Neuropsychopharmacology (2012) 37, 2671–2683

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PI3K/Akt Signaling Pathway in the Basolateral Amygdala Mediates the Rapid Antidepressant-like Effects of Trefoil Factor 3

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Depression is one of the most common and debilitating psychiatric illnesses around the world, but the current antidepressants used to treat depression have many limitations. Progressively more studies have shown that neuropeptide systems are potential novel therapeutic targets for depression. However, whether the neuropeptide trefoil factor 3 (TFF3) participates in the development of depression has not been examined. In the current experiments, we assessed the antidepressant effects of TFF3 using the forced swim test (FST), tail suspension test (TST), and chronic mild stress (CMS) paradigm. Furthermore, we determined the mechanism that underlies the antidepressant-like effects of TFF3 in the rat FST. TFF3 dose-dependently reduced immobility time in both FST and TST. CMS elevated plasma TFF3 and decreased basolateral amygdala (BLA) TFF3 levels in rats, and acute TFF3 (0.1 mg/kg, i.p.) treatment reversed the depressive-like behaviors induced by CMS. Furthermore, TFF3 (0.1 mg/kg, i.p.) significantly increased Fos expression in the BLA, medial prefrontal cortex, and hypothalamus in rats subjected to the FST. Intra-BLA infusions of TFF3 (1 ng/side) exerted rapid antidepressant-like effects in the rat FST. Additionally, acute systemic TFF3 administration increased the level of phosphorylated-Akt (p-Akt) in the BLA. Finally, intra-BLA infusions of LY294002 (5 mM/side), a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, significantly blocked the antidepressant-like effect of TFF3. Our results demonstrated that TFF3 exerts antidepressant-like effects that might be mediated by the PI3K/Akt signaling pathway in the BLA. These findings suggest a novel neuropeptide system target in the development of new antidepressants.
Neuropsychopharmacology (2012) **37,** 2671–2683; doi:10.1038/npp.2012.131; published online 25 July 2012

Keywords: depression; neuropeptide; trefoil factor 3; basolateral amygdala; phosphatidylinositol 3-kinase; chronic mild stress

INTRODUCTION

Depression is a serious mental disorder that affects approximately 16% of the population and is one of the top three most widespread and debilitating illnesses worldwide (Kessler *et al*, 2003). Current treatments are dominated by conventional antidepressants that exert their therapeutic effects through an interaction with serotonin and norepinephrine systems. Unfortunately, not all depressed patients respond to existing antidepressants, and months of treatment are usually required for a full therapeutic response (Cassano and Fava, 2004). Although newer types of antidepressants are better tolerated and safer than older tricyclic compounds, they still produce troublesome side effects (Schatzberg, 2007; Uher *et al*, 2009). Thus, better antidepressants from the perspectives of efficacy and safety are needed.

Neuropeptides that act as neurotransmitter/neuromodulators or growth factors have been shown to participate in many important physiological and pathologic processes, such as sleep, emotion, memory, and the immune response (Shiba *et al*, 2010; Stengel and Tache, 2010; Thorsell, 2010). Numerous studies suggested that many neuropeptides participate in stress-related disorders and exert antidepressant effects at both the preclinical and clinical levels (Alldredge, 2010; Lu *et al*, 2006; Maruyama *et al*, 2004; Rotzinger *et al*, 2010; Schmidt and Duman, 2010; Sergeyev *et al*, 2005; Thorsell, 2010). Therefore, neuropeptide systems may be novel therapeutic targets for depression (Holmes *et al*, 2003; Madaan and Wilson, 2009; Mathew *et al*, 2008).

Neuropeptide trefoil factors (TFFs) are major secretory products of mucin-producing cells and can be synthesized in the brain (Griepentrog *et al*, 2000). Three types of TFFs

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Received 9 March 2012; revised 25 May 2012; accepted 18 June 2012

have been characterized in mammals and humans: TFF1 (also designated pS2), TFF2 (hSP), and trefoil factor 3 (TFF3) (hP1.B/hITF). Evidence has shown that TFF3 can act on the epidermal growth factor receptor (EGFR), which then activates several downstream signaling pathways, including mitogen-activated protein kinases and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Baus-Loncar and Giraud, 2005). TFF3 stimulated epithelial migration by phosphorylating EGFRs and then activating downstream pathways (Peterson *et al*, 2009). The previous studies of TFF3 have focused on its maintenance of mucosal integrity and the promotion of repair. Evidence suggests that TFF3 serves as an initiator of mucosal healing to enable the acute restoration of cellular continuity, and TFF3-deficient mice showed an impaired capacity for mucosal recovery after colonic injury even after exposure to antineoplastic agents and total body radiation (Beck *et al*, 2004; Mashimo *et al*, 1996). Additionally, a recent clinical report showed that a recombinant human TFF3 (rhTFF3) oral spray formulation was safe and effective for the treatment of chemotherapy-associated oral mucositis in patients with colorectal cancer (Peterson *et al*, 2009).

The neural expression of TFF3 has been observed in the hypothalamus (ie, magnocellular neurons of the supraoptic and paraventricular nuclei) and amygdala, which were revealed by immunohistochemistry (IHC) and real-time polymerase chain reaction (Probst *et al*, 1996; Suemori *et al*, 1991; Wang *et al*, 2010). However, the role of TFF3 in the central nervous system (CNS) has not yet been clarified. Previous studies suggested that TFF3 influences the development of the CNS. Lack of TFF3 resulted in hearing impairment and accelerated presbyacusis (Lubka *et al*, 2008). However, some reports found no obvious neural abnormalities in transgenic TFF3 knockout mice (Mashimo *et al*, 1996). TFF3 administration induced Fos protein expression in magnocellular oxytocin neurons in the hypothalamus (Derbyshire and Ludwig, 2004). TFF3 was found to colocalize with oxytocin in the paraventricular nuclei of the hypothalamus, suggesting similar roles for TFF3 and oxytocin (Griepentrog *et al*, 2000; Jagla *et al*, 2000). However, few studies have examined the role of TFF3 in the regulation of mood-related disorders. Using the passive avoidance test and elevated plus maze, Schwarzberg and colleagues found that bilateral application of TFF3 into the amygdala exerted anxiolytic-like effect at a low dose and an anxiogenic-like effect at a high dose (Schwarzberg *et al*, 1999), but the effect of TFF3 on major depression has not yet been elucidated.

The present study examined the effects of TFF3 on depressive-like behaviors in both acute and chronic stress models of depression. To investigate the underlying mechanisms, we evaluated critical brain areas and the role of the PI3K/Akt signaling pathway in the antidepressant-like effects of TFF3.

MATERIALS AND METHODS

Animals

Male ICR mice, weighing 18–22 g upon arrival, and male Sprague–Dawley rats, weighing 220–240 g upon arrival, were individually housed under a constant temperature (23 ± 2 °C) and maintained on a 12 h/12 h light/dark cycle (lights on at 2000 pm and off at 0800 am) with free access to food and water. All of the procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Local Animal Use Committee. All of the behavioral tests and drug administrations were performed during the animals' dark phase.

Drugs

Recombinant human TFF3 (rhTFF3) (purity > 98%) was purchased from Beijing Yong Kang Jia Xin Science and Technology Development (China) and freshly prepared in saline. Murine TFF3 has been shown to share 70% inferred amino acid identity with human TFF3 (Mashimo *et al*, 1995). We sought to develop a safe and effective antidepressant treatment for depressed patients; therefore, rhTFF3 was the optimal target for our investigation rather than rat or mouse TFF3. Desipramine (DMI), a serotonin and norepinephrine reuptake inhibitor, was purchased from Sigma (St Louis, MO, USA) and dissolved in saline to a final volume of 15 mg/ml before the experiment. LY294002, a specific PI3K inhibitor, was purchased from Tocris Bioscience (Bristol, UK), dissolved in dimethyl sulfoxide (DMSO) to 25 mM, and then dissolved in ethanol to 5 mM before use.

