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GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR IS CRUCIAL FOR LONG-TERM MAINTENANCE OF THE NIGROSTRIATAL SYSTEM

N. NEVALAINEN,[1] M. CHERMENINA,[1] A. REHNMARK, E. BERGLÖF, F. MARSCHINKE AND I. STRÖMBERG*

Department of Integrative Medical Biology, Umeå University, SE-90187 Umeå, Sweden

Abstract—Glial cell line-derived neurotrophic factor (GDNF) is a potent factor for the ventral mesencephalic dopamine neurons. However, studies on the *Gdnf* gene deleted (*Gdnf*$^{-/-}$) mouse have been limited to fetal tissue since these mice die prematurely. To evaluate long-term effects of *Gdnf* gene deletion, this study involves co-grafts of ventral mesencephalon (VM) and lateral ganglionic eminence (LGE) derived from different *Gdnf* genotypes. The VM/LGE co-grafts were evaluated at 3, 6, and 12 months for tyrosine hydroxylase (TH) -positive cell survival and nerve fiber formation in the LGE co-transplant, visualized by dopamine- and cyclic AMP-regulated phosphoprotein relative molecular mass 32,000 (DARPP-32) -immunoreactivity. Cell counts revealed no difference in TH-positive neurons between *Gdnf* genotypes at 3 months postgrafting. At 6 months, a significant reduction in cell number was observed in the *Gdnf*$^{-/-}$ grafts. In fact, in the majority of the *Gdnf*$^{-/-}$ VM/LGE transplant had degenerated. At 12 months, a reduction in cell number was seen in both *Gdnf*$^{-/-}$ and *Gdnf*$^{+/-}$ compared to wild type transplants. In the *Gdnf*$^{-/-}$ grafts, TH-negative inclusion-like structures were present in the cytoplasm of the TH-positive neurons at 3 months. These structures were also found in the *Gdnf*$^{+/-}$ transplants at 12 months, but not in *Gdnf*$^{+/+}$ controls at any time point. In *Gdnf*$^{+/+}$ grafts, TH-positive nerve fiber innervation of the striatal co-grafts was dense and patchy and overlapped with clusters of DARPP-32-positive neurons. This overlap did mismatch in the *Gdnf*$^{+/-}$ grafts, while the TH-positive innervation was sparse in the *Gdnf*$^{-/-}$ transplants and the DARPP-32-positive neurons were widespread distributed. In conclusion, GDNF is essential for long-term maintenance of both the VM TH-positive neurons and for the striatal tissue, and appears crucial for generation of a proper organization of the striatum.

Key words: GDNF, transplant, substantia nigra, striatum, DARPP-32, *Gdnf* knockout.

The development of the dopamine neurons in the nigrostriatal system has been well characterized: generation occurs during the early development and after generation they migrate into a ventral-lateral direction upon leaving the proliferative zone (Olson and Seiger, 1972; Lauder and Bloom, 1974; Vitalis et al., 2005; Gates et al., 2006). After migration, the nigral neurons extend their axons to enter the striatal anlage, the lateral ganglionic eminence (LGE). The dopamine nerve fiber innervation is developed along the lateral border of the striatum, and then in dopamine-dense islands in the striatal anlage (Olson et al., 1972; Voorn et al., 1988). These dopamine-dense patches are overlapping with specific areas that correspond to the developing striatal striosomes (Graybiel, 1984; van der Kooy and Fishell, 1987). The areas between these striosomes are developed into the matrix compartment of the striatum, although it takes place at a later stage than the development of the striosomes (Fishell and van der Kooy, 1987).

Many studies have been devoted to find attractants that promote the dopamine nerve fiber growth, especially in the context of promoting regeneration from dopamine neurons in Parkinson's disease. Among numerous neurotrophic factors that have been evaluated for their effects on the ventral mesencephalic (VM) dopamine neurons, glial cell line-derived neurotrophic factor (GDNF) has gained great attention. GDNF was purified and cloned in 1993 (Lin et al., 1993), and results have demonstrated to improve graft survival as well as exert neuroprotective and neurorestorative properties on the midbrain dopamine neurons (Strömberg et al., 1993; Beck et al., 1995; Tomac et al., 1995a; Rosenblad et al., 1996; Sinclair et al., 1996; Granholm et al., 1997; Yurek, 1998). Indeed, GDNF infused into the brains of parkinsonian patients significantly reversed the symptoms with improved activities of daily living, increase in [^{18}F]-dopamine uptake, and sprouting of dopamine nerve fibers in the putamen (Gill et al., 2003; Love et al., 2005; Slevin et al., 2005). Thus, GDNF appears as a potential candidate for treatment in Parkinson's disease in terms of rescuing the reminiscent dopamine neurons and inducing dopamine nerve fiber sprouting, although the role of GDNF during normal adulthood remains unknown.

