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Research report

Infusion of BDNF into the nucleus accumbens of aged rats improves cognition and structural synaptic plasticity through PI3K-ILK-Akt signaling

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ABSTRACT

To investigate the involvement of the nucleus accumbens (NAc) in cognitive impairment and the therapeutic effects of brain-derived neurotrophic factor (BDNF) in an animal model of cognitive deficit, we infused BDNF into the NAc of cognitively impaired aged rats. Cognition was evaluated by Morris water maze test. Structural synaptic plasticity was measured by Golgi staining. Brain tissue homogenization was used to measure the changes in signal molecules. Cultured PC-12 cells expressing tyrosine kinase receptor (Trk) B/p75 neurotrophin receptor ($p75^{NTR}$), $p75^{NTR}$ or TrkA/$p75^{NTR}$ receptors were used for BDNF stimulation assays. Significant decreases in the levels of BDNF, phosphatidylinositol-3-kinase (PI3K) and integrin-linked kinase (ILK) activity, protein kinase B (Akt) Ser^{473} phosphorylation, dendritic branching, and density of dendritic spines on medium spiny neurons were observed in the NAc. Importantly, infusion of BDNF restored cognition, synaptic plasticity, and cell signaling. In cultured PC-12 cells, BDNF activated PI3K/Akt signaling through the TrkB receptor, whereas stimulation of ILK/Akt occurred through TrkA/$p75^{NTR}$ heteroreceptor. Our study suggested that the decreased BDNF level and its downstream signaling as well as loss of synaptic plasticity in the NAc are associated with cognitive impairments in aged rats. The BDNF-activated PI3K-Akt and ILK-Akt signaling play a key role in structural synaptic plasticity. Our study also suggested that BDNF could be a mechanism-based treatment for dementia.

1. Introduction

Memory and cognitive deficits are widely thought to be caused by dysfunction and death of neurons in specific regions/circuits such as the prefrontal cortex (PFC) and hippocampus, which are mainly subordinate to memory and cognition [1]. However, recent studies have demonstrated that information transferred from the hippocampus and PFC to the nucleus accumbens (NAc) plays a central role in several types of cognitive activities [2]. NAc is also involved in drug-related memories [3]. Therefore, pathological changes in the NAc may lead to impairments in memory and cognition [4]. The degree of surface deformity of the accumbens, rostral medial caudate nucleus, and ventral lateral putamen were found to be associated with the severity of cognitive impairment [5]. However, there is no direct evidence for the role of the NAc in memory and cognitive deficits.

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor that plays a pivotal role in synaptic plasticity and cognition [5]. Recent findings have suggested that a decrease in BDNF levels within the PFC and hippocampus is related to cognitive deficits in Alzheimer's disease (AD) animal models [6]. Moreover, serum BDNF concentration is significantly correlated with the severity of dementia in patients [7]. Strikingly, BDNF administration has been shown to reverse synapse loss and restore learning and memory in several animal models of AD, including a cognitively impaired aged rat model [8]. BDNF exerts its effects through TrkB-mediated activation of various signal transduction pathways, including pathways that involve PI3K, mitogen-activated kinase (MAPK), and phospholipase C-γ [9]. In addition, anti-AD drugs rasagiline and ladostigil enhanced the expression levels of BDNF accompanied by activation of PI3K, protein kinase and MAPK signaling [10]. Observations on brain activity of AD patients also indicate a decrease in TrkA in the cortex and nucleus basalis [11]. These observations gave rise to questions regarding TrkA's involvement in the activation of BDNF-associated signaling and whether infusion of BDNF could activate these signal pathways.

