Neuroscience 147 (2007) 712–719

DIFFERENTIAL EFFECTS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR ON A9 AND A10 DOPAMINE NEURON SURVIVAL *IN VITRO*

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Abstract—Glial cell-line derived neurotrophic factor (GDNF) enhances dopamine (DA) cell survival and fiber outgrowth, and may be beneficial in enhancing cell restorative strategies for Parkinson's disease (PD). However, GDNF may have different roles for transplanted DA cell sub-types. The present *in vitro* study investigated the effect of GDNF on the survival of rat DA cells displaying a phenotype consistent with either the substantia nigra [A9 cells immunopositive for tyrosine hydroxylase (TH) and G-protein-gated inwardly rectifying potassium channel subunit 2 (GIRK2)] or with the ventral tegmental area [A10 cells immunopositive for TH and calbindin]. It was found that a single exposure of GDNF enhanced the number of DA cells of an A9 phenotype, without affecting DA cells of an A10 phenotype. Conversely, repeated GDNF exposure did not alter the survival of A9 phenotypic cells, but doubled the percentage of A10 cells. It was concluded that GDNF administration may affect dopaminergic cells differently depending on time and degree of GDNF exposure. For cell transplantation in PD, long-term GDNF administration may result in detrimental effects for transplanted A9 TH+ cells as this may introduce competition with A10 TH+ cells for survival and fiber outgrowth into the host striatum. These results may have important implications for clinical neural transplantation in PD. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: GDNF, VM tissue, substantia nigra, VTA, dopamine, transplantation.

Transplantation of dopaminergic fetal ventral mesencephalic (VM) cells for Parkinson's disease (PD) has the potential to restore dopaminergic function to the brain but is limited by poor survival (Barker et al., 1995; Sinclair et al., 1999; Sortwell, 2003; Bartlett et al., 2004) and insufficient fiber outgrowth into the adult host striatum. Previous research has demonstrated that the use of several trophic factors, particularly glial cell line-derived neurotrophic factor (GDNF), can enhance dopaminergic cell survival and neurite extension (Lin et al., 1993; Hoffer et al., 1994; Bowenkamp et al., 1995; Tomac et al., 1995; Rosenblad et al., 1996; Sinclair et al., 1996; Apostolides et al., 1998; Mehta et al., 1998; Sautter et al., 1998; Yurek, 1998; Connor et al., 1999). In combination with VM transplantation in the 6-hydroxydopamine hemiparkinsonian rat model, GDNF improves the rate of recovery for amphetamine-induced rotational behavior (Rosenblad et al., 1996; Sautter et al., 1998; Tang et al., 1998; Yurek, 1998), and improves reflexive but not skilled motor ability (Mehta et al., 1998; Schneider and Peacock, 1998; Kirik et al., 2001; Winkler et al., 2006). Clinical results from double-blind, placebo-controlled randomized trials have demonstrated no functional improvements for i.c.v. or intrastriatal GDNF recipients, despite increased fluorodopa uptake (Nutt et al., 2003; Lang et al., 2006a,b; Sherer et al., 2006).

Skilled motor ability may require endogenously controlled dopamine (DA) release (Björklund et al., 1987; Dunnett et al., 1987; Mandel et al., 1990; Olsson et al., 1995; Barnéoud et al., 2000; Metz et al., 2001), which may depend upon the normal development of dopaminergic synaptic pathways. In the developing striatum, GDNF levels peak at birth (Schaar et al., 1993; Ikeda et al., 1999) and are minimal as early as postnatal day (P) (7; Strömberg et al., 1993). VM cells are generally harvested on embryonic day (E) 14 (Sinclair et al., 1999), therefore 1 week of GDNF infusion is equivalent to the developmental time point of P0 that marks the beginning of GDNF down-regulation. The common pre-clinical strategy of prolonging high levels of striatal GDNF (Sautter et al., 1998; Yurek, 1998; Kirik et al., 2004; Ahn et al., 2005) is not consistent with levels of GDNF in the developing striatum. Furthermore, this unnatural developmental cue may alter the growth of DA neurons within the developing VM tissue. Over-expressed striatal GDNF *in vivo* does not enhance the number of nigral DA cells surviving into adulthood (Kholodilov et al., 2004) but does enhance DA neurons in the ventral tegmental area (VTA). Dopaminergic neurons in the VTA are anatomically referred to as A10 neurons, and are located adjacent to the substantia nigra pars compacta (SNc). DA neurons in the VTA project to the nucleus accumbens via the mesolimbic pathway and are associated with reward. This pathway does not involve the nigrostriatal dopaminergic pathway, which projects from the ventral SNc to the motor striatum (Joel and Weiner, 2000). It is the A9 neurons of the ventral SNc that are preferentially lost in PD (Fearnley and Lees, 1991) likely due to