Forced Swim Test

The mouse forced swim test (FST) was modified from a previous protocol (Porsolt *et al*, 1977). Briefly, the mice were placed in a 20 cm diameter × 35 cm height plastic cylinder filled to a depth of 20 cm with 23–25 °C water. The test was videotaped, and immobility time was measured. The definition of immobility was the absence of all movements with the exception of motions required to maintain the animal's head above the water. The results are expressed as the time spent immobile during the last 5 min of the 6-min session. The observers were blind to the treatment design.

The rat FST was similar to the protocol used previously (Lucki, 1997; Porsolt *et al*, 1978). Briefly, the rats were placed for 15 min into a 25 cm diameter × 65 cm height plastic cylinder filled to a depth of 45 cm with 23–25 °C water. The rats were then removed, dried, and returned to their home cage. They were placed again in the cylinders 24 h later, and a 5-min swim test was conducted and videotaped. Immobility was defined as the minimum movement required to passively keep the animal's head above the water without other motions. Climbing was defined as the upward-directed movement of the forepaws against the wall. The results are expressed as the time (in seconds) that the animals spent immobile and climbing during the 5-min test.

Tail Suspension Test

The mouse tail suspension test (TST) was performed according to a previous publication (Steru *et al*, 1985). Briefly, the mice were suspended 50 cm above the floor with adhesive tape placed ~1 cm from the tip of the tail. The test was videotaped, and immobility time was measured for 6 min. Immobility was defined as the absence of any limb or

body movements, with the exception of those required for respiration, when the mouse hung passively and completely motionless. During the test, the mice were separated from each other to prevent visual and acoustic associations. The number of seconds spent immobile was recorded. The observers were blind to the treatment groups.

Locomotor Activity Test

Locomotor activity in mice was assessed using an activity-monitoring system as described previously (Chen *et al*, 2011). The apparatus consisted of a Perspex cycloid box (12 cm height × 24 cm diameter) with six horizontal infrared beams arranged at 60° angles. Each mouse was placed in the center of the open-field apparatus and monitored for 10 min. The number of crossings and total distance traveled were recorded by a video camera connected to a computer and scored to evaluate locomotor activity. After the completion of the experiment, the animals were returned to their home cages.

Locomotor activity in rats was measured in the open field. Briefly, the open-field apparatus consisted of a 75 × 75 × 40 cm square arena that was divided into 25 equal squares (15 × 15 cm) on the floor. Each rat was placed in the center of the apparatus, and the number of crossings into adjacent squares was counted for 5 min to assess locomotor activity.

Chronic Mild Stress

The chronic mild stress (CMS) protocol was adapted from our and others' previous reports with minor modifications (Willner *et al*, 1987; Zhu *et al*, 2011, 2012). Briefly, the rats were subjected to different mild stressors for 21 consecutive days. The stressors included the following: Day 1 (cold immobilization for 1 h at 4 °C, tilted cages 45° for 24 h), Day 2 (immobilization for 1 h, crowding for 24 h), Day 3 (forced cold swim for 5 min, soiled bedding for 24 h), Day 4 (immobilization for 1 h, vibration for 1 h), Day 5 (tilted cages 45° for 24 h, cold immobilization for 1 h at 4 °C), Day 6 (forced cold swim for 5 min at 4 °C, crowding for 24 h), and Day 7 (vibration for 1 h, soiled bedding for 24 h). This schedule was repeated two more times.

Sucrose Preference Test

The sucrose preference test was used to assess anhedonia induced by the CMS protocol. The assessment of sucrose preference was adapted from previous studies (Lu *et al*, 2006; Zhang *et al*, 2010a). Briefly, rats were trained to be adapted to a 1% sucrose solution (*w*/*v*) for 48 h at the start of the experiment, in which two bottles of 1% sucrose solution were placed in each cage. After adaptation, the rats were deprived of water for 4 h, followed by the sucrose preference test, in which the rats were housed in individual cages for 1 h and had free access to two bottles that contained 1% sucrose or tap water. After 1 h, sucrose and water consumption was measured, and sucrose preference was calculated as the following percentage: *sucrose consumption*/(*sucrose consumption* + *water consumption*). To eliminate the instability of sucrose preference, we measured sucrose preference in three consecutive tests. For the first test, after 4 h water deprivation, a free choice between plain water and 1% sucrose solution was provided to each animal. Water intake and sucrose intake were measured for 1 h in the first test, immediately followed by duplicate 3 h water deprivation and a 1-h test without any treatment in the second and third tests. The sucrose preference values from the three tests were averaged.

Novelty Suppressed Feeding Test

The novelty suppressed feeding (NSF) test was adapted from previous studies (Bodnoff *et al*, 1988; Hamani *et al*, 2010; Zhu and Tan, 2005). The rats were deprived of food for 24 h before the test in their home cages. On the test day, the rats were individually placed in an open-field arena (75 × 75 × 40 cm) with several pellets of food placed on a piece of white paper (10 × 10 cm) in the center. The animal was placed in a corner of the cage. The latency to approach the food and begin eating was recorded (in seconds) as the main test parameter. The maximum time was 10 min. To exclude the possibility that TFF3 affects normal appetite and feeding, we also measured the food consumption (mg/kg bodyweight) for each rat during the first 5 min in their home cage, immediately after they were removed from the open-field arena.

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was adapted from a previous study (Vestergaard *et al*, 2002). Briefly, ELISA plates were coated overnight at 8 °C with the primary TFF3 antibody. After an extensive wash, plasma or brain homogenates were added in three dilutions (1 : 50, 1 : 200, and 1 : 1000), and the plates were incubated at 37 °C for 1 h. The plates were washed again. After adding the secondary antibody (ie, horseradish peroxidase goat anti-rat immunoglobulin G (IgG)), they were further incubated at 37 °C. The enzyme substrate, 3′,5,5′-tetramethylbenzidine (Sigma-Aldrich), produces a colored end product that can be read spectrophotometrically at 450 nm. Titers were determined as the dilution that gave a positive signal (ie, the signal measured from control sera plus two SD).

Immunohistochemistry

IHC for Fos in brain tissue sections was carried out using antiplasma (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to previous studies with minor modifications (Dong *et al*, 2005; Sundquist and Nisenbaum, 2005). One hour after the behavioral tests, rats were perfused with 4% paraformaldehyde, and the brains were removed and postfixed for 24 h. The brains were then sectioned coronally with a microtome into 30 μm thick sections. Every third serial section was collected on gelatin-coated microscope slides. All of the sections were placed in a freshly prepared methanol–H_2O_2 solution for 10 min to block endogenous peroxidase activity. After incubation with rabbit anti-Fos (Santa Cruz Biotechnology; 1 : 200 dilution in phosphate-buffered saline (PBS), 30 min, 37 °C), the tissue sections were washed three times in PBS, followed by an additional 10-min incubation with biotin-conjugated second antibody and three washes with PBS. The sections

were then incubated for 10 min in streptavidin-peroxidase and washed three times in PBS. The sections were then reacted with a 0.05% solution of 3,3′-diaminobenzidine (DAB, Beijing Zhongshanjinqiao Biological Technology, Beijing, China) and 0.01% H_2O_2 in 0.1-M PBS. Incubation times varied from 3 to 10 min, depending on the expression levels of the DAB reaction product determined by microscopy. To exclude the possibility that different developmental procedures across treatment groups would confound the Fos comparisons, we incubated sections from different groups for one specific region for an equal time. To identify the sensitive area for TFF3, we assessed Fos protein expression in the posterior hypothalamic area, basolateral amygdala (BLA), central nucleus of the amygdala (CeA), and medial prefrontal cortex (mPFC), including infralimbic and prelimbic regions, in different treatment groups. The number of Fos-positive cells in these brain regions was counted according to a previous report from our laboratory (Jiang *et al*, 2011), in which two or three sections from each brain region for each rat were selected. The cell numbers on either side of the specific brain region were averaged and taken as the positive immunoreactive cell number for each rat. The number of Fos-labeled cells was measured using a cast-grid microscope (MetaMorph/DP10/Bx41, UIC/Olympus, US/JP) with an image-analysis program (MetaMorph, version 4.65). Under $\times 100$ magnification, two images were taken for each specimen.

