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A novel therapeutic approach to depression *via* supplement with tyrosine hydroxylase

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Abstract

Tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2, TH) is the rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters, dopamine (DA), noradrenaline (NE), and adrenaline, in the neurons. The regulated activity of TH is thought to play a critical role in modulating the functional activity of catecholaminergic neuronal systems in the brain. It is well known that the catecholaminergic neuronal systems are associated with depression. Here we showed that TH, delivered by protein transduction domain (PTD), passed through the blood–brain barrier and entered the neurons. Systemic TH treatment improved the behavioral despair in the forced swim test (FST) and the tail suspension test (TST), the two models widely used to screen the potential anti-depressant efficacy. The results indicated a novel and potential therapeutic use of TH in the depression disorder.

Keywords: Tyrosine hydroxylase; Depression; Anti-depressant; Protein transduction domain; Protein therapy

Tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2, TH) is the rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters, dopamine (DA), noradrenaline (NE), and adrenaline. TH is an ≈60 kDa protein abundant in noradrenergic and dopaminergic cell bodies and distributed throughout the brain in terminal fields of these catecholaminergic systems. The regulated activity of the enzyme is thought to play a critical role in modulating the functional activity of catecholaminergic neuronal systems in the brain [1].

It is well known that catecholaminergic neuronal systems are associated with depression [2]. According to the catecholamine theory of mood [3], the major symptoms of depression arise primarily from a deficiency in catecholamine neurotransmitters in the synaptic cleft, and those that increase the availability of catecholamines might improve mood and act as anti-depressants [4]. Traditional anti-depressants, such as the tricyclic anti-depressants (TCAs) and monoamine oxidase inhibitors (MAOIs), increase the concentration of catecholamine neurotransmitters in the brain by either inhibiting neurotransmitter reuptake or inhibiting their degradation. Moreover, it is reported that NE-deficient mice lack responses to anti-depressant drugs, including selective serotonin reuptake inhibitors (SSRIs) [5].

TH catalyzes the synthesis of catecholaminergic neurotransmitters in neurons, and the level of neurotransmitters could be increased by supplement with TH. To study whether supplement with TH was effective in the treatment of depression, we applied protein transduction technique to delivery TH into brain because the delivery of proteins across the blood–brain barrier is severely limited by the proteins' size and biochemical properties. Protein transduction is an approach to deliver therapeutic peptides or proteins into brain by linking TH to so-called protein transduction domains (PTD, an undecapeptide, YGRKK RRQRRR, derived from the human immunodeficiency virus TAT protein), which is able to deliver macromolecules to pass

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the blood–brain barrier and cell membranes [6,7]. Here, we show that a recombinant PTD-TH fusion protein manifests the anti-depressant-like effect in mice.

Materials and methods

Preparation of recombinant PTD-TH. The preparations of recombinant TH and PTD-TH were described previously [8]. In brief, the plasmid pET3c-TH bearing the human TH gene was used as the template. Primer pairs were listed as follows: P1: 5′ GCAGAATTCATGCCCACCCCC GACG 3′; P2: 5′ GCTGTCGACCTAGCCAATGGCACTCA 3′ (the restriction sites are underlined). The pfu DNA polymerase was used for amplification of the TH gene fragments. PCR products were digested with *Eco*RI and *Sal*I, and cloned into the plasmid pET28a (pET-TH), then the nucleotide sequence of encoding the 11 amino acids of the HIV-1 TAT protein (the sense chain: 5′ GATCCTATGGTCGTAAAAAGCGACG CCAACGTAGACGTG 3′; the antisense chain: 5′ AATTCACGTCTA CGTTGGCGTCGCTTTTTACGACCATAG 3′) was incorporated in the enzymatic sites (*Bam*HI and *Eco*RI) of pET-TH to construct the pET-PTD-TH. The nucleotide sequence of the plasmids was analyzed for correctness by Bioasia Co. (Shanghai, China). The recombinant prokaryotic plasmids (pET-TH or pET-PTD-TH) were transformed into *Escherichia coli* BL21, then the bacteria were induced by 1 mM isopropyl-D-thiogalactoside to express the fusion protein. The bacteria were then harvested and sonicated. The fusion protein was purified by using Ni–NAT column at room temperature. The protein concentration was measured according to Lowry [9].