Studies concerning the presence or absence of GDNF have been performed using the *Gdnf* knockout (*Gdnf*$^{-/-}$) mice. However, these mice lack kidneys, ureters, and the enteric system, and therefore die shortly after birth (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996). Consequently, it has not been possible to study GDNF-dependency on the dopamine system during adulthood. However, studies on the *Gdnf* heterozygous (*Gdnf*$^{+/-}$) mice, which have reduced striatal GDNF levels, demonstrate accelerated loss of mesencephalic dopamine neurons compared to normal aging (Airavaara et al., 2004; Boger et al., 2006). To further explore the importance of

[1] Equal contribution.

*Corresponding author. Tel: +46-90-786-6585; fax: +46-90-786-6696.
E-mail address: ingrid.stromberg@histocel.umu.se (I. Strömberg).

Abbreviations: ALDH1, aldehyde dehydrogenase 1A1; DARPP-32, dopamine and cyclic AMP-regulated phosphoprotein relative molecular mass 32,000; GDNF, glial cell line-derived neurotrophic factor; GIRK2, G-protein activated inwardly rectifying potassium channel 2; LGE, lateral ganglionic eminence; TH, tyrosine hydroxylase; VM, ventral mesencephalic.

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GDNF on the nigrostriatal system, transplanting *Gdnf*$^{-/-}$ tissue enable studies on GDNF-dependency from development to adulthood. In the present study, fetal VM and LGE was co-grafted into the lateral ventricles of wild type mice to create a nigrostriatal microcircuit that was developed and maintained in the absence or presence of GDNF. The co-grafts were morphologically evaluated at 3, 6, and 12 months postgrafting.

EXPERIMENTAL PROCEDURES

Animals

Gdnf$^{-/-}$ mice on a C57Bl/6J background were used for this study (Pichel et al., 1996). *Gdnf*$^{+/-}$ mice were mated to obtain *Gdnf*$^{+/+}$, *Gdnf*$^{+/-}$, and *Gdnf*$^{-/-}$ fetuses. Graft recipients were *Gdnf*$^{+/+}$ female mice around 3–4 months old. Animals were housed in a temperature controlled and 12 h light/dark cycle environment with free access to food and water. The animal experiments had been approved by the local ethics committee, which is in the accordance with NIH guidelines.

Intracranial transplantation

For collecting tissue for transplantation, pregnant *Gdnf*$^{+/-}$ mice at embryonic day (E) 14 were deeply anesthetized with 4% isofluran (Baxter Medical AB, Kista, Sweden) using Univentor 400 anaesthesia machine (AgnThos, Stockholm, Sweden). Following dislocation of the neck, the fetuses were obtained from the abdominal cavity and placed in a sterile Petri dish. The tip of the tails were collected from all fetuses and kept on ice during the operation procedure. The VM and the LGE were dissected from the fetuses and kept in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) aseptically. Meanwhile, adult *Gdnf*$^{+/+}$ mice were anesthetized with isofluran and placed in a stereotaxic frame. A hole was drilled in the cranium at 0.8 mm in a mediolateral direction, at the bregma level. The VM and LGE tissues were inserted into the lateral ventricle with a push-pull cannula that was lowered 3.5 mm below the dura mater. Every VM/LGE co-transplant originated from the same fetus, such that co-grafts from *Gdnf*$^{+/+}$ (n=32), *Gdnf*$^{+/-}$ (n=40), *Gdnf*$^{-/-}$ (n=18) were included in this study. Graft survival time was 3 (*Gdnf*$^{+/+}$ n=16, *Gdnf*$^{+/-}$ n=7, *Gdnf*$^{-/-}$ n=8), 6 (*Gdnf*$^{+/+}$ n=11, *Gdnf*$^{+/-}$ n=17, *Gdnf*$^{-/-}$ n=6), and 12 (*Gdnf*$^{+/+}$ n=5, *Gdnf*$^{+/-}$ n=16, *Gdnf*$^{-/-}$ n=4) months.

Tissue preparation

For evaluating tissue, mice with grafts were deeply anesthetized with pentobarbital (60 mg/mL, i.p.), and sacrificed by intracardial perfusion. Ca^{2+}-free Tyrode solution was used to rinse the blood system followed by fixation of the tissue with 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The brains were postfixed in 4% paraformaldehyde for 1–2 h after dissection and then transferred to 10% sucrose in 0.1 M phosphate buffer with 0.01% sodium azide. The sucrose solution was changed for several times to rinse paraformaldehyde from the tissue.

For evaluating fetal brains, the whole head was immersion fixed in 4% paraformaldehyde over night. The tissue was collected from E19 fetuses of *Gdnf*$^{+/+}$, *Gdnf*$^{+/-}$, and *Gdnf*$^{-/-}$ mutant mice (n=3 for each genotype), and the tails from each fetus were collected to be genotyped. After fixation, the tissue was rinsed in sucrose solution and then processed as described for transplanted brain tissue.