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Abbreviations: CB, calbindin; CR, calretinin; DA, dopamine; DMEM, Dulbecco's modified essential medium; E, embryonic day; GDNF, glial cell line-derived neurotrophic factor; GDNF H, ventral mesencephalon with glial cell line-derived neurotrophic factor hibernation; GDNF +, ventral mesencephalon with glial cell line-derived neurotrophic factor hibernation and repeated exposure in culture; GIRK2, G-protein-gated inwardly rectifying potassium (K) channel subunit 2; KPBS, potassium phosphate-buffered saline; NGS, normal goat serum; P, postnatal day; PD, Parkinson's disease; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VM, ventral mesencephalon/mesencephalic; VTA, ventral tegmental area.

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doi:10.1016/j.neuroscience.2007.03.057

differential expression of intracellular molecules (Chung et al., 2005) and thus the replacement of this specific subtype of DA cell may yield a more effective therapeutic outcome following transplantation in PD (Isacson et al., 2003). The results by Kholodilov et al. (2004) describing enhanced long-term A10 but not A9 cell survival due to over-expressed GDNF suggest differential effects of GDNF on dopaminergic neurons from these regions. Transplanted VM cells are dissected from an area of the fetal brain which is destined to include both the SNc and the VTA. It is therefore critical to determine specific developmental roles of GDNF on each of these dopaminergic cell subtypes in order to correctly simulate normal developmental cues for transplanted A9 and not A10 cells. This study was designed as an *in vitro* analysis of the effects of GDNF exposure on the survival of A9 versus A10 DA cell subtypes contained within a VM cell preparation. Tyrosine hydroxylase (TH)+ A9 neurons were identified by the presence of the G-protein-gated inwardly rectifying potassium channel subunit 2 (GIRK2), which is present in the ventral SNc but not the VTA (Schein et al., 1998; Mendez et al., 2005; Thompson et al., 2005), and TH+ A10 neurons were identified by the co-localization of calbindin (CB), which is present on dopaminergic neurons of the VTA but not the ventral SNc (Gerfen et al., 1987; González-Hernández and Rodríguez, 2000; Thompson et al., 2005).

EXPERIMENTAL PROCEDURES

Cell culture protocol

Four cell culture treatment groups were investigated: VM alone (VM), VM with GDNF hibernation (GDNF H), VM with GDNF hibernation plus single exposure at culture initiation (GDNF), and VM with GDNF hibernation and repeated exposure in culture (GDNF +). VM tissue was dissected under aseptic conditions from gestational day 14 Wistar rat fetuses (Charles River, St. Constant, QC, Canada) in Dulbecco's modified essential medium (DMEM; Hyclone, Logan, UT, USA), and incubated overnight at 4 °C in a calcium-free phosphate-buffered hibernation medium [30 mM potassium chloride (BDH Inc., Toronto, ON, Canada), 5 mM glucose (Sigma Chemical Co., St Louis, MO, USA), 0.24 mM magnesium chloride (Sigma), 20 mM lactic acid (Sigma), 32.18 mM potassium hydroxide (Merck KGaA, Darmstadt, Germany), 164.7 mM sorbitol (Sigma)] at pH 7.4. VM tissue was then dissociated for 20 min in 0.1% trypsin (Sigma)/0.05% deoxyribonuclease (DN-25; Sigma)/DMEM, and mechanically triturated resulting in a homologous cell suspension of 50,000 cells/150 μl. Cell viability was assessed using the Trypan Blue dye exclusion method. All GDNF groups were hibernated overnight in hibernation media containing 1 μg/ml GDNF (PeproTech Canada Inc., Ottawa, ON, Canada). VM cells were then plated on 16-well glass chamber slides (Laboratory-Tek; Nalge Nunc Int., Rochester, NY, USA) previously coated with poly-L lysine (0.01%; Sigma) at a cell density of 50,000 cells/well. Cells were cultured for 8 days in at 37 °C in differentiation medium [3:1 DMEM: F-12 (HyClone); 2% B-27 (Invitrogen Canada Inc., Burlington, ON, Canada); 1% PenStrep (HyClone); 1% fetal bovine serum (HyClone)]. GDNF exposure consisted of the addition of 10 μl of GDNF in dissociation media (final well concentration of 1 ng/ml) on culture days 0, 3, 5 and 7 for the 'GDNF +' group, and on culture day 0 for the 'GDNF' group. The 'VM' and 'GDNF H' treatment groups were not supplemented with GDNF but were infused with 10 μl of dissociation media at the same time as GDNF administration.