Western Blot Assays

The rats were decapitated, and brains were extracted based on our previous study (Zhu *et al*, 2012). Bilateral tissue punches of the BLA and CeA (12-gauge) were obtained and homogenized with RIPA lysis buffer (Beyotime Biotechnology, Haimen, Jiangsu Province, China). The protein concentrations of all of the samples were determined using the BCA assay kit (Beyotime Biotechnology). The samples were subjected to SDS-polyacrylamide gel electrophoresis (12.5% acrylamide/0.27% *N*,*N*′-methylenebisacryalamide resolving gel) for $\sim$30 min at 80 V in stacking gel and $\sim$1 h at 120 V in resolving gel. Proteins were transferred electrophoretically to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA) at 0.25 A for 3 h. The membranes were washed with TBST (Tris-buffered saline plus 0.05% Tween-20, pH 7.4) before dipping in blocking buffer (5% skimmed dry milk in TBST) overnight at 4 °C. The membranes were then incubated for 1 h at room temperature with primary antibody against phosphor-Akt-Ser473 (1 : 1000, Cell Signaling, Danvers, MA, USA), Akt-ser473 (1 : 1000, Cell Signaling), and β-actin (1 : 2000, A5316; Sigma) in TBST plus 5% bovine serum albumin. After the membranes were shaken in 4×6 min washes in TBST buffer, the blots were incubated for 45 min at room temperature with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or mouse IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA, and Vector Labs, Burlingame, CA, USA, respectively), diluted 1 : 5000 in blocking buffer. The blots were then shaken in 4×6 min washes in TBST. Afterward, the blots were incubated with a layer of Super Signal enhanced chemiluminescence substrate mixture (Pierce Biotechnology, Rockford, IL, USA) for 1 min at room temperature. Finally, the blots were exposed against X-ray film (Eastman Kodak Company). Band intensities were quantified using Quantity One software (version 4.0.3) from Bio-Rad (Hercules, CA, USA).

Intracerebral Cannula Implantation and Intracranial Injections

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and permanent guide cannulas (23-gauge; Plastics One, Roanoke, VA, USA) were implanted bilaterally 1 mm above the BLA. The coordinates (Lubka *et al*, 2008; Wang *et al*, 2008) for the BLA were the following: anterior/posterior, -2.9 mm; medial/lateral, ± 5.0 mm; dorsal/ventral, -8.5 mm (Kessler *et al*, 2003). The cannulas were anchored to the skull with stainless-steel screws and dental cement. A stainless-steel stylet blocker was inserted into each cannula to maintain patency and prevent infection.

The injections were performed with Hamilton syringes that were connected to 30-gauge injectors (Plastics One). TFF3 or LY294002 was infused into the BLA bilaterally (0.5 µl per side) at a rate of 0.5 µl/min. The injection needle was kept in place for another 1 min to allow the drug to completely diffuse from the injector tips (Chen *et al*, 2010; Lu *et al*, 2007). The dose of LY294002 was based on a previous report (Zhang *et al*, 2010b), and LY294002 was infused 10 min before the TFF3 injection.

Data Analysis

The data are expressed as mean $\pm$ SEM. The statistical analyses were performed using an unpaired Student's *t*-test and one- or two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test (see Results for details). Values of $p < 0.05$ were considered statistically significant.

RESULTS

TFF3 Administration Exerted Antidepressant-Like Effects in Mice

Using the FST and TST in mice, we first assessed the antidepressant-like effects of acute TFF3 administration. The mice received intraperitoneal injections of TFF3 (0, 0.01, 0.1, 0.5, 1.0, and 2.0 mg/kg, i.p.) or subcutaneous injections of DMI (15 mg/kg, s.c.) 30 min before the behavioral test. Each group of mice was divided into two subgroups ($n = 10$–12 per group) for different tests. One subgroup was tested for locomotor activity for 10 min, immediately followed by the TST (Figure 1a). The other subgroup was tested in the FST (Figure 1c). One-way ANOVA revealed that TFF3 significantly decreased immobility time ($F_{6,78} = 62.6$, $p < 0.005$) in the TST. TFF3, at doses of 0.1 mg/kg (77 ± 9 s), 0.5 mg/kg (72 ± 11 s), and 1.0 mg/kg (102 ± 11 s), and DMI (89 ± 8 s) significantly reduced immobility time, but TFF3 at doses of 0.01 mg/kg (151 ± 13 s) and 2.0 mg/kg (140 ± 19 s) had no effects on immobility compared with saline-treated mice (168 ± 18 s; Figure 1b). The results from the FST showed that TFF3 significantly decreased floating time ($F_{6,64} = 9.27$, $p < 0.005$). TFF3, at doses of 0.01 mg/kg (64 ± 5 s), 0.1 mg/kg (32 ± 5 s), and 0.5 mg/kg (152 ± 9 s), and DMI (59 ± 5 s) significantly reduced floating time, but TFF3 at doses of

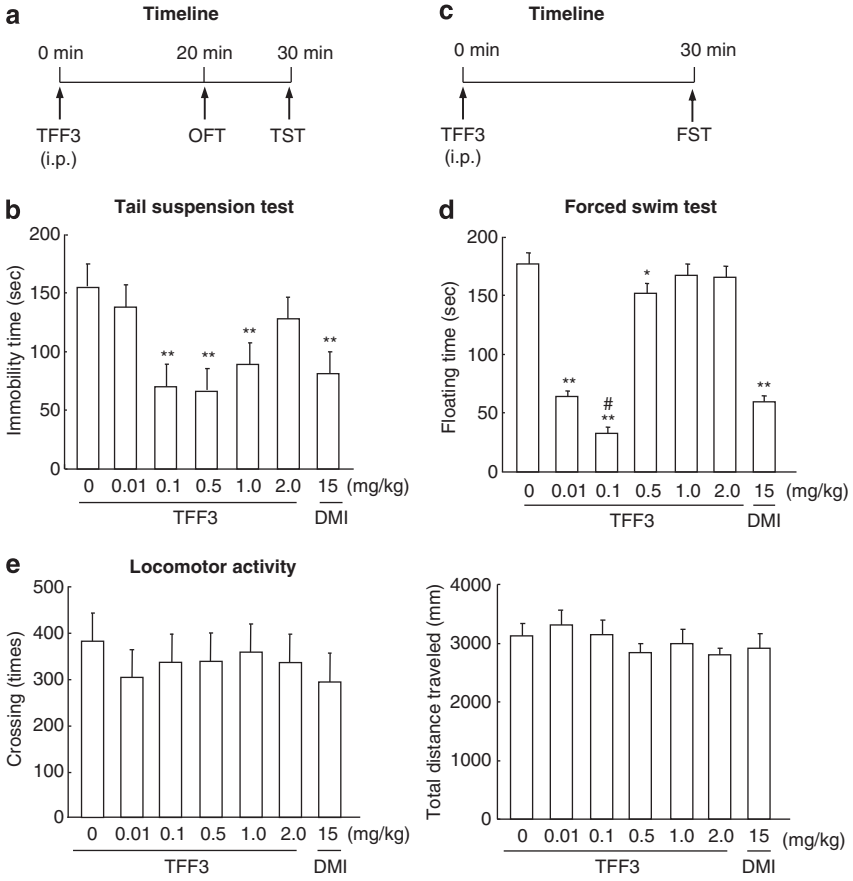

Figure 1 Antidepressant-like effect of acute trefoil factor 3 (TFF3) administration in mice. (a) Experimental procedure for the OFT and tail suspension test (TST). The TST was conducted 30 min after a single injection of saline or TFF3 (i.p.) or Desipramine (DMI) (s.c.). (b) Acute TFF3 administration significantly decreased immobility time in the TST. (c) Experimental procedure for the FST. (d) Acute TFF3 administration significantly reduced floating time in the FST. (e) Acute TFF3 administration had no effects on locomotor activity, reflected by the number of crossings (*left*) and total distance traveled (*right*) in mice. Locomotor activity was monitored for 10 min. The data are expressed as mean ± SEM ($n = 9$–12 per group). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with saline-treated group; $^{\#}p < 0.05$, compared with DMI-treated group. DMI, desipramine; FST, forced swim test; OPT, open field test; TFF3, trefoil factor 3; TST, tail suspension test.

