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Research article

Striatal hypodopamine phenotypes found in transgenic mice that overexpress glial cell line-derived neurotrophic factor

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HIGHLIGHTS

- The expression of GDNF transgene increases RET phosphorylation in the brain.
- The chronic increase in glial GDNF reduces the dopamine content and tyrosine hydroxylase activity.
- Extracellular concentrations of dopamine and its metabolism decrease in the striatum of transgenic mice.
- The alteration of locomotor traits of transgenic mice is consistent with dopaminergic changes.

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) positively regulates the development and maintenance of *in vitro* dopaminergic neurons. However, the *in vivo* influences of GDNF signals on the brain dopamine system are controversial and not fully defined. To address this question, we analyzed dopaminergic phenotypes of the transgenic mice that overexpress GDNF under the control of the glial *Gfap* promoter. Compared with wild-type, the GDNF transgenic mice contained higher levels of GDNF protein and phosphorylated RET receptors in the brain. However, there were reductions in the levels of tyrosine hydroxylase (TH), dopamine, and its metabolite homovanillic acid in the striatum of transgenic mice. The TH reduction appeared to occur during postnatal development. Immunohistochemistry revealed that striatal TH density was reduced in transgenic mice with no apparent signs of neurodegeneration. In agreement with these neurochemical traits, basal levels of extracellular dopamine and high K+-induced dopamine efflux were decreased in the striatum of transgenic mice. We also explored the influences of GDNF overexpression on lomomotor behavior. GDNF transgenic mice exhibited lower stereotypy and rearing in a novel environment compared with wild-type mice. These results suggest that chronic overexpression of GDNF in brain astrocytes exerts an opposing influence on nigrostriatal dopamine metabolism and neurotransmission.

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) belongs to the transforming growth factor-beta (TGFβ) superfamily whose members include neurturin, artemin, persephin, and others [1]. These neurotrophic factors bind to GDNF family receptors (GFR) and activate its co-receptor (RET tyrosine kinase) to transduce their signals [2]. *In vitro* culture and slice studies reveal that GDNF and its derivatives in the TGFβ family act on brain dopamine neurons and promote their development and regeneration [3–5]. Further, these neurotrophic factors enhance cell autonomous firing and dopamine release and reuptake from their terminals [6–8], supporting their neurotrophic role in dopaminergic development and maintenance [9]. In animal models for Parkinson's disease, the gene delivery of GDNF efficiently prevents midbrain dopaminergic neurons from their neurodegeneration [10–12]. Gene knockout studies of the

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GDNF signal transducer RET also indicate its neurotrophic function in the dopaminergic system [13,14].

However, various *in vivo* findings contraindicate the above neurotrophism of GDNF. Analyses of GDNF-full deficient mice reveal that the no apparent decreases of dopaminergic cells, fibers, and monoamines are detected in these mutant mice under certain genetic backgrounds or breeding conditions [15]. Further, mice with heterozygous mutations in the GDNF gene rather exhibit increased levels of dopamine and tyrosine hydroxylase (TH) [16]. A similar discrepancy associated with GDNF neurotrophism occurs when exogenous GDNF is provided [17]. Overexpression of the GDNF gene from a viral vector down-regulates dopaminergic neurotransmission [18–22]. In this context, *in vivo* effects of GDNF hypersignaling on brain dopaminergic system remain to be controversial.

What causes the distinct and/or discrepant actions of GDNF hypersignals on the dopaminergic system? In the present investigation, we exmamined the neurotrophic actions of a GDNF transgene *in vivo* and attempted to illustrate the *in vivo* role of GDNF in the nigrostriatal dopamine system.

2. Materials and methods

2.1. Animals

Mice were housed in plastic cages (200 mm × 300 mm × 140 mm) with food and water available *ad libitum*. Each cage contained 2–4 mice and was kept in a temperature-controlled room (22 ± 1 °C) under a 12–12-h light–dark cycle (8:00 on, 20:00 off). Only male mice were used for experiments to avoid the infleunces of female estrus cycle. The animal experiments described here were approved by the Animal Use and Care Committee of Niigata University and were performed in accordance with the Guiding Principles for Care and Use of Laboratory Animals (NIH, USA).