Immunocytochemistry

Post-culture, cells were fixed with 4% paraformaldehyde for 0.5 h and rinsed three times with 0.1 M phosphate buffer in preparation for immunostaining.

CB/TH staining

VM cells were washed three times with 0.1 M potassium phosphate-buffered saline (KPBS) and then incubated in blocking buffer for 1 h [5% normal goat serum (NGS; Pel Freez, Rogers, AR, USA), 0.25% Triton X-100 (Sigma) in 0.1 M KPBS]. Cells were then incubated overnight at room-temperature in fresh blocking buffer solution containing mouse anti-TH (1:1000; Chemicon Int., Temecula, CA, USA) and rabbit anti-CB (1:1000; Chemicon) primary antibodies. Cells were then rinsed three times with 0.1 M KPBS, and then incubated for 1 h in 0.1 M KPBS blocking buffer (2% NGS) containing fluorophore-tagged secondary antibodies Alexa 488 goat anti-mouse IgG (1:1000; Molecular Probes, Eugene, OR, USA) and Cy3-conjugated goat anti-rabbit IgG (1:1000; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). Cells were again rinsed for three times in 0.1 M KPBS, after which time the chambers were removed and the slides were coverslipped using Citifluor glycerol/PBS solution (Marivac Ltd., Montreal, QC, Canada).

GIRK2/TH staining

VM cells were washed three times in a 0.5 M Tris buffer (pH 7.4), and then incubated in blocking buffer for 1 h [3% bovine serum albumin (Sigma), 0.5% Triton X-100 (Sigma), in 0.5 M Tris–saline (Tris (BDH Inc.) with 0.9% NaCl, pH 7.4)]. A high concentration of Tris was required to reduce high background (Schein et al., 2005). After blocking, cells were incubated overnight at room temperature in fresh blocking buffer solution containing mouse anti-TH (1:1000; Chemicon) and rabbit anti-GIRK2 (1:50; Alomone Laboratories, Jerusalem, Israel) primary antibodies. The next day, cells were rinsed three times in 0.5 M Tris and incubated for 1 h in fresh blocking solution containing fluorophore-tagged secondary antibodies Alexa 488 goat anti-mouse IgG (1:1000; Molecular Probes) and Cy3-conjugated goat anti-rabbit IgG (1:1000; Jackson Laboratories). Cells were again rinsed three times in 0.5 M Tris, after which time the chambers were removed and the slides were coverslipped using Citifluor glycerol/PBS solution (Marivac). Primary antibody specificity was assessed for the rabbit anti-GIRK2 by pre-incubation with the GIRK2 control antigen (Kir3.2-GST Fusion Protein, Alomone) prior to following the normal staining protocol.

Cell counts

Fluorescent cells were counted using a light-microscope (Leica DM6000 B) and band-pass 480/40 and 545/30 filters (Leica Microsystems). All TH+ cells within each of the 16 wells were counted by an experimenter blinded to treatment group, and the numbers of TH+ cells and of co-labeled GIRK2+ or CB+ cells were recorded. Data were presented as total TH+ cells as well as the percentage of co-labeled cells.

Digital imaging

Image frame size was 1300×1030 pixels and images were saved as 8-bit TIFF files. For Figs. 1 and 2, red and green channel digital images were photographed separately and manipulated in Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA) as follows: corresponding pictures were overlaid and cropped. For the entire cropped image, background levels were adjusted to black, with contrast and brightness enhanced as needed.

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