1.0 mg/kg (167 ± 9 s) and 2.0 mg/kg (165 ± 10 s) had no effect on floating compared with saline-treated mice (177 ± 10 s; Figure 1d). To exclude the possibility that TFF3 induced locomotor alterations in these behavioral tests, we measured the effects of TFF3 on locomotor activity before the TST. The mice treated with TFF3 (0.01, 0.1, 0.5, 1.0, and 2.0 mg/kg) and DMI (15 mg/kg) did not differ from saline-treated mice in the number of crossings or total distance traveled ($p > 0.05$; Figure 1e), indicating that reductions in immobility and floating in the two models were not attributable to alterations in locomotor activity.

TFF3 Administration Produced Antidepressant-Like Effects in Rats

We determined the potential antidepressant-like effect of TFF3 in rats. Based on the results from mice, we chose 0.1 mg/kg as the TFF3 treatment dose. Three groups of rats ($n = 9$–12 per group) were treated with TFF3 (0 and 0.1 mg/kg, i.p.) or DMI (15 mg/kg, s.c.), 30 min before the FST (Figure 2a). One-way ANOVA showed that both TFF3 (85 ± 20 s, $p < 0.01$) and DMI (100 ± 19 s, $p < 0.01$) significantly reduced immobility time compared with saline-treated control rats (169 ± 12 s; Figure 2b). No significant difference in climbing time was found between the TFF3-treated group (47 ± 7 s) and saline-treated control rats (30 ± 6 s), whereas DMI (113 ± 24 s) significantly increased climbing time compared with saline-treated control rats (Figure 2c). We also used three separate groups of rats treated with saline, TFF3 (0.1 mg/kg, i.p.), or DMI (15 mg/kg, s.c.), respectively. Thirty minutes after drug administration, the rats were placed in the open-field apparatus to assess locomotor activity. The results showed that acute TFF3 and DMI treatment did not affect normal locomotion, reflected by the number of crossings in the open field (Figure 2d). Additionally, we measured the time course of brain TFF3 levels to determine whether TFF3 crosses the blood–brain barrier and is centrally available after systemic administration. The BLA was selected because of its important role in emotional processing. The results showed that TFF3 levels were significantly increased in the BLA 30 min after systemic TFF3 treatment (Figure 2e), suggesting that systemic TFF3 crosses the blood–brain barrier. The elevation of TFF3 levels in the BLA was transient and decreased to baseline levels 2 h after TFF3 administration (Figure 2e), demonstrating that the antidepressant-like effect of TFF3 is attributable to central regulation rather than peripheral actions.

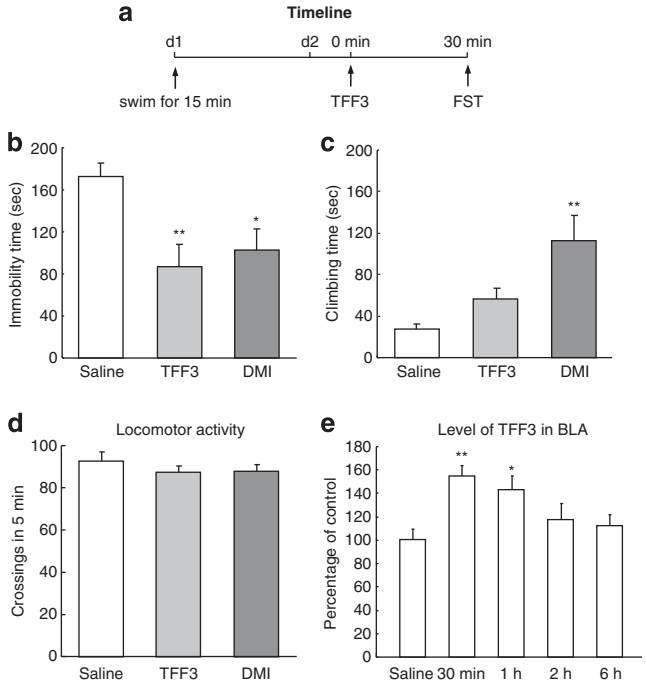

Figure 2 Antidepressant-like effects of acute trefoil factor 3 (TFF3) administration in rats. (a) Experimental procedure. A single trefoil factor 3 (TFF3) injection (0.1 mg/kg, i.p.) was administered 30 min before the forced swim test (FST). Acute TFF3 administration significantly decreased immobility time (b) but had no significant effects on climbing time (c). (d) Acute TFF3 or Desipramine (DMI) (15 mg/kg, s.c.) administration had no effect on locomotor activity in the open field test ($n=9$–12 per group). (e) The level of TFF3 in the basolateral amygdala (BLA) was elevated 30 min after systemic administration and returned to baseline levels 2 h after TFF3 administration ($n=6$ per group). The data are expressed as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, compared with saline-treated group. DMI, desipramine; FST, forced swim test; TFF3, trefoil factor 3.

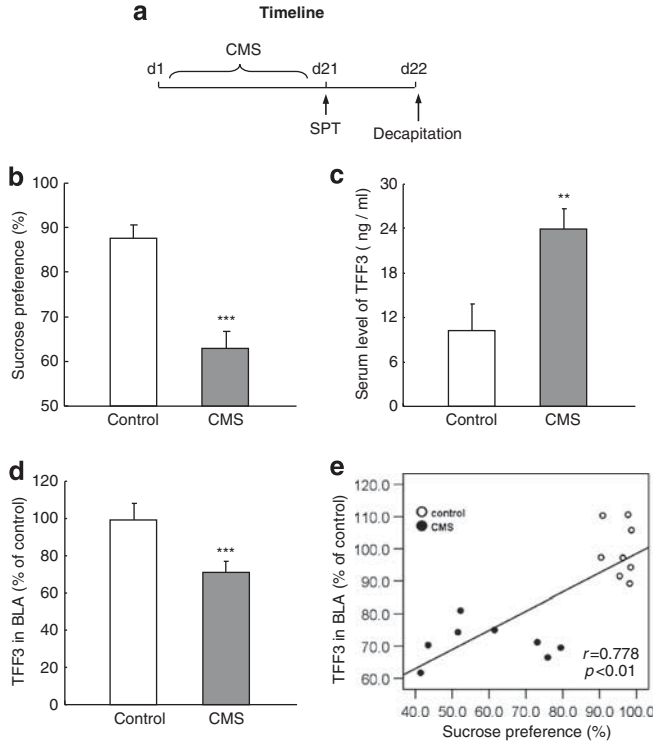

Figure 3 Effects of chronic mild stress on depressive-like behavior and plasma and basolateral amygdala trefoil factor 3 (BLA TFF3) levels in rats. (a) Experimental procedure. Rats were exposed to chronic mild stress (CMS) for 21 days, and sucrose preference was tested on day 21. The rats were then decapitated to assay plasma and BLA TFF3 levels by enzyme-linked immunosorbent assay (ELISA). (b) CMS significantly decreased sucrose preference ($n=8$). CMS increased plasma TFF3 levels (c) and decreased BLA TFF3 levels (d) ($n=8$). (e) A significant, positive linear correlation was found between BLA TFF3 levels and sucrose preference test (SPT) in control and CMS-treated rats ($r=0.778$, $p<0.01$; $n=16$). The data are expressed as mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$, compared with control group. BLA, basolateral amygdala; CMS, chronic mild stress; SPT, sucrose preference test.

Chronic Mild Stress Increased Plasma TFF3 Levels and Decreased Brain TFF3 Levels in Rats

To investigate whether CMS alters plasma and central TFF3 levels in rats, two groups of rats (control and CMS, $n=8$ per group) were used. After 21 days of CMS treatment, the rats were killed by decapitation on day 22, and blood and the BLA were collected for the detection of plasma and brain TFF3 by ELISA (Figure 3a). The *t*-test revealed that sucrose preference, which has been used to reflect the core symptom of anhedonia in depressed individuals, was significantly decreased by chronic stress ($p<0.001$; Figure 3b). The data also showed that plasma TFF3 levels increased in CMS-treated rats (24 ± 5 ng/ml) compared with control rats (10 ± 7 ng/ml; $p<0.01$; Figure 3c). The ELISA showed that TFF3 levels in the BLA were reduced by CMS ($p<0.001$; Figure 3d). To further determine the possible correlation between TFF3 levels in the BLA and the degree of anhedonia-like behavior, we explored the correlation between TFF3 levels in the BLA and sucrose preference in control and CMS rats. The results showed that sucrose preference positively correlated with TFF3 levels in the BLA ($r=0.778$, $p<0.01$; Figure 3e). These findings suggest that central TFF3 decreases the magnitude of anhedonia.

