levels at 2 \((P < 0.01)\) and 4 h \((P < 0.001)\) post-conditioning increased significantly, compared with that in the naïve control group. Thus, there was an up-regulation of BDNF mRNA transcription and protein translation during consolidation of fear memory.

### 2.2 Up-regulation of BDNF expression occurs only after CS-US paring

The present experiments show that there was a temporal change in BDNF expression in the ACC after fear conditioning. However, as we all know, three different stimuli existed in the conditioning procedure, i.e., the context, the tone (CS) and the foot shock (US). Then, which factor was the critical one that induced the change of BDNF expression? To address this question, rats were separated into the five groups: naïve group, context group, CS-alone group, US-alone group, and CS-US pairing group. Rats of these groups were either

---

**Fig. 1.** Temporal change of BDNF expression after fear conditioning. A: Change of BDNF mRNA levels in the ACC after conditioning, as normalized to naïve control. B: BDNF protein levels in the ACC at 0.5, 1, 2, 4 and 6 h post-conditioning. Mean ± SEM, \( n = 4 \). **P < 0.01, ***P < 0.001 vs naïve control.

**Fig. 2.** Fear memory is established only after CS-US pairing. A: Freezing scores of different groups in the contextual fear memory test. B: Freezing scores of different groups in the auditory fear memory test. Mean ± SEM, \( n = 4 \). ***P < 0.001 vs naïve group.
sacrificed 4 h after training/pseudo-training, or tested 24 h later for the presence of freezing response. As shown in Fig. 2, only those animals that had received CS-US paring showed a significantly higher freezing score, as compared with naïves (Fig. 2A, contextual fear memory, ANOVA: $F_{4,15} = 189.653, P < 0.001$ for CS-US pairing vs naïve; Fig. 2B, auditory fear memory, ANOVA: $F_{4,15} = 142.404, P < 0.001$ for CS-US pairing vs naïve), indicating that fear response to the context or the tone could be acquired only when the CS and US was paired.

Animals were sacrificed 4 h post-training/pseudo-training, and brains were prepared for real-time quantitative PCR and ELISA analysis with the probes described above. The time point was so chosen because we previously found that BDNF expression was up-regulated 4 h post-conditioning (Fig. 1). As shown in Fig. 3A, BDNF mRNA level was elevated only in the CS-US paring group (ANOVA: $F_{4,15} = 12.339, P < 0.001$ for CS-US pairing vs naïve). Consistently, BDNF pro-

![Fig. 3. CS-US pairing induces BDNF expression in the ACC. A: Relative levels of BDNF mRNA after context, CS-alone, US-alone, and CS-US paring treatments. B: Relative levels of BDNF protein after context, CS-alone, US-alone, and CS-US paring treatments. Mean ± SEM, n = 4. ***P < 0.001 vs naïve group.](image)

![Fig. 4. Antibody blockade of BDNF in the ACC impairs fear memory. Rats received bilateral intra-ACC infusions of BDNF antibody or 0.01 mol/L PBS (vehicle) 15 min prior to conditioning. Contextual and auditory fear memory tests were performed 24 h post-conditioning. A: Freezing scores in contextual fear memory test. B: Freezing scores in auditory fear memory test. Mean ± SEM, n = 8. *P < 0.05 vs control (vehicle). C: Representative microphotograph and reconstruction of the infusion sites in the ACC. Open circles: vehicle infusion; filled circles: BDNF antibody infusion. Scale bar, 600 μm.](image)
tein level was increased only in the CS-US paring group (Fig. 3B; ANOVA: $F_{4,15} = 9.209, P < 0.001$ for CS-US pairing vs naïve). These results further confirmed that CS-US pairing was the only critical factor that triggered the up-regulation of BDNF expression in the ACC.

2.3 Antibody blockade of BDNF in the ACC impairs fear memory

Although BDNF expression was regulated after fear conditioning, it was unclear if endogenous BDNF in the ACC is functionally necessary for the formation of fear memory. To address this question, we bilaterally infused BDNF antibody into the ACC 15 min prior to fear conditioning. The same volume of vehicles was infused as control. When tested 24 h post-conditioning, the BDNF antibody group exhibited significantly lower freezing score relative to the vehicle group (Fig. 4A, contextual fear memory, $P < 0.05$; Fig. 4B, auditory fear memory, $P < 0.05$, unpaired t-test). The placements of cannulae are shown in Fig. 4C. Thus, BDNF in the ACC is required for the formation of both contextual and auditory fear memories.

