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Research report

G-protein-coupled receptor kinase interactor-1 serine 419 accelerates premature synapse formation in cortical neurons by interacting with Ca(2+)/calmodulin-dependent protein kinase IIβ

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ABSTRACT

In the present study, we investigated the mechanisms of brain derived neurotrophic factor (BDNF) in regulating cortical neuron premature synapse formation. KN-93, a specific inhibitor of Ca(2+)/calmodulin-dependent protein kinase II (CaMKII), and G-protein-coupled receptor kinase interactor-1 (G1T1) siRNA were utilized, and the premature synapse formation of cortical neurons was detected under BDNF stimulation. Plasmids HA-GIT1, HA-GIT1 (ΔSLD), HA-GIT1 (S419A) and Flag-CaMKIIβ were constructed. The interaction between GIT1 and CaMKIIβ, and their influence on the premature synapse formation of BDNF-stimulated cortical neurons were examined. BDNF-stimulated cortical neurons were associated with increased premature synapse formation, the enhancement of phosphorylation for CaMKIIβ, and the combination of GIT1 and p-CaMKII^{thr286}. G1T1 siRNA and KN-93 inhibited premature synapse formation in cortical neurons. The interaction between GIT1 and CaMKIIβ required SLD domain and serine 419 in GIT1. BDNF-induced CaMKIIβ phosphorylation and premature synapse formation were suppressed in GIT1 (S419A) transfected cortical neurons. By interacting with CaMKIIβ, G1T1 (S419) were shown to participate in BDNF-induced premature synapse formation within cortical neurons.

1. Introduction

Neural networks are built by synaptic connections, which play important roles in physiological and pathological processes of the central nervous system (CNS). Synapse formation is regulated by multiple factors such as ephrins, integrins, astrocytes, schwann cells and neural growth factors (Bamji et al., 2006; Segura et al., 2007; Suzuki et al., 2007; Ullian et al., 2004; Webb et al., 2007). Studies have confirmed that brain-derived neurotrophic factor (BDNF) is involved in the regulation of synapse formation (Bamji et al., 2006; Suzuki et al., 2007; Zhang et al., 2013). Luikart et al. found that short-term BDNF stimulation of neurons could increase the synapse number through the TrkB/PI3K pathway (Luikart et al., 2008). Alonso et al. observed that long-term BDNF stimulation of neurons (e.g., 12–24 h) regulated synapse formation through the TrkB/ERK1/2 pathway (Alonso et al., 2004). It is possible that BDNF enhances synapse formation by disrupting the cadherin–β-catenin interactions (Bamji et al., 2006), and accelerates the synthesis of synapse protein by affecting the biological formation of cholesterol in hippocampal and cortical neurons (Suzuki et al., 2007).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is highly expressed in the CNS of animals. In advanced vertebral animals, CaMKII is a polymer with 12 subunits; the CNS is comprised primarily of α and β subunits. The α and β subunits selectively form α and β enzymes or isomerases, and have completely different roles in neural plasticity (Fink et al., 2003; Schworer et al., 1993; Tombes et al., 2003). The α subunit mainly regulates synaptic strength, while the β subunit mainly influences dendritic growth, filopodia extension and synapse formation (Fink et al., 2003; Okamoto et al., 2007). Recent studies suggest that CaMKII regulates phosphorylation of histone deacetylase 5 (HDAC5) in vascular smooth muscle cells (VSMC) by interacting with G protein-coupled receptor kinase 2-interacting protein 1 (GIT1) (Pang et al., 2008). However, further research is required to elucidate the mechanisms of GIT1 and β subunits in synapse formation.

GIT1 is mainly located in focal adhesions of cytomembrane and cytoplasmic composite structures such as endosomes. Although GIT1 itself has no catalytic capability, it can induce intracellular kinase activity by autophosphorylation, and thus generate a series of intracellular biological effects. Our previous studies suggested that GIT1 was involved in the regulation of cell migration by tyrosine phosphorylation (Wang et al., 2010). A recent study by Ren

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et al. showed that the interaction of GIT1 tyrosine 321 phosphorylation with focal adhesion kinase (FAK) regulated cell migration stimulated by platelet-derived growth factor (PDGF) (Ren et al., 2012). GIT1 serine/threonine sites also have an important regulatory role e.g., regulating neural cell migration (Webb et al., 2006a), and neural synapse formation (Zhang et al., 2003), however, the specific mechanisms underlying GIT1 regulation of neural synapse formation are unclear.

Our study found that BDNF affected premature synapse formation by accelerating the interaction between GIT1 and CaMKIIβ. GIT1 S419 was important not only for interacting with CaMKIIβ, but it was also important for BDNF-stimulated CaMKIIβ phosphorylation and premature synapse formation.

2. Materials and methods

2.1. Reagents

Anti-rat GIT1, synapsin I and CaMKIIβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). p-CaMKII^{thr286} and GFP antibodies were from Cell Signaling Technology (Danvers, USA). Flag and HA antibodies were from Sigma–Aldrich (St. Louis, USA). All the secondary antibodies used in this study were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). BDNF was from PeproTech (Rocky Hill, USA). KN-93 was from Merck (Darmstadt, Germany). Rat GIT1 siRNA (AAGCTGCCAAGAAGAAGCTAC) and non-silencing control siRNA (AATTCTCCGACACGTGTCACT) were synthesized by Invitrogen (Carlsbad, USA) according to the published sequences (Cavet et al., 2008). The siRNA sequences were cloned into pGCsi3.0 GFP plasmid vector (Genechem, Shanghai, China) respectively. The immunofluorescence detection of GFP was carried out to choose the positively transfected neurons.

