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Distinct signaling pathways of precursor BDNF and mature BDNF in cultured cerebellar granule neurons

Hisatsugu Koshimizu[a,1,2], Shunsuke Hazama[a,b,1], Tomoko Hara[a,c], Akihiko Ogura[b], Masami Kojima[a,c,*]

[a] Research Institute for Cell Engineering (RICE), National Institute of Advanced Industrial Science and Technology (AIST), Ikeda 563-8577, Japan
[b] Graduate School of Frontier Bioscience, Osaka University, Suita 565-0871, Japan
[c] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Kawaguchi 332-0012, Japan

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ABSTRACT

Recent studies have focused on a distinctive contrast between bioactivities of precursor brain-derived neurotrophic factor (proBDNF) and mature BDNF (matBDNF). In this study, using a proteolytic cleavage-resistant proBDNF mutant (CR-proBDNF), signaling mechanisms underlying the proapoptotic effect of proBDNF and antiapoptotic effect of matBDNF on the low potassium (LK)-inducing cell death of cultured cerebellar granule neurons (CGNs) were analyzed. A time course study demonstrated that unlike matBDNF, CR-proBDNF failed to induce TrkB phosphorylation for up to 360 min. CR-proBDNF did not activate ERK-1, ERK-2 and Akt, which are involved in TrkB-induced cell survival signaling, while matBDNF activated these kinases. On the other hand treatment of CGNs with CR-proBDNF led to a rapid activation of Rac-GTPase and phosphorylation of JNK which are involved in $p75^{NTR}$-induced apoptosis. In addition, a JNK-specific inhibitor, SP600125, inhibited the CR-proBDNF-induced apoptosis but did not affect the antiapoptotic effect of matBDNF. CR-proBDNF treatment led to an earlier appearance of active caspase-3. In contrast, matBDNF dramatically postponed the appearance of active caspase-3. Not like other signaling molecules, activation of caspase-3 was conversely regulated by both CR-proBDNF and matBDNF. These results thus suggest that in CGNs proBDNF elicits apoptosis via activation of $p75^{NTR}$, Rac-GTPase, JNK, and caspase-3, while matBDNF signals cell survival via activation of TrkB, ERKs and Akt, and deactivation of caspase-3.

BDNF plays pivotal roles in the development and functioning of mammalian CNS neurons [2]. BDNF is first synthesized as a precursor (proBDNF) and is subsequently cleaved either intracellularly by prohormone convertases (PCs) and/or furin, or extracellularly by plasmin and matrix metalloproteases (MMPs) to form the mature protein (matBDNF). This pathway is common to many peptide hormones, growth factors and other neurotrophins, such as nerve growth factor (NGF), neurotrophin-3 (NT-3) and NT-4/5 [15]. matBDNF and other mature forms of neurotrophins elicit their biological actions by binding to the Trk family of receptor-type tyrosine kinases [11]. On the other hand, as recent studies revealed, precursor neurotrophins such as proNGF and proBDNF also have unique bioactivities which are opposite to those of their respective mature forms, mediated by $p75^{NTR}$ instead of Trk [1,5,9,16,19].

We have recently demonstrated that single nucleotide polymorphism (SNP) variants of *human BDNF*, which have amino acid substitution(s) near the cleavage site between the pro- and mature-domain of BDNF: R125M, R125L, R125M/R125L, leads predominantly to the secretion of protease cleavage-resistant proBDNF (CR-proBDNF) from cultured CNS neurons [7]. Because only trace amount of matBDNF is made from CR-proBDNF, CR-proBDNF has an advantage for the identification of proBDNF-specific signaling [7]. Cultured CGNs are widely used as a model system for studying neuronal apoptosis [20]. After maturation by culturing in high K^+ (HK) medium, changing to LK medium rapidly induces neuronal apoptosis [20]. Using CR-proBDNF we have previously revealed that in the presence of proBDNF, the LK-inducing apoptosis of rat CGNs was enhanced, while the presence of matBDNF inhibited the cell death [7]. In addition we have also demonstrated that proBDNF has other actions on CNS neurons in a cell type-specific manner; proBDNF dramatically reduced the number of cholinergic fibers of basal forebrain cholinergic neurons (BFCNs) and dendritic spines of hippocampal neurons, without affecting the survival of these neurons, while matBDNF increased

* Corresponding author at: Research Institute for Cell Engineering (RICE), National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31 Midorigaoka, Ikeda 563-8577, Japan. Tel.: +81 727 51 9081; fax: +81 727 51 9628.
E-mail address: m-kojima@aist.go.jp (M. Kojima).
[1] These authors collaborated equally.
[2] Current address: Section on Cellular Neurobiology, NICHD, NIH, Bethesda, MD, USA.