Genotyping

DNA was extracted from tail tissue of fetal donors and analyzed by using polymerase chain reaction (PCR) to determine the *Gdnf* genotype. To extract DNA from the cells, each tail tissue sample was homogenized with 200 μl lysis-buffer (25 mM NaOH and 0.2 mM EDTA; pH=12) and placed in a thermomixer (Thermomixer Compact, Eppendorf, Hornsholm, Denmark) for 1 h at 95 °C. Thereafter, samples were cooled to 4 °C and 200 μl of a neutralizing buffer (40 mM Tris–HCl; pH=5) was added. DNA samples were stored at −20 °C. When preparing for the PCR reaction, each reaction tube was loaded with a total volume of 20 μl containing 13 μl dH_2O, 0.4 μl TAQ polymerase 5U (Fermentas, Helsingborg, Sweden), 2 μl 10× buffer (Fermentas), 2 μl $MgCl_2$ from 25 mM (Fermentas, Helsingborg, Sweden), 0.3 μl sense primer and 0.3 μl antisense primer (Promega, Nacka, Sweden), 0.5 μl dNTP 10 mM (Fermentas, Helsingborg, Sweden), and finally 1.5 μl of DNA sample or dH_2O for negative controls. Two sets of primers were used to identify the presence of *Gdnf*$^{+/+}$ gene and *Gdnf*$^{-/-}$ gene, respectively. For detection of the *Gdnf*$^{+/+}$ genes, primer sense – 5′-CCA GAG AAT TCC AGA GGG AAA GGT C-3′ and antisense– 5′-CAG ATA CAT CCA CAC CGT TTA GCG G-3′ was added. To detect *Gdnf*$^{-/-}$ gene, primer sense – 5′- CGG AGC CGG TTG GCG CTA CCG G-3′ and antisense – 5′-ACG ACT CGG ACC GCC ATC GGT G-3′ were utilized. PCR was performed in a thermal cycler (PTC-200, MJ Research, Inc., Waltham, MA, USA) programmed to amplify the DNA during 40 cycles totally. The program was initiated with a denaturation step at 92 °C for 4 min and thereafter followed by 40 cycles where each cycle consisted of 1 min denaturation at 92 °C, 1 min annealing at 56 °C and 2 min elongation at 72 °C. PCR products were stored at −20 °C until analyzed on a 2% agarose gel (Fermentas, Helsingborg, Sweden) by gel electrophoresis. A 100 basepair (bp) DNA ladder (Fermentas, Helsingborg, Sweden) was applied as a reference. DNA fragments consisting of 344 bp was detected for the *Gdnf*$^{+/+}$ allele and fragments of 255 bp indicated *Gdnf*$^{-/-}$. For *Gdnf*$^{+/-}$, one DNA product of each reaction and size was received.

Immunohistochemistry

The brains were frozen with gaseous CO_2 and cut into 14 μm thick sections using a cryostat. The tissue sections were thawed onto slides coated with gelatin/chrome alune. The sections were then washed in phosphate buffered saline (PBS; 0.1 M, pH=7.4) for 15 min and subsequently mounted with 90% glycerol in PBS until processed for antibody incubations. Sections were incubated with primary antibodies, raised in mouse or rabbit against the indirect dopamine marker tyrosine hydroxylase (TH; mouse-anti-TH, 1:1500; Immunostar, Inc; Hudson, WI, USA; rabbit-anti-TH, 1:300; Pel-Freeze; Rogers, AR, USA) and the striatal cell marker dopamine and cyclic AMP-regulated phosphoprotein relative molecular mass 32,000 (DARPP-32; raised in rabbit, 1:600; Cell Signaling Technology; Danvers, MA, USA). To distinguish between subtypes of dopamine neurons antibodies against G-protein Activated Inwardly Rectifying Potassium Channel 2 (GIRK2; raised in rabbit, 1:25; Millipore, Solna, Sweden), aldehyde dehydrogenase 1A1 (ALDH1; raised in rabbit; 1:100, Abcam, Cambridge, UK), and calbindin (raised in mouse; 1:100; Sigma, Saint Louis, MO, USA) were used in the combination with antibodies against TH. Incubation in antibodies raised against Iba1 in rabbit (1:1000; Wako Pure Chemical Industries, Ltd, Osaka Japan) and glucose transporter 1 (Glut1; raised in rabbit; 1:250; Abcam, Cambridge, UK) were utilized to visualize microglia and blood capillaries, respectively. In addition, antibodies against α-synuclein (raised in rabbit; 1:50; Cell Signaling Technology, Danvers, MA, USA) were combined with TH-immunohistochemistry. The sections were incubated with primary antibodies for 48 h at 4 °C. Before addition of the secondary antibodies, sections were incubated for 15 min with 5% goat serum in PBS to block unspecific binding. Incubation with the secondary antibodies were performed at room temperature for 1 h, and the secondary antibodies used were Alexa Fluor® 594 (Invitrogen, Molecular probes; Eugene, OR, USA) goat anti-