Animals. Male ICR mice (20–24 g, Grade II) were provided by the Shanghai Experimental Animal Center, Chinese Academy of Sciences. Animal studies were approved by the Animal Care and Use Committee, Ministry of Science and Technology, China. Animals were housed in plastic cages (420 × 240 × 170 mm) with free access to standard laboratory food and water, and kept in a regulated environment (23 ± 1 °C) in a 12-h light/dark cycle (lights off at 18:00 h).

Immunofluorescence staining. Immunofluorescence staining in the prefrontal cortex and the midbrain was assayed 5 h after intravenous (iv) injection by the tail veins of PTD-TH or TH (5 mg/kg) in a volume of 10 ml/kg (sterilized saline as solvent). Mice were anesthetized and fixed by intracardical perfusion of 4% paraformaldehyde in PBS (pH 7.4). The brains were cryoprotected in sucrose at 4 °C. The slices of the prefrontal cortex and the midbrain (30 μm) were, respectively, incubated stepwise in 10% normal goat serum diluted in PBS at 4 °C overnight, in the rabbit anti-TH polyclonal antibody (Bioss Biotech. Co., Beijing, China) diluted in PBS (1:1000) for 48 h at 4 °C, then in the Texas red-labeled goat anti-rabbit IgG (Sigma Co., USA) diluted in PBS (1:200) for 1 h. The PBS was used to wash the slices before each addition. Following the final wash, the slices were placed on coverslips using a fluorescent mounting medium. Brain sections were examined under fluorescence microscope to determine whether TH could enter the neurons.

Western blotting analysis. Mice were anesthetized and decapitated after iv injection PTD-TH by the tail veins. The prefrontal cortex and the midbrain of each mice were homogenized, respectively, in the lysis buffer (5 mM EDTA, 1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 10 mM CHAPS, 1% Triton X-100, 0.25 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 50 mM Tris–HCl, pH 8.2). Briefly, the supernatant fluids were collected after centrifugation at 10,000 rpm for 15 min at 4 °C. Samples were subjected to preparative SDS–PAGE in a 10% gel and electrophoretically transferred to the polyvinyl difluoride membranes. The membranes were blocked in the blocking solution (5% skimmed milk, 10 mM Tris–HCl, 100 mM NaCl, and 0.01% Tween 20) for 2 h at room temperature to block non-specific binding and immersed with the anti-TH antibody overnight at 4 °C. Membranes were washed twice for 15 min each in TBST (10 mM Tris–HCl, 100 mM NaCl, and 0.01% Tween 20) and incubated for 2 h with the horseradish peroxidase-conjugated secondary antibody, goat anti-rabbit IgG (1:1000). After washing twice for 15 min each in TBST, the signal was detected by the ECL system. A Western blot of GAPDH was performed in the same way, using a monoclonal anti-GAPDH antibody as the first antibody and a goat anti-mouse-horseradish peroxidase antibody (1:5000) as the second antibody. Bands were quantified using Bandscan software.

Forced swim test (FST). This test consists of a 15-min pretest swim and a 6-min test swim 24 h later [10]. The stressful pretest swim facilitates the development of immobility during the test session and increases the sensitivity to anti-depressants. On the first day of the experiment, the mice were placed in a cylindrical tank (20 cm tall × 14 cm in diameter) for the 15-min pretest, the tank containing 10-cm depth water at a temperature of 25 ± 1 °C. Animals received iv injections by the tail veins of saline, recombinant human TH or PTD-TH (5 mg/kg, 5 h before the test) in a volume of 10 ml/kg, or clomipramine (20 mg/kg), a tricycle anti-depressant used as a positive control, 2 h before the testing session. Water was changed between subjects. Immobility was defined as floating or no active movements made other than those necessary to keep the nose above the water. The tests were recorded by a video camera, which was positioned on the top of the tanks. The immobility time was scored at the final 4-min during the 6-min swimming. After the test, the animals were returned to their home cages and remained undisturbed.