TFF3 Administration Reversed Depressive-Like Behavior Induced by CMS in Rats

This experiment investigated the effects of TFF3 on depressive-like behaviors induced by CMS. Four group of rats were used ($n=8$–12 per group): (1) rats not subjected to CMS and treated with a single saline injection, (2) rats not subjected to CMS and treated with a single TFF3 injection, (3) rats subjected to CMS and treated with a single saline injection, and (4) rats subjected to CMS and treated with a single TFF3 injection. Saline (1 ml/kg, i.p.) and TFF3 (0.1 mg/kg, i.p.) were administered 30 min before each behavioral test (Figure 4a). A two-way ANOVA was performed for this experiment, with CMS (no stress and stress) and treatment (saline and TFF3) as the between-subjects factors. In the sucrose preference test, the two-way ANOVA revealed a significant effect of CMS treatment ($F_{1,34}=10.0$, $p<0.01$) on sucrose preference in the 1-h test during the dark phase and a significant CMS treatment × TFF3 treatment interaction ($F_{1,34}=4.4$, $p<0.05$; Figure 4b). The results showed that TFF3 reversed the anhedonic-like state induced by CMS in rats. Furthermore, we assessed water intake in the sucrose preference test in

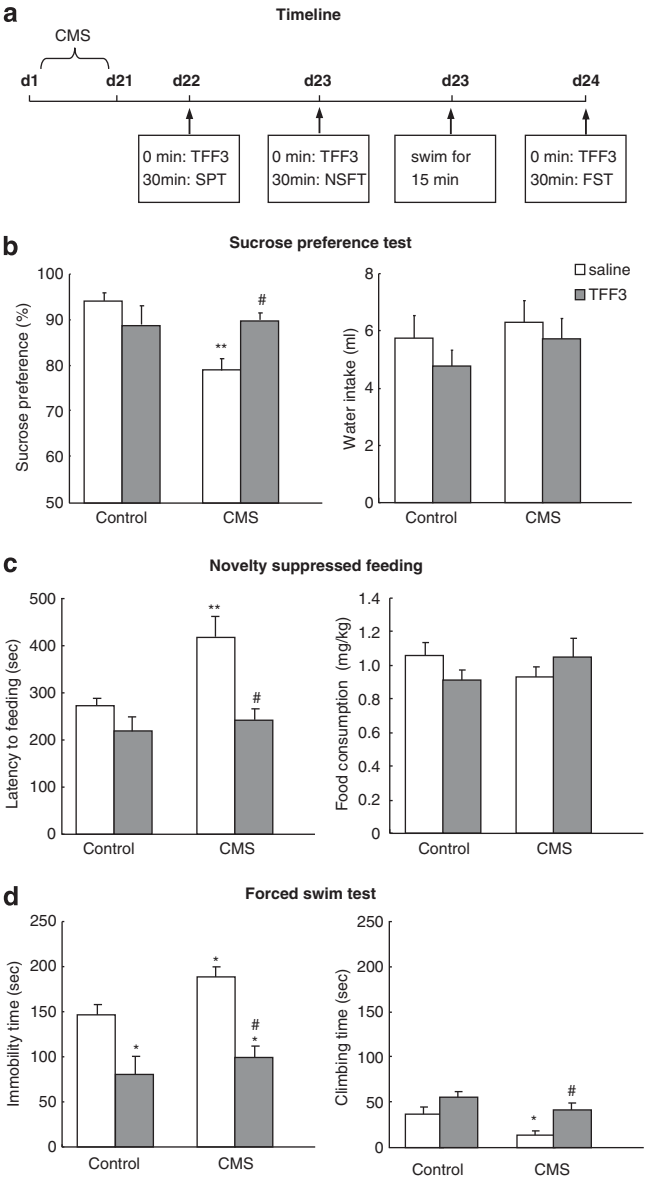

Figure 4 Effects of acute trefoil factor 3 (TFF3) administration on depressive-like behavior, induced by chronic mild stress in rats. (a) Behavioral test procedure. (b) Acute TFF3 administration increased sucrose preference test (SPT) (*left*), with no change in total water intake (*right*) in a 1 h test during the dark phase in rats subjected to chronic mild stress (CMS). (c) TFF3 administration significantly reversed the increased latency to feed (*left*) induced by CMS and had no effects on home cage food consumption (*right*). (d) Acute TFF3 administration reversed the increased immobility time and decreased climbing time induced by CMS. The data are expressed as mean ± SEM ($n = 8$–12 per group). $^{*}p < 0.01$, $^{**}p < 0.05$, compared with saline-control group; $^{\#}p < 0.05$, compared with saline-CMS group. CMS, chronic mild stress; FST, forced swim test; NSFT, novelty-suppressed feeding test; SPT, sucrose preference test; TFF3, trefoil factor 3.

rats in different groups. The data showed no significant differences between the TFF3- and vehicle-treated groups (Figure 4b).

In the NSF test, the two-way ANOVA revealed significant effects of CMS treatment ($F_{1,37} = 6.5$, $p < 0.05$) and TFF3 treatment ($F_{1,37} = 12.0$, $p < 0.01$) on the latency to feed (Figure 4c), suggesting that TFF3 can reverse anxiety-like behavior induced by CMS. To exclude the possibility that TFF3 affects normal appetite and feeding, we also measured food consumption in each rat during the first 5 min in their home cage, immediately after the rats were removed from the open field. The results showed that acute TFF3 treatment did not change home cage food consumption compared with vehicle-treated rats (Figure 4c). These findings suggest that acute TFF3 administration did not affect either feeding or drinking in rats.

In the FST, the statistical analysis revealed significant effects of CMS treatment ($F_{1,36} = 4.1$, $p < 0.05$) and TFF3 treatment ($F_{1,36} = 27.0$, $p < 0.001$) on immobility time in the FST (Figure 4d). The statistical analysis also revealed significant effects of CMS treatment ($F_{1,38} = 15.0$, $p < 0.001$) and TFF3 treatment ($F_{1,38} = 12.0$, $p < 0.01$) on climbing time in the FST and no significant CMS treatment × TFF3 treatment interaction ($F_{1,38} = 0.1$, $p = 0.747$). The results showed that TFF3 reversed behavioral 'despair' not only in control rats but also in CMS rats.

TFF3 Administration Increased Fos Protein Expression in Several Brain Areas in the FST in Rats

To assess whether the antidepressant-like effect of TFF3 observed in the FST is associated with alterations in the activation of specific brain areas, we examined the protein expression of *c-fos*, an immediate-early gene used as a neuronal activation marker, in rats exposed to the FST. Three groups of rats ($n = 4$–5 per group) were used: (1) saline group (rats treated with a single saline injection), (2) TFF3 group (rats treated with a single TFF3 injection (0.1 mg/kg, i.p.)), and (3) DMI group (rats treated with a single DMI injection (15 mg/kg, s.c.)). These three groups of rats are subjected to the FST and drugs were administered 30 min before the FST. One hour later, the rats were perfused to detect the protein expression of Fos by IHC. The *t*-test showed that Fos expression was significantly increased by TFF3 in the hypothalamus (79 ± 8), mPFC (70 ± 9), and BLA (62 ± 10) compared with saline treatment (hypothalamus, 21 ± 1; mPFC, 36 ± 3; BLA, 11 ± 4, respectively; Figure 5a and b). DMI significantly increased Fos expression in the hypothalamus (62 ± 10) and mPFC (78 ± 12), but not BLA (18 ± 10) compared with saline-treated control rats (Figure 5a and b).