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the number of cholinergic fibers and hippocampal dendritic spines [7,17]. However signaling mechanisms underlying the contrasting events remain unsolved. In this study taking advantage of CR-proBDNF, the proapoptotic action of proBDNF and antiapoptotic action of matBDNF on the signaling of LK-inducing CGN cell death were studied.

Recombinant proBDNF (CR-proBDNF, BDNF-R125M/R127L) was prepared using an *E. coli* expression system as previously described [7]. Recombinant matBDNF was kindly provided by Sumitomo Pharmaceuticals (Osaka, Japan). The anti-BDNF (N-20; used at 1:1000 dilution), anti-ERK (K-23; 1:3000), and anti-phospho-ERK (E-4; 1:2000) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-phospho-Trk (Y490; 1:1000), anti-Akt (1:1000), anti-phospho-Akt (1:1000), anti-JNK (c-Jun N-terminus kinase; 1:200), anti-phospho-JNK (1:1000), and anti-cleaved caspase-3 (1:1000) antibodies were from Cell Signaling (Beverly, MA); the anti-$p75^{NTR}$ (1:1000) antibody was from Promega; the anti-TuJ1 (class III–tubulin; 1:1000) antibody was from BAbCo (Richmond, CA); the anti-TrkB ($TrkB_{out}$-specific antibody: clone 47; 1:1000) antibody was from BD Biosciences (San Jose, CA); the anti-mouse IgG (1:1000) and anti-rabbit IgG (1:1000) secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch (West Grove, PA). 4′,6′-Diamidino-2-phenylindole dihydrochloride (DAPI) was from Molecular Probes (Eugene, OR); the JNK inhibitor, SP600125, was from Tocris Bioscience (llisville, MO). Wistar ST rats and C57BL/6 mice were from NIPPON SLC (Hamamatsu, Japan); B6C3F1 mice were from the Charles River Laboratory (Yokohama, Japan); the $p75^{NTR}$-knockout mice ($ngfr^{tm1Jae}$) were from The Jackson Laboratory (Bar Harbor, MA). All animal experiments were strictly in accord with the protocols approved by the Institutional Animal Care and Use Committee of the AIST.

Primary cultures of dissociated rat and mouse CGNs were prepared from the cerebella of P9 rats, P6–7 wild-type ($p75^{NTR+/+}$) and knockout ($p75^{NTR-/-}$) mice, according to a previous report [8]. Briefly, cells were dissociated using a plastic pipette after digestion with papain (90 units/mL, Worthington) at 37 °C and were then cultured in minimum essential medium (MEM, Gibco, Carlsbad, CA) that contained 5% fetal bovine serum (FBS), 5% horse serum (HS), to a final cell density of 5×10^5 cells/cm^2 on polyethyleneimine-coated culture plates. After culturing for 1 day in a humidified CO_2 (5%) incubator, the medium was changed to MEM that contained 26 mM potassium (high K^+, HK) and was supplemented with 5% HS and 1 mM cytosine arabinoside (AraC). After 4 days in culture, cells were placed in serum-free MEM containing 5.4 mM potassium (LK) or HK-MEM, for experimentation.

The lysates of cultured neurons were prepared as described previously [7]. Briefly, cultured cells were washed three times with ice-cold PBS and were quickly lysed in cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 10 mM $Na_2P_2O_7$, 100 μM phenylarsine oxide, and 1% protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Hertforshire, UK). Lysates were boiled for 5 min at 100 °C and were then sonicated. The lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were electrotransferred to polyvinylidene fluoride membranes (Immobilon P membrane, Millipore, Bedford, MA), which were blocked in Tris-buffered saline (TBS) containing 0.2% Tween-20 (TBS-T) and 5% BSA or Block Ace (Dainippon Pharmaceutical, Osaka, Japan) and were then incubated with the indicated primary antibodies in TBS-T containing 0.5% BSA (TBS-TB) or 1% Block Ace at room temperature for 90 min. After washing three times with TBS-T, membranes were incubated with peroxidase-conjugated secondary antibodies in TBS-TB at room temperature for 30 min, washed three times with TBS-T, and chemical luminescence signals were detected using the ImmunoStar Reagents (Wako, Tokyo, Japan) or the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). Anti-TuJ1 antibody was used for the normalization (data not shown).