Tail suspension test (TST). TST was similar to that described by Steru and his colleagues [11]. Mice were suspended on the edge of a shelf 5 cm above a tabletop by adhesive tapes, placed approximately 2 cm from the tip of the tail. They were allowed to hang for 6 min, and the duration of immobility was recorded during the last 5 min of the test. Mice were considered immobile only when they were hung passively and completely motionless. Testing was performed 5 h after the administration of the TH or PTD-TH.

Locomotor activity. This test is used as a measure of general activity level. Mouse was placed in a clear cage (420 × 240 × 170 mm) for 25 min to track down its locomotor activity after 5 h iv injected TH, PTD-TH (5 mg/kg) or the equivalent volume of saline (10 ml/kg). The locomotor activities were recorded by a video camera, which was positioned on the top of the cages. The activity counts were calculated and pooled over 5 min.

Statistical analysis. Data were expressed as means ± SEM and were evaluated for statistical significance with two-way ANOVA followed by Duncan's multiple range tests.

Results

TH mediated by PTD enters the neurons

The mesoprefrontal system plays an important role in the modulation of behavior and involves in a series of neuropsychiatric disorders [12,13], thus we selected two regions of focus, the prefrontal cortex, and the midbrain. Immunofluorescence assay in the prefrontal cortex and the midbrain was used 5 h after injection of PTD-TH or TH (5 mg/kg) to determine whether the systemic injection of PTD-TH or TH could enter the neurons. The results showed that the fluorescence was detected in the cytoplasm, particular rich in the cell bodies after injection of PTD-TH. However, the fluorescence could not be detected after injection of TH without bearing PTD (Fig. 1). The result indicated that the recombinant TH mediated by PTD could penetrate the blood–brain barrier and enter the neurons.

TH level in the prefrontal cortex and the midbrain was elevated after PTD-TH administration

Western blotting analysis was employed to determine the TH protein level in the prefrontal cortex and midbrain,