Intra-BLA Infusion of TFF3 Exerted Antidepressant-Like Effects in Rats

The BLA is the central subregion involved in mood and psychiatric disorders, and we investigated the role of the BLA in the antidepressant-like effect of TFF3. Furthermore, the BLA is the specific brain region among the four tested brain regions where TFF3, but not DMI, increased Fos expression (Figure 5). Therefore, the BLA is the only region we selected to assess the antidepressant-like effect of acute local TFF3 administration. Four groups of rats ($n = 8$–9 per group) were used to investigate the behavioral effects of intra-BLA infusion of TFF3 using the rat FST. Intra-BLA infusions of TFF3 (0, 0.01, 1.0, and 100 ng/side) were administered 30 min before the behavioral test. A schematic representation of the BLA injection sites is shown in Figure 6a. The experimental procedure was performed according to Figure 6b. One-way ANOVA revealed that TFF3 significantly altered immobility

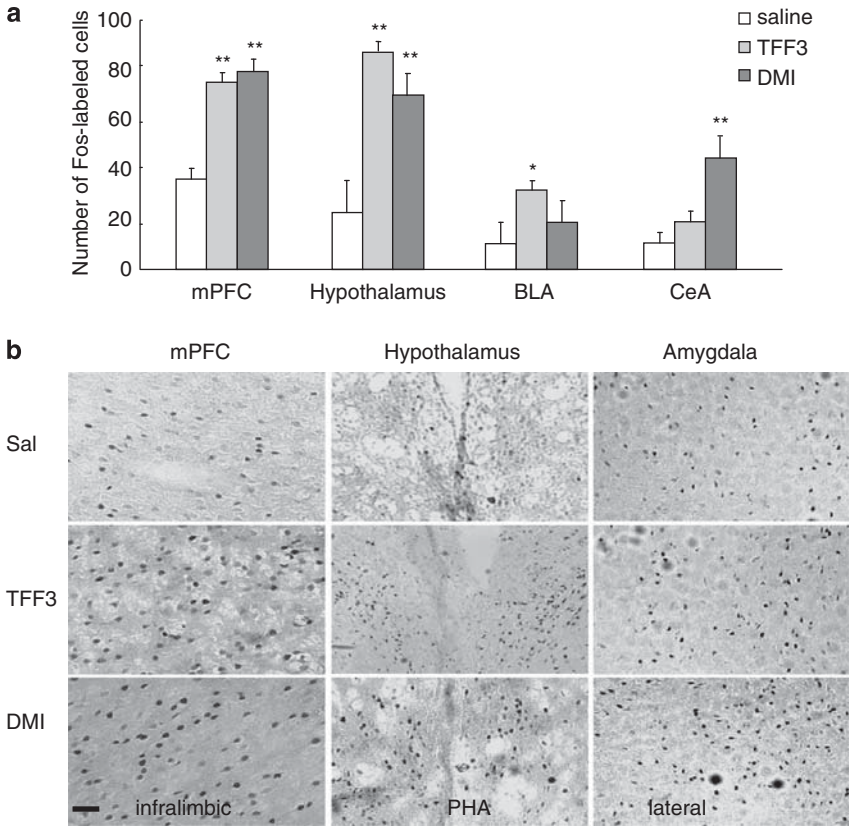

Figure 5 Effects of trefoil factor 3 (TFF3) administration on Fos induction in rats subjected to the forced swim test (FST). Rats were injected with TFF3 (0.1 mg/kg, i.p.), DMI (15 mg/kg, s.c.), or saline 30 min before the FST. The number of Fos-labeled cells was counted in the mPFC, hypothalamus, basolateral amygdala (BLA), and central nucleus of the amygdala (CeA). (a) Effects of acute saline, TFF3, and Desipramine (DMI) administration on Fos expression in the hypothalamus, medial prefrontal cortex (mPFC), and BLA. (b) Representative images show TFF3 expression in the hypothalamus, mPFC, and BLA. Scale bar = 20 μm. The data are expressed as mean ± SEM ($n = 4$–5 per group). *$p < 0.05$, **$p < 0.01$, compared with saline group. BLA, basolateral amygdala; CeA, central nucleus of the amygdala; DMI, desipramine; mPFC, medial prefrontal cortex; TFF3, trefoil factor 3.

time ($F_{3,35} = 8$, $p < 0.001$). The infusion of TFF3 at a dose of 1 ng/side (39 ± 6 s) significantly reduced immobility time ($p < 0.05$), whereas 100 ng/side (128 ± 14 s) significantly increased immobility time ($p < 0.05$). No significant difference was found at 0.01 ng/side (88 ± 19 s) compared with saline-treated rats (82 ± 13 s; Figure 6c). TFF3 at doses of 0.01, 1, and 100 ng had no significant effects on climbing time (23 ± 10 s, 43 ± 9 s, and 15 ± 6 s, respectively) compared with saline-treated rats (27 ± 9 s; Figure 6d). The differential behavioral response to different doses of TFF3 in the present study are consistent with the bidirectional characteristics of the anxiolytic effects of TFF3 demonstrated in a previous report, in which bilateral application of TFF3 into the amygdala exerted anxiolytic-like effects at a low dose and an anxiogenic-like effect at a high dose (Schwarzberg *et al*, 1999).

Inhibition of PI3K Activity in the BLA Blocked the Antidepressant-Like Effects of TFF3 in Rats

This experiment investigated the role of the PI3K/Akt signaling pathway in the BLA in the antidepressant-like effects of TFF3. We first determined the effects of FST on the phosphorylated protein levels of Akt (phosphorylated-Akt (p-Akt)), a downstream molecule of PI3K. Two groups of rats (control and FST, $n = 4$–5 per group) were used for the FST and were decapitated 1 h later for western blot assay. The *t*-test revealed significantly decreased levels of p-Akt in the BLA ($p < 0.05$), but not CeA ($p > 0.05$) in the FST rats compared with control rats (Figure 7a). We then measured the effects of acute systemic administration of TFF3 (0.1 mg/kg, i.p.) on p-Akt in the BLA and CeA without behavioral testing using two groups of rats (saline and TFF3, $n = 6$ per group). The results from the western blot assay revealed that p-Akt levels increased in the BLA (*t*-test, $p < 0.05$), but not CeA, 30 min after acute TFF3 administration (Figure 7b). To further clarify whether FST-induced decreases in p-Akt in the BLA can be blocked by systemic TFF3, we injected rats with TFF3 (0.1 mg/kg, i.p.), 30 min before the FST and assayed p-Akt levels ($n = 6$ per group). The results showed that the reduction of p-Akt induced by the FST in the BLA was reversed by acute TFF3 administration (Figure 7c). Subsequently, we determined the role of the PI3K pathway in the antidepressant-like effects of TFF3 using the PI3K inhibitor LY294002. Four groups of rats were used ($n = 8$–9 per group): (1) rats infused with saline and vehicle into the BLA, (2) rats infused with TFF3 and vehicle, (3) rats infused with saline and LY294002, and (4) rats infused with TFF3 and LY294002. LY294002 (5 mM/side) or its vehicle (DMSO + ethanol, 0.5 μl/side) was infused into the BLA, 30 min before the FST (Figure 7d). The dose of LY294002 was based on a previous study (Zhang *et al*, 2010b). Ten minutes after LY294002 administration, saline