2.2. Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37.8 °C in 5% CO_2. HEK293 T cells were transfected by LipofectAMINE/plus (Invitrogen, Carlsbad, USA). Cortical neurons were isolated from Sprague Dawley rat embryos at the 18th day of gestation (E18) according to a few modifications of Meng's method (Meng et al., 2005). Briefly, under the aseptic condition, the cerebral hemisphere of the E18 embryos was dissociated, digested with 0.05% Trypsin/10 mg DNase I (Thermo Scientific, Logan, USA) for 10 min at 37.8 °C in an incubator with 5% CO_2 After ceasing the digestion, the cell suspensions were passed through a 70-μm mesh Falcon nylon filter. The filtered medium was centrifuged at 1000 rpm for 10 min. The cell pellet was resuspended in Neurobasal medium (Invitrogen) supplemented with 2%

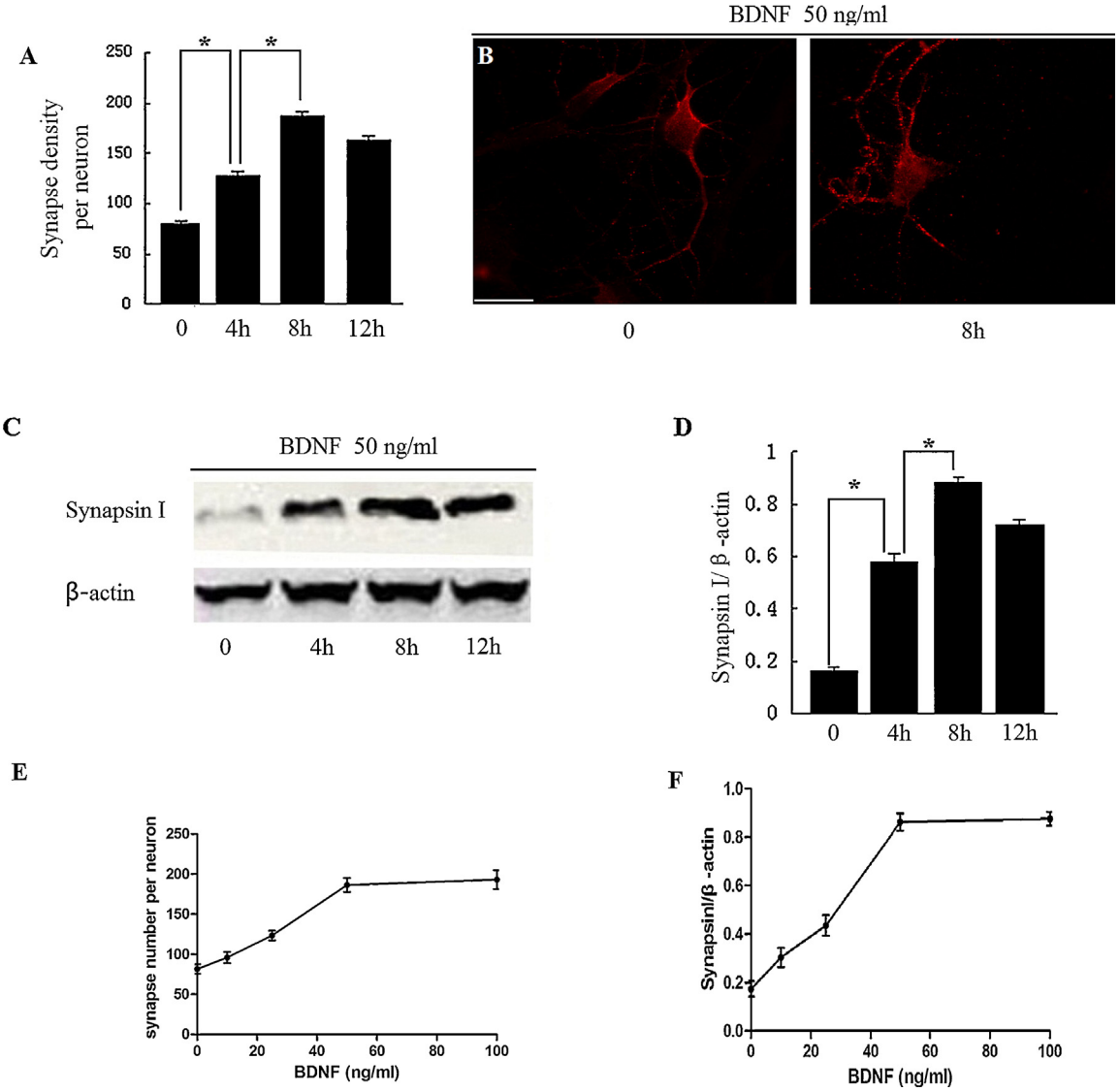

Fig. 1. The effect of BDNF on premature synapse formation of cortical neurons. BDNF was added to the medium on DIV 12. (A) Statistical result of synapsin I density after BDNF stimulation (50 ng/ml) for 0 h, 4 h, 8 h and 12 h respectively. (B) The synapsin I density of cortical neurons after BDNF stimulation (50 ng/ml) for 0 h and 8 h respectively. (C) The synapsin I expression of cortical neurons after BDNF stimulation for 0 h, 4 h, 8 h and 12 h respectively. (D) Statistical result of C. (E) The synapsin I density curve to BDNF stimulation for 8 h with 0 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml respectively. (F) The synapsin I expression curve to BDNF stimulation for 8 h with 0 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml respectively. *$P < 0.05$. Scale size is 40 μm.