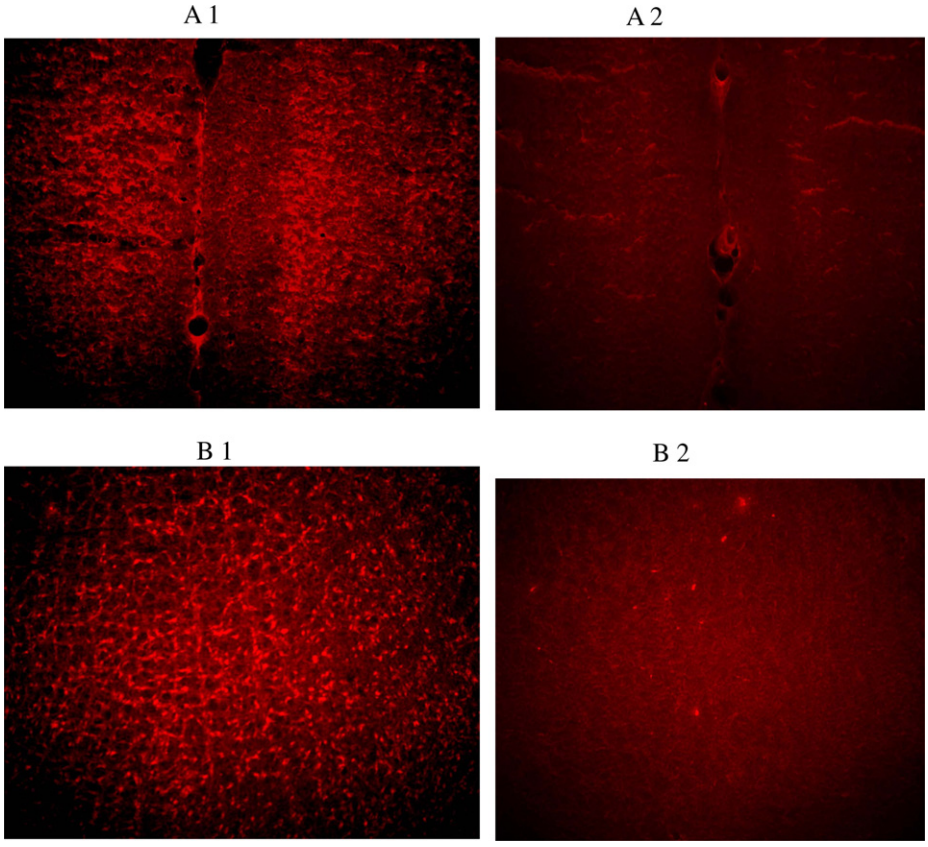

Fig. 1. Immunofluorescence analysis of TH mediated by PTD into the neurons. (A1) The prefrontal cortex of 5 h after recombinant PTD-TH (5 mg/kg) injection. (A2) TH (5 mg/kg) injection. (B1) The midbrain of 5 h after PTD-TH injection. (B2) TH injection. The slices were magnified 100.

using an anti-TH antibody. The prefrontal cortex and midbrain were dissected at different time points (0, 1, 2, 5, 8, 12, 23, or 31 h). As shown in Fig. 2, the TH protein level was significantly increased along with time, peaked at 5–8 h, and then declined gradually after iv injection 5 mg/kg PTD-TH by tail veins. The protein levels of TH at the 5 and 8 h increased significantly as compared with that of TH at the 0 h ($P < 0.05$). These results indicated that the level of endogenous TH could be regulated by systemic PTD-TH administration.

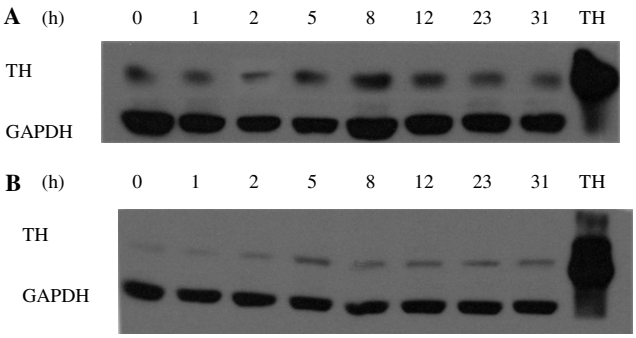

Fig. 2. Transient increase of TH level in the prefrontal cortex and the midbrain of mice after PTD-TH injection (5 mg/kg). The data are expressed as $\bar{x} \pm$ SEM, $n = 4$. *$P < 0.05$.

Anti-depressant-like effects of PTD-TH in the FST

The most widely used behavioral assay for detecting potential anti-depressant-like activity is the FST, which was originally developed by Porsolt and his colleagues [14]. This test had been shown to have high predictive validity for anti-depressant activity. In this test, animals displayed "despair" behavior as indicated by immobility. To evaluate further whether PTD-TH possesses anti-depressant-like activity, behavioral effects of PTD-TH in the FST were measured. PTD-TH-treated mice produced a significant reduction in the duration of immobility (45.2 ± 9.0 s) as compared with that of saline control (108.5 ± 13.3 s) in the FST (Fig. 3A), while TH-treated mice did not show the difference of immobility compared with that of saline (110.7 ± 15.5 s). Clomipramine, the positive control, significantly decreased immobility time as expected (57.9 ± 11.0 s), and the magnitude of anti-immobility effect of PTD-TH at 5.0 mg/kg was comparable to treatment of clomipramine at 20 mg/kg.

Anti-depressant-like effects of PTD-TH in the TST

The TST is another "behavioral despair" paradigm used to identify anti-depressants [15]. Thus, we tested whether immobility time in the TST was consistent with those