3 DISCUSSION

The present study provided evidence that BDNF in the ACC was involved in fear memory formation. BDNF exhibited a temporally specific increase in both mRNA and protein levels after CS (tone) and US (foot shock) paring. Such increase did not occur after rats were exposed to CS or US alone. Furthermore, the antibody blockade of BDNF in the ACC impaired both contextual and auditory fear memories.

Evidence from both human and animal studies have demonstrated that the ACC consists of two functionally distinct regions: the rostral ACC (rACC) and the caudal ACC (cACC) . Experiments of fMRI studies in human subjects indicate that the rACC is activated by emotional tasks, whereas the cACC is activated by cognitive tasks . Experiments investigating the effects of rACC and cACC lesions in rats suggest that, the rACC, but not the cACC, is involved in aversive learning . Therefore, the present study only focused on the rACC.

We found that BDNF expression was temporally induced in the ACC after fear conditioning. Both BDNF mRNA and protein levels in the ACC increased at 2 h post-conditioning, peaked at 4 h and returned to control level at 6 h post-conditioning. This suggests that BDNF in the ACC contributes to the consolidation of fear memory. Elevated BDNF expression levels in the ACC only occurred after CS-US pairings, but not after treatment with CS or US alone, indicating that an interaction of the representations of the CS and US is obligatory for BDNF involvement in fear memory formation. These results are consistent with a number of previous studies that report a selective induction of BDNF expression in the hippocampus and amygdala during contextual fear condition and fear-potentiated startle learning . Thus, our results suggest that BDNF gene is up-regulated at the level of transcription and translation as well, and such regulation is closely related with fear memory formation.

We next found that, bilateral intra-ACC infusion of BDNF antibody prior to fear conditioning impaired both contextual and auditory fear memories. This is in agreement with previous pharmacological study showing that inactivation of the ACC impaired both contextual and auditory fear memories . Anatomical studies have shown that the prefrontal cortex receives a direct projection from the hippocampal CA1/subicular region . The ACC is highly interconnected with other sub-regions of the prefrontal cortex, such as the prelimbic and infralimbic cortical areas , and with the amygdala . It is known that the amygdala is central for the CS to acquire aversiveness and the hippocampus primarily processes the information associated with fear context . Thus, it is possible that the ACC participates in the formation of contextual and auditory fear memories through its anatomical and functional connections with the hippocampus and amygdala.

It is well established that BDNF signaling is mediated by two different receptors: TrkB and p75NTR . So far, most synaptic effects of BDNF are coursed by TrkB activation. BDNF binds to TrkB, triggering the activation of several signaling pathways involving phosphatidylinositol 3 kinase (PI3K), phospholipase C gamma (PLCγ), and mitogen-activated protein kinase (MAPK) . Accumulating evidence suggests that BDNF/TrkB signaling in the hippocampus and amygdala plays a crucial role in learning and memory. For example, BDNF/TrkB/PI3K signaling in the hippocampus is activated when rats received radial arm maze training for spatial reference and working memory . Moreover, BDNF/TrkB/MAPK signaling appears to be activated in the hippocampus during acquisition of fear
memory. In addition, BDNF enhances fear-potentiated startle response likely via TrkB-mediated MAPK and PI3K signaling pathways in the amygdala. Considering that BDNF in the ACC exerts a vital role in the formation of fear memory, it is of importance for future studies to determine which, of the TrkB-mediated signaling cascades, is required for the formation of fear memory.

ACKNOWLEDGEMENTS: We gratefully acknowledge the technical assistance of XU Kai-Jing, HUANG Qian, and SUN Yue at the Fudan University Institute of Neurobiology and State Key Laboratory of Medical Neurobiology.

REFERENCES


Chen J, Kitanishi T, Ikeda T, Matsuki N, Yamada MK. Contextual learning induces an increase in the number of hippocampal CA1 neurons expressing high levels of BDNF. Neurorobiol Learn Mem 2007; 88(4): 409–415.