PI3K-mediated full activation of Akt requires phosphorylation on both the Thr^{308} and Ser^{473} residues. Parenthetically, the upstream kinases responsible for these two residues' phosphorylation are different and each residue can be phosphorylated

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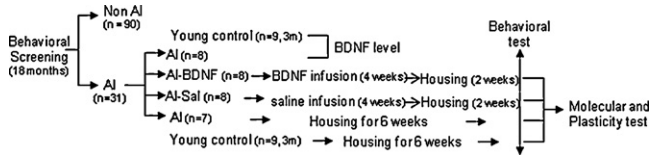

Fig. 1. Experiment design and treatments of different groups. 3 m: 3 months; AI: cognitively impaired aged rats. The rats in young control group were 4.5 months old and rats in AI groups were 19.5 months.

independently. Accordingly, Ser^{473} phosphorylation may occur in the absence of Thr^{308} and provide kinase activity to Akt [12]. An upstream kinase responsible for Akt Ser^{473} phosphorylation is the integrin-linked kinase (ILK) [13]. Although ILK has been studied primarily for its contribution to oncogenic transformation and cell survival [14], this kinase is localized in neuronal cell bodies and dendrites in various brain regions including the cerebral cortex, striatum and NAc [15]. Furthermore, ILK may play key roles in processes as diverse as neurite outgrowth following growth factor or integrin receptor stimulation, survival of hippocampal neurons under apoptotic conditions, and central nervous system myelination by oligodendrocytes [15–17].

In this study, we investigated whether infusion of BDNF into the NAc of the cognitively impaired aged rats could restore cognition and synaptic plasticity, and improve cell signaling, such as the PI3K-ILK-Akt pathway. We further identified the potential subtypes of tyrosine kinase receptors (Trk) which are involved in the BDNF stimulation.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats at the age of 3 months were provided by the animal center of Shanghai Biological Science Institution and housed in rooms under standard lighting conditions (12:12 h light–dark cycle) and temperature (22–23 °C). Water and food were provided *ad libitum*. All animal experiments were conducted under an approved protocol from Central South University and performed in accordance with the animal care guidelines of the Chinese Council.

2.2. Behavioral screening and Morris water maze test

Aged rats were screened for learning and memory impairment by Morris water maze test as previously described [18]. The Morris water maze test was performed as previously described [19]. The time rats took to find the platform was analyzed. The cognitive status of an aged animal was determined on the basis of its mean latencies to find the platform on day 4 of testing in Morris water maze relative to the latency of young controls. An aged impaired (AI) rat was defined as one whose latency differed by >3.0 SDs from young controls [18]. One hundred twenty one aged rats (18 months old) were screened and 31 rats were found to fit the AI rat criteria. The AI rats were randomly divided into 4 groups and subjected to BDNF measurement of the NAc tissue ($n = 8$), BDNF infusion ($n = 8$), saline infusion ($n = 8$), and no infusion ($n = 7$), respectively (Fig. 1). Eight young rats were continually housed ($n = 8$). Nine young rats and 8 AI rats were euthanized under deep isoflurane anesthesia and brains were rapidly removed (Fig. 1).

2.3. BDNF protein

To quantitatively analyze BDNF levels in brain tissue lysates, sandwich-style ELISAs were performed using the BDNF Emax ImmunoAssay System kit (Promega, Madison, WI) according to the manufacturer's instructions. The homogenate was diluted 1:4 with 1× sample buffer provided in the kit. BDNF concentrations were determined using a standard curve run for each plate (linear range: 7.8–500 pg/ml BDNF). These values were normalized to the respective total protein concentration. Samples from a given brain area were determined in single assays.

2.4. Surgery

One day after behavioral screening, rats in BDNF infusion and saline infusion groups were anesthetized with 60 mg/kg of sodium pentobarbital (i.p.) and surgery was performed using aseptic techniques. Rats were then mounted onto a Kopf stereotaxic frame and bilateral stainless steel cannulae (26 gauge; Plastics One) were implanted 2 mm above the NAc target region (AP, 2.5; ML, 0.6, relative to bregma) [20]. Cranioplastic cement anchored by three steel machine screws was applied to permanently secure the NAc cannulae to the skull.

2.5. NAc intracranial infusions and behavioral test

Two days after the surgery, infusion cannulaes (33 gauge) were inserted bilaterally into the guide cannulae, such that 1 mm of the infusion cannulae extended past the end of the guide cannulae. Five microliters (150 ng) of human recombinant BDNF (R&D Systems) or saline was infused per side into the NAc at a rate of 0.5 μl/min. The infusion was precisely controlled by an infusion pump (Harvard Apparatus). The infusion cannulae remained in the guide cannulae for 1 min before and after the infusion. Infusions were given 3 times per week for a total of 4 weeks. Two weeks after the last infusion, young rats (4.5 months) and all aged rats (BDNF infusion, saline infusion, and no infusion; 19.5 months) were subjected to Morris water maze test as described above (Fig. 1). The time rats took to find the platform was analyzed.