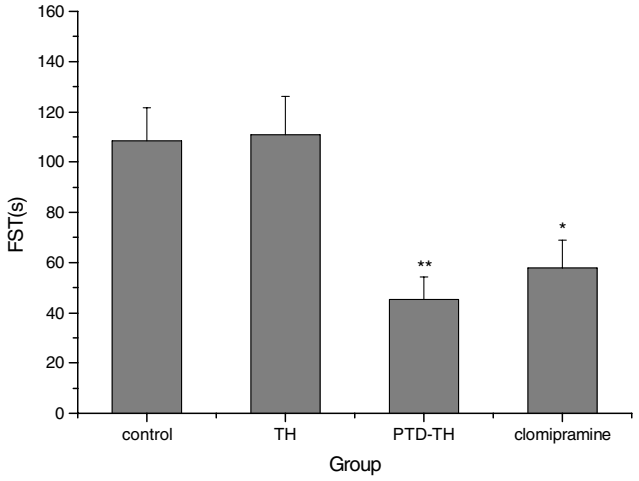

Fig. 3. Anti-depressant-like effect in the FST induced by iv injection of PTD-TH. $\bar{x} \pm$ SEM, $n = 8$. *$P < 0.05$, **$P < 0.01$.

obtained in the FST (Fig. 4). The result showed that the immobility times were about 2–3-fold shorter in PTD-TH (39.2 ± 8.9 s) and clomipromine (51.4 ± 6.6 s) than control mice (115.9 ± 7.2 s) during TST, while TH without PTD did not significantly decrease the duration of immobility (108.9 ± 9.2 s) compared with the control mice. The result was consistent with that of FST, suggesting that PTD-TH possessed the ability of anti-depression as potent as clomipramine.

Locomotor activity

To rule out non-specific motor effects of PTD-TH that could influence activity in the FST and TST, locomotor activity of mice was evaluated for 25 min after administration of PTD-TH or TH. PTD-TH showed no significant effects on locomotor activity as compared with that seen in the control and TH mice (Supplementary Fig. 5). Such data implied that the immobility-antagonizing in TST and swimming-enhancing effects of PTD-TH in the FST are not related to its increasing locomotor activity.

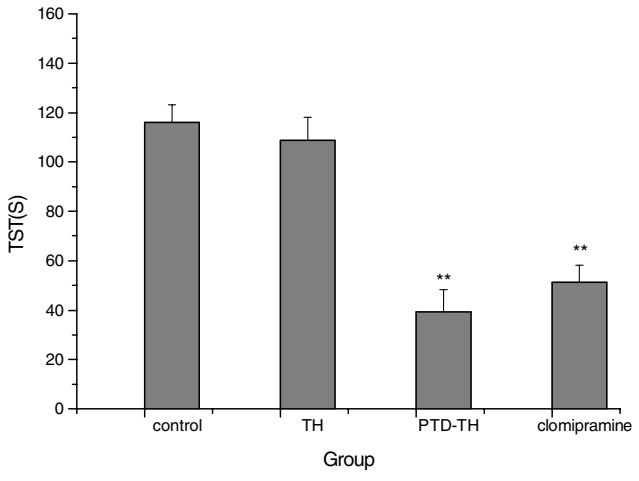

Fig. 4. Anti-depressant-like effect in the TST induced by iv injection of PTD-TH. $\bar{x} \pm$ SEM, $n = 8$. **$P < 0.01$.

Discussion

The present study demonstrated that endogenous TH, the key enzyme that synthesized catecholamines, could be increased after PTD-TH injection. Furthermore, the PTD-TH administration produced the anti-depressant-like activity in the FST and TST, supporting the idea that PTD-TH possessed the anti-depressant potential.

PTD-mediated delivery of biologically active proteins across the blood–brain barrier represents a novel and promising strategy to treat neuropsychic diseases [16]. TH is an ≈60 kDa globulin, thus it cannot pass the biological membranes and enter the cells. In our study, TH was confirmed that it could not get through the blood–brain barrier by the immunofluorescence assay. Supplement with TH without PTD had not affected the TH levels in the brains and animal behaviors. However, protein transduction of PTD-TH was successfully transduced into the CNS, increasing the levels of TH in the brains by systemic delivery without special preparation of the subject. The position of PTD in PTD-TH protein was predicted by computer software Insight II. The picture showed that PTD peptide stretched from the TH protein due to its highly cationic nature (Supplementary Fig. 6), which was advantageous to cargo protein into the cells. TH includes two separate domains, one regulatory domain (N-terminal), and one catalytic domain (C-terminal) [17]. The N-terminal domain lacks any catalytic activity, and all of the catalytic residues of TH are located in the C-terminal 330 amino acids. Furthermore, TH can be phosphorylated by protein kinases at serine residues, such as Ser 8, Ser 19, Ser 31, and Ser 40 [18,19]. From the homology model of the monomer of PTD-TH, the PTD may not alter/affect 3-D structure of TH because the PTD locates N-terminal of TH, which does not influence the TH enzyme activity, and TH does not include phosphorylation sites (serine residues), which cannot be phosphorylated. Moreover, the TH tetramer (whole enzyme) is formed by the hydrophobic bond, thus the highly cationic PTD peptide cannot effect the tetramer formation.