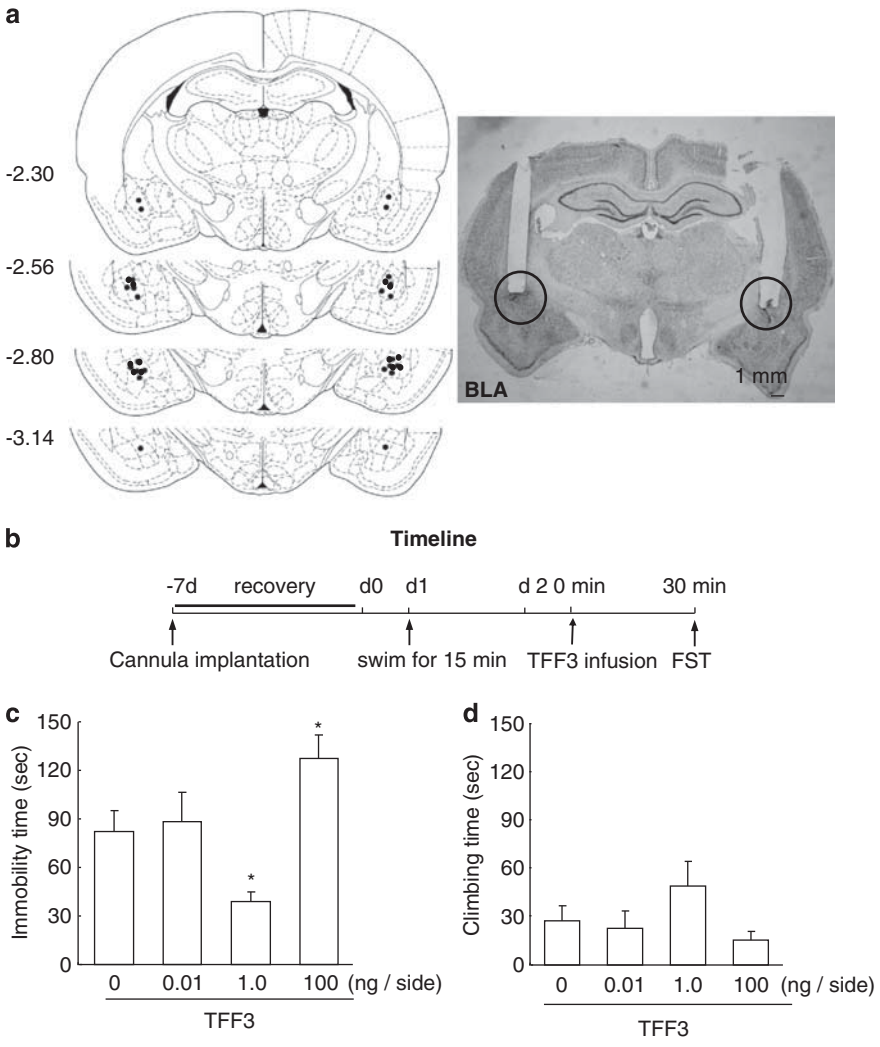

Figure 6 Effects of intra-BLA infusions of trefoil factor 3 (TFF3), on depressive-like behavior in rats. (a) Schematic representation of the injection sites in the basolateral amygdala (BLA). (b) Experimental procedure. Rats were microinjected with TFF3 (0.01, 1, and 100 ng/side) or saline into the BLA 30 min before the forced swim test (FST). Immobility time (c) and climbing time (d) were assessed. The data are expressed as mean ± SEM ($n = 8$–9 per group). *$p < 0.05$, compared with saline group. BLA, basolateral amygdala; FST, forced swim test; TFF3, trefoil factor 3.

(0.5 μl/side) or TFF3 (1 ng/side) was infused. Two-way ANOVA revealed significant effects of TFF3 treatment ($F_{1,35} = 5.5$, $p < 0.05$), but not LY294002 treatment ($F_{1,35} = 0.9$, $p = 0.33$) on immobility time and a significant TFF3 treatment × LY294002 treatment interaction ($F_{1,35} = 3.8$, $p < 0.05$; Figure 7e). No significant effects of TFF3 treatment ($F_{1,\ 34} = 1.5$, $p = 0.24$) or LY294002 treatment ($p > 0.05$) were found on climbing time, with no significant TFF3 treatment × LY294002 treatment interaction ($p > 0.05$; Figure 7e).

DISCUSSION

In the present study, we investigated the antidepressant-like effects of TFF3 and the underlying molecular mechanism. The main findings of the present study were the following: (1) systemic TFF3 administration produced rapid antidepressant-like effects in the FST and TST, and significantly increased TFF3 levels in the BLA, 30 min after administration, (2) serum TFF3 levels were elevated and TFF3 levels in the BLA were reduced in CMS-treated rats, and acute TFF3 administration reversed depressive-like behavior induced by CMS, (3) systemic administration of TFF3 increased Fos expression in the BLA, mPFC, and hypothalamus, (4) acute TFF3 administration increased the level of p-Akt in the BLA but not CeA, (5) infusion of TFF3 into the BLA exerted antidepressant-like effects in the FST, and (6) inhibition of PI3K activity blocked the antidepressant-like effect of TFF3.

Homeostatic Adaption of Neuropeptide TFF3 in Response to Stress

Previous studies showed that stress, especially chronic stress, acts as a predisposing factor that participates in the onset of depression in humans (Krishnan and Nestler, 2010). Animals exposed to chronic stress develop a series of behavioral and hormonal alterations that are closely related to the clinical symptoms of depression and have been used as an experimental model of depression (Bortolato *et al*, 2007; Gersner *et al*, 2010; Jayatissa *et al*, 2006; Wu and

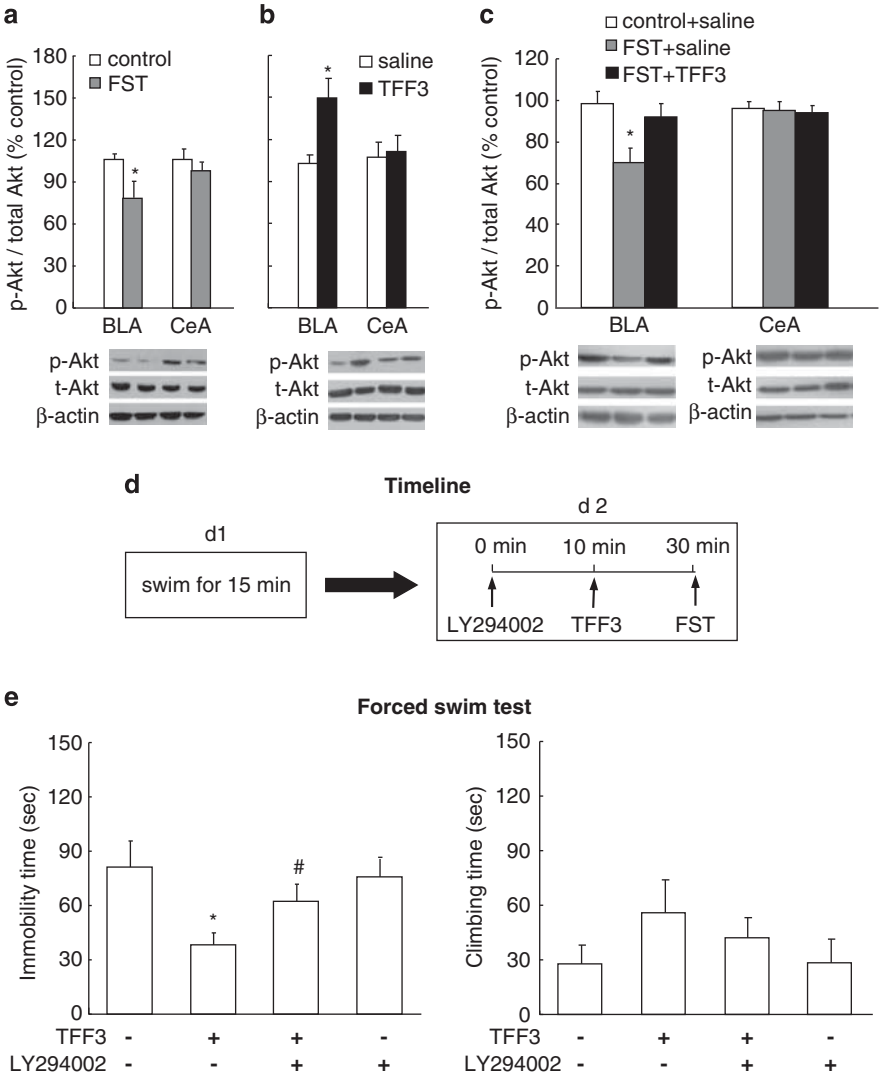

Figure 7 Phosphatidylinositol 3-kinase/ (PI3K)/Akt activity mediates the antidepressant-like effects of trefoil factor 3 (TFF3) in rats. (a) Akt and phosphorylated-Akt (p-Akt) protein levels in the basolateral amygdala (BLA) and central nucleus of the amygdala (CeA) were measured in the forced swim test (FST). The rats were decapitated 1 h after exposure to the FST ($n = 4$–5). *$p < 0.05$, compared with control group. (b) The rats were decapitated 30 min after TFF3 injection, and Akt and p-Akt protein levels in the BLA and CeA were measured by western blot ($n = 6$). *$p < 0.05$, compared with saline group. (c) The FST-induced decrease in p-Akt in the BLA was blocked by systemic TFF3 treatment. Rats were injected with TFF3 (0.1 mg/kg, i.p.) 30 min before the FST and were assayed for p-Akt levels ($n = 6$ per group). Representative Western blot bands of p-Akt, total-Akt, and β-actin are shown. (d) Experimental procedure for the assessment of the role of PI3K/Akt activity in the BLA in the effects of TFF3. Rats were injected with LY294002 (5 mM/side) followed by TFF3 (1 ng/side) into the BLA 30 min before the FST. (e) Immobility and climbing time were measured. Pretreatment with LY294002 reversed the reduction of immobility induced by TFF3. The data are expressed as mean ± SEM ($n = 8$–9 per group). *$p < 0.05$, compared with saline group; $^{\#}p < 0.05$, compared with TFF3 group. BLA, basolateral amygdala; CeA, central nucleus of the amygdala; FST, forced swim test; TFF3, trefoil factor 3.