Cell viability was quantified by counting the number of dead cells using DAPI as previously described [7]. Briefly, cells were fixed using 4% paraformaldehyde (PFA)/PBS for 20 min and were then stained with 100 ng/mL DAPI in PBS for 15 min. The ratio of condensed to intact nuclei was quantified based on observations using a fluorescence microscope.

Cultured CGNs were maintained in HK-MEM for 4 days. Cells were then incubated for 5 min in LK medium, in the presence or absence of the indicated agents. Cells were lysed in 400 μL of buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM Tris–HCl (pH 7.5), 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO), and 1% phosphatase inhibitor cocktail 2 (Sigma–Aldrich). GTPγS (1 mM) and GDP (1 mM) were added into mock lysates as positive and negative controls, respectively, followed by a 15 min incubation at 30 °C. To perform the pull-down experiment, 10 μL of the p21-binding domain of human PAK-1 (PAK-1 PBD, which is predicted to interact with all mammalian Rac and cdc21 proteins) conjugated to glutathione agarose beads (Rac/cdc42 assay reagent, Upstate, Temecula, CA) was added to the lysates and the mixture was rotated at 4 °C overnight. The agarose beads were pelleted and the bound protein was analyzed by Western blot using an anti-Rac antibody (Upstate).

Significance was analyzed by one-way ANOVA followed by Tukey's *post hoc* test. In all graphs, data were presented as means ± standard error of the mean.

First, to determine whether the proBDNF-mediated apoptosis observed in CGNs is mediated by $p75^{NTR}$, we compared the effects of proBDNF in CGNs derived from $p75^{NTR}$-knockout mice ($p75^{NTR-/-}$) to its effect in CGNs from their wild-type littermates. CGNs were incubated with 5 mM potassium (low K^+, LK) medium with 100 ng/mL proBDNF (CR-proBDNF, BDNF-R125 M/R127L) or 100 ng/mL matBDNF. Forty-eight hours after the treatment DAPI staining was performed as previously described [7], revealing that treatment with CR-proBDNF enhanced cell death in CGNs from wild-type mice (Fig. 1, left, $p75^{NTR+/+}$, $^*P < 0.05$, compared to mock), which was consistent with the data obtained from rat CGNs [7]; however, treatment of CGNs derived from $p75^{NTR-/-}$ mice with CR-proBDNF failed to induce cell death (Fig. 1, right, $p75^{NTR-/-}$). On the other hand matBDNF inhibited the cell death in both genotypes (Fig. 1, $^*P < 0.05$, compared to mock).

To identify the mat/proBDNF signaling pathways we analyzed what signaling molecules are activated by matBDNF or

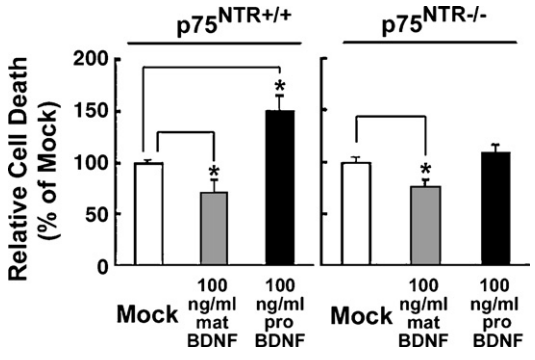

Fig. 1. The proapoptotic effect of proBDNF is dependent on $p75^{NTR}$. After 4 days of culture in HK medium, CGNs from $p75^{NTR+/+}$ (left) or $p75^{NTR-/-}$ [16] mice were treated with CR-proBDNF (proBDNF)- or matBDNF-containing 5 mM potassium (low K^+, LK) medium for 48 h and the cell viability was quantified using DAPI nuclear staining as previously described [7]. $^*P < 0.05$, one-way ANOVA followed by Tukey's *post hoc* test, $n = 4$ independent culture dishes. Results were replicated in at least three independent experiments. Note that CR-proBDNF did not induce apoptosis in CGNs derived from $p75^{NTR-/-}$ mice.