2.6. Brain dissection and protein extraction

Two weeks after infusion, animals were euthanized under deep isoflurane anesthesia and brains were rapidly removed. NAc from one hemisphere was dissected, rapidly frozen on dry ice, and stored at −80 °C for protein extraction. The other hemisphere was dissected for Golgi staining. Tissues for protein extraction were homogenized in 400 μl ice-cold homogenate buffer containing proteinase inhibitor cocktail [21]. Samples were sonicated and centrifuged to remove large cell debris. Protein concentrations were determined by Bradford assay.

2.7. Western blots

Western blots were performed as previously described [21]. The primary antibodies [anti-TrkA, anti-TrkB, anti-p75NTR, anti-phospho-Akt (Ser^{473}), anti-phospho-Akt (Thr^{308}), anti-Akt, phospho-(Tyr) PI3K p85α binding motif, 1:1000 dilutions] and peroxidase-labeled secondary antibody (1:2000 dilution) were purchased from Cell Signaling Inc. (Berverly, MA). The PI3 kinase activity was determined by the level of p85α phospho-tyrosine binding motif detected by Western blot [13]. The blot was developed with chemiluminescent substrate (Santa Cruz Biotechnology, Santa Cruz, CA). To control loading efficiency, blots were stripped and re-probed with α-tubulin antibody (1:1000 dilution; Sigma, St. Louis, MO) and expression levels of TrkA, TrkB, $p75^{NTR}$, total Akt, and phospho-Akt proteins were normalized relative to α-tubulin.

2.8. Golgi staining

Golgi staining was performed as previously described [20]. Briefly, a 1 cm section around the infusion site in the brain was dissected and transferred to a vial containing solution provided by the FD Rapid GolgiStain kit (Ellicott City, MD). The dendritic density in the NAc was analyzed at a lower magnification (20×). To minimize possible divergence in location, sections were numbered sequentially and similarly numbered sections from different rats were compared. The number of medium spiny neurons was counted by two "blinded" researchers. To measure the dendritic density, the densitometries from 10 photographs per rat were scanned and averaged, then normalized to the control.

2.9. Cell culture

Rat $PC12^{nnr5}$ cells (tyrosine kinase deficient mutant, $p75^{NTR}$ only), TrkA-PC12 cells ($PC12^{nnr5}$ cells over-expressing human TrkA receptor), and nnr5-TrkB cells ($PC12^{nnr5}$ cells stably transfected to express rat TrkB receptors) were grown in DMEM with 3% FBS and 5% horse serum [22–27]. $PC12^{nnr5}$-TrkA (B5) cells ($PC12^{nnr5}$ cells stably transfected to express rat TrkA receptors) were grown in RPMI1640 with 3% FBS and 5% horse serum [28]. All cells were grown at 37 °C, 5% CO_2. One hundred ng/ml of recombinant human BDNF and/or 60 μM LY294002 (PI3K inhibitor) were added to the cultures for 2 h. Cells were collected, triturated and proteins were extracted in 200 μl ice-cold homogenization buffer for Western blot and enzymatic activity assay.

2.10. ILK activity assay

Cells were lysed with homogenization buffer [21] and ILK activity assay was conducted as previously described [13], with modifications. Briefly, cells or NAc tissue lysates (100 μg) were incubated with 2 μg of anti-ILK antibody (Millipore, Temecula, CA) or IgG (as a negative control) overnight at 4 °C. Then, 50 μl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) were added and continually incubated for 2 h at 4 °C. The immune complex was isolated by centrifugation, washed 5× with 1 ml homogenate buffer, and subsequently washed 2× with kinase reaction buffer (25 mM Tris–HCl, pH7.5, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM NaF). Kinase activity was determined by incubating the immunoprecipitated complex with 1 μg inactive Akt-GST agarose (Millipore) and ATP (final concentration: 200 μM) in reaction buffer for 1 h at 30 °C. ILK activity was determined by Western blot of Akt-GST phosphorylation using the site-specific anti-phospho-Akt (Ser^{473}) antibody (cell signaling).