Wang, 2010). Neuropeptides are considered the largest class of neuromessengers in the CNS, and they are induced by high-frequency neuronal activity. Neuropeptides are highly plastic and responsive to stimuli, modulate classic neurotransmitter systems, and exert trophic effects (Rotzinger *et al*, 2010). Neuropeptides are ubiquitously distributed and most often associated with one or more classic neurotransmitters, supporting their role as modulatory factors. These properties suggest that neuropeptides mediate adaptations and responses to homeostatic challenges (Alldredge, 2010; Holmes *et al*, 2003; Madaan and Wilson, 2009).

The present data showed that systemic TFF3 administration significantly increased TFF3 levels in the BLA, 30 min after administration, suggesting that systemic TFF3 can cross the blood--brain barrier and that the antidepressant-like effect of TFF3 is attributable to central regulation rather than peripheral actions. The elevation of TFF3 levels in the BLA was transient and returned to baseline levels, 2 h after TFF3 injection. Furthermore, plasma TFF3 levels increased in rats subjected to chronic stress, and systemic administration of TFF3 reversed the depressive-like behaviors induced by chronic stress. The results suggested that increased plasma TFF3 levels in response to chronic stress may be a homeostatic adaptation that helps an individual cope with chronic stress (Lutter *et al*, 2008; Takebayashi *et al*, 2010), indicating that TFF3 might be a resilience factor

in response to chronic stress or accelerate recovery from chronic stress. Based on the present results, we cannot conclude that different serum levels of TFF3 produce differential effects. However, an interesting result from our study is that intra-BLA infusion of TFF3 at different doses (1.0 and 100 ng/side) produced contrasting behavioral effects in rats. Furthermore, we hypothesized that the antidepressant-like action of TFF3 is attributable to its central regulation. We measured brain TFF3 levels after the CMS paradigm. The results showed that TFF3 levels in the BLA were reduced by CMS (Figure 3d). This finding suggests that CMS induced depressive-like behavior that might be related to the downregulation of TFF3 concentrations in the BLA. Although serum TFF3 levels were elevated by CMS, this elevation did not block the reduction of TFF3 in the BLA. Therefore, the elevation of central TFF3 levels after systemic TFF3 administration reversed the behavioral phenotype induced by chronic stress.

Role of the BLA in the Regulation of Depressive-like Behavior by TFF3

Recent evidence suggests that the neural networks that putatively modulate normal emotional behaviors, including the hippocampus, amygdala, hypothalamus, prefrontal cortex, and other regions, participate in the pathophysiology of depression (Krishnan and Nestler, 2010; Nestler and Carlezon, 2006; Shelton, 2007). Most studies have focused on specific regions of the prefrontal cortex and hippocampus that might regulate the cognitive component of depression, whereas the amygdala has been recognized for its central role in mood and emotional behavior (Drevets *et al*, 2008). Increasing evidence has shown that certain psychiatric illnesses induced by stress might be caused by pathophysiological alterations in neuronal excitability in the amygdala (Knoll *et al*, 2011; Liu *et al*, 2011). Repeated stress results in a state of hyperexcitability in the BLA, and might subsequently modulate efferent projections from the amygdala to the hypothalamus, locus coeruleus, and raphe to organize the neuroendocrine, neurotransmitter, and behavioral responses to stressors and emotional stimuli (Aroniadou-Anderjaska *et al*, 2007; Drevets *et al*, 2008). Stress-induced impairment in the noradrenergic facilitation of γ-aminobutyric acid release in the BLA may be the underlying mechanism of amygdala hyperexcitability in certain stress-related mood disorders (Aroniadou-Anderjaska *et al*, 2007; Drevets *et al*, 2008). Furthermore, previous studies showed that the neural expression of TFF3 includes the hypothalamus and amygdala (Hinz *et al*, 2004). Our results also showed that Fos expression, an indicator of neuronal activation, was increased in the BLA by systemic TFF3 administration. Furthermore, intra-BLA infusions of TFF3 exerted rapid antidepressant-like effects in the chronic stress procedure. Altogether, our results indicate that the BLA might be a critical brain site that mediates the effects of TFF3 in models of depression.

PI3K/Akt Signaling Pathway in the BLA Mediates the Antidepressant-Like Effects of TFF3

Several intracellular signal pathways, such as mammalian target of rapamycin, extracellular signal-regulated kinase (ERK), glycogen synthase kinase 3β (GSK-3β), PI3K, and cyclin-dependent protein kinase 5, have been implicated in the adaptive response to stress, and are engaged in the development of mood-related disorders (Bohus *et al*, 1993; Chen *et al*, 2010; Schmidt and Duman, 2010; Zhu and Tan, 2005). Many studies showed that TFF3 regulates multiple downstream pathways in central and peripheral tissues. The effects of TFF3 are transmitted to signaling cascades by still unknown adaptor proteins. Despite the absence of an identified cell surface receptor for TFF3, TFFs can act through the EGFR to activate several downstream effector pathways, including ERK1/2, Jun *N*-terminal kinase, PI3K, and signal transducers and activation of transcription 3 (Baus-Loncar and Giraud, 2005). As the downstream regulatory signaling pathway of TFF3, PI3Ks are a large family of intracellular signal transducers. The role of the PI3K pathway has been implicated in the regulation of cell growth, survival, proliferation, and movement (Astle *et al*, 2011; Bun Chan *et al*, 2011; Nedachi *et al*, 2011). Numerous studies have also implicated PI3K in depression and anxiety (Ackermann *et al*, 2008; Kelly and Lynch, 2000; Sanna *et al*, 2002). For example, the PI3K signaling pathway mediates the stress-induced modification of hippocampal synaptic plasticity (Zhang *et al*, 2010b). In the present study, we found that FST significantly decreased phosphorylated Akt protein levels in the BLA, but not CeA. We also found that acute TFF3 administration increased the level of p-Akt in the BLA. Furthermore, intra-BLA infusions of LY294002, a specific PI3K inhibitor, blocked the antidepressant-like effects of TFF3 in the rat FST, whereas pharmacological inhibition of BLA PI3K alone did not induce depressive-like behavior, suggesting that the antidepressant-like effects of TFF3 may be mediated by the PI3K/Akt signaling pathway in the BLA.

Conclusion

In summary, our results revealed that acute systemic TFF3 administration exerted rapid antidepressant-like properties in mouse and rat models of depression. The data suggest that deficits in PI3K/Akt activity in the BLA have a key role in depression-related behavioral disturbances and indicate that activation of the BLA PI3K/Akt signaling pathway is involved in the antidepressant activity of TFF3. Our findings might contribute to the development of novel rapid antidepressants that target the TFF3 neuropeptide system and downstream PI3K/Akt signaling pathway to achieve therapeutic effects.

ACKNOWLEDGEMENTS

This work was supported in part by the National Basic Research Program of China (No. 2009CB522004), National Natural Science Foundation of China (No. 30800362 and 81071079), Specific Foundation for Doctoral Institute Program from the State Education Ministry of China (No. 20121323120002 to Dr HS Shi) and National Science and Technology Major Project (2012ZX09103301).

DISCLOSURE

The authors declare no conflict of interest.

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