2.2. Glial cell line-derived neurotrophic factor (GDNF) transgenic mice

A full-length mouse GDNF cDNA was inserted into the plasmid cassette *pGFAP-hGH*, containing a 5′-flanking region of human glial fibrially protein gene (*Gfap)* and a 3′-flanking region of the human growth hormone gene, including its polyadenylation signal sequence [23]. Prototype transgenic mice were generated by pronuclear injection of the DNA fragment into fertilized mouse eggs (B6/CBA strain) and were bred by crossing with wild-type mice at least five times (C57 B6/N strain; CLEA Japan Inc., Tokyo Japan). The pure genetic background of C57B6/N transgenic mice produced the psychobehavioral abnormality resembling Tourette syndrome or obsessive-compulsive disorder. The mice frequently performed head grooming by themselves and produced head skin lesion, leading to its infection. Thus, we used the sperm of the C57B6/N transgeneic mice to inseminate the eggs of wild-type mice (FVB/N strain; CLEA Japan Inc.) to generate F1 heterozygous mice carrying both C57B6/N and FVB/N genetic backgrounds equally. These F1 mice were genotyped using PCR with forward (5′-AGCTCACTGCAGCCTCAACCTACT-3′) and reverse (5′-CAGGCATATTGGAGTCACTGG-3′) primers and subjected to the following experiments. Unless the age of mice was specified, 8–12 week old mice were subjected to the following experiments.

2.3. Quantification of GDNF

Mice were anesthetized using isoflurane and then decapitated. After removing the brain, 1-mm slices were prepared, and the striatum and ventral midbrain were dissected on ice. Each tissue was stored at −80 °C until use. Brain tissues were homogenized in 50 mM Tris-buffered saline containing 1% NP-40, 1% glycerol, and protease inhibitor cocktail (Roche Diagnosis Japan, Tokyo, Japan). Brain homogenates were centrifuged at 15000 rpm for 30 min at 4 °C, and the supernatants were harvested. GDNF content in the supernatant was determined using an enzyme immunoassay kit (Biogenesis, Thebarton, SA, Australia). Protein concentrations in the samples were determined using a Micro BCA kit (Pierce, Rockland, IL, USA). The average of two measurements per sample was normalized and used.

2.4. Immunoblotting

Brain tissues were homogenized in a 10-fold volume of lysis buffer containing 2% sodium dodecylsulfate (SDS), 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine, and protease inhibitor cocktail (Roche Diagnosis). After centrifugation at 12,500 rpm for 20 min, the supernatants were harvested, and the protein concentrations were determined using a Micro BCA kit (Pierce Chemical, Rockland, IL). Sample buffer [5x; 0.31-M Tris-HCl (pH 6.8), 5% SDS, 50% glycerol, 25% dithiothreitol] was added to the supernatants. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (ADVANTEC, Tokyo, Japan) at 4 °C for 12 h. The membrane was probed with an anti-TH antibody (1:1000; EMD Millipore, Billerica, MA), an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:500; Santa Cruz Biotechnology, Dallas, TX). Alternatively we used the anti-phospho Ret (Tyr 1062) antibodiy (1:1000; Santa Cruz Biotechnology, sc-20252R) and the anti-total Ret protein antibody (1:1000; Santa Cruz Biotechnology, H-300) that have been validated previously [24,25]. Immunoreactivity was detected using an anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratory, West Grove, PA) followed by chemiluminescence detection of immune complexes (Immunostar, WAKO, Tokyo, Japan).

2.5. Immunohistochemistry

Mice were anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Brains were removed and then fixed in the same solution for 24 h at 4 °C. Fixed brains were embedded in paraffin. Sections were cut with 0.4 μm thickness. After rinsing in Tris-buffered saline [TBS; 0.1 M Tris-HCl (pH 7.4), 150 mM NaCl] containing 0.2% Triton X-100, sections were treated with 6% bovine serum albumin and 0.2% Triton X-100 in TBS and then probed with the anti-TH antibody. After rinsing three times in TBS/0.2% Triton X-100, sections were incubated with a biotinylated anti-rabbit immunoglobulin antibody. Immunoreactivity was visualized using a Vectastin Elite ABC kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the substrate.

2.6. Determination of tissue content of dopamine and its metabolites

Brain tissues were homogenized in an extraction solution (0.1 M perchloric acid, 0.1 mM EDTA). Dopamine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid levels in the homogenates were determined using high-performance liquid chromatography (HPLC) and electrochemical detection as described previously [26]. Tissue pellets were homogenized in 0.5 N NaOH, and the protein concentration was determined using Micro BCA kit (Pierce). Monoamine contents of tissues were normalized to those of the protein concentrations.