TH is the first and rate-limiting enzyme in the biosynthesis pathway of the catecholamine neurotransmitters: DA, adrenaline, and NE. These neurotransmitters are among the most widely used and universally distributed neurochemical systems in the brain. According to the catecholamine theory of mood suggested by Schildkraut and Kety [3], depression is associated with a catecholamine deficiency in the brain. Inhibition of the TH activity, such as α-methyl-para-tyrosine (AMPT), could stop the catecholamine synthesis and produce depression. Furthermore, large numbers of studies had reported that increment of TH level in the brain could be used to treat depression [20–23]. For example, electroconvulsive shock (ECS) was a highly effective therapy for the treatment of major

depression, mechanisms of this action were that the TH gene expression was elevated whether a single interaural ECS or repeated daily treatments [20]; administration of the cofactor of TH, tetrahydrobiopterin (BH4), had been reported to improve the clinical depression by increasing TH activity [21]; NADH could stimulate TH activity and was used to therapy depression [22]; furthermore, TH positive neurons were transplanted into catecholamine deficient patients to upregulate TH activity and thus treat the catecholamine-related diseases including Parkinson's disease and manic depression [23].

In the patients of depression, TH level was unbalance in the locus coeruleus (LC), the major noradrenergic nucleus in brain. But the results were inconsistent. Zhu and his colleagues [24] reported that the TH level was elevated in the LC in major depression. They found that amounts of TH-immunoreactivity (TH-ir) in the rostral, middle, and caudal levels of the LC from major depressive subjects were significantly higher than that of matched control subjects. However, Baumann and his colleagues [25] indicated that TH-ir in the LC was remarkably reduced in depressed non-suicidal patients but normal in depressed suicide patients (the results of studies about the TH levels in the LC of suicide victims remained controversial) [26,27], and implied that traditional anti-depressants could enhance noradrenergic activity of the LC in depressed patients. In the present study, systemic TH treatment could improve the behavioral despair in the forced swim test (FST) and the tail suspension test (TST), the two models widely used in screening potential anti-depressant efficacy, indicating that supplement with TH could be used to treat depression. These results were consistent with the theory that depression is associated with a catecholamine deficiency in the brains.

The current pharmacological treatment of patients with depressive disorders with anti-depressant drugs results in clinically significant improvement in 65–75% of patients and complete recovery in only 40–50% of patients, so better anti-depressants of the perspectives of efficacy are needed. In the previous studies [28,29], the rats were treated chronically with the representatives of all major classes of anti-depressant medication, including TCAs desipramine; serotonin and NE reuptake blocker imipramine; NE reuptake blocker nortriptyline; MAOIs tranylcypromine; SSRIs fluvoxamine and fluoxetine; atypical anti-depressants bupropion and iprindole; ECS, were found to decrease levels of TH-ir by 40–70% in the LC. Decreased levels of TH-ir were shown to be associated with equivalent decreases in enzyme mRNA levels. This might be the part reasons of ineffectiveness of anti-depressants for some depressive patients. Replacement remedy by PTD-TH or combination PTD-TH with other types of anti-depressants may be potential ways to improve the depressive disorder in patients.

The major finding of our study is that TH, the key enzyme of synthesis catechoamine, mediated by PTD, can increase TH level in neurons, therefore regulating mood disorder. PTD-TH treatment is effective in the two behavioral models of depression, the FST and TST. Thus, PTD-TH may function as a novel anti-depressant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.013.

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