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FETAL STRIATUM- AND VENTRAL MESENCEPHALON–DERIVED EXPANDED NEUROSPHERES RESCUE DOPAMINERGIC NEURONS *IN VITRO* AND THE NIGRO-STRIATAL SYSTEM *IN VIVO*

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Abstract—The pathogenesis of Parkinson's disease (PD) involves ongoing apoptotic loss of dopaminergic neurons in the substantia nigra pars compacta. Local delivery of the trophic factors can rescue dopaminergic neurons and halt the progression of PD. In this study we show that fetal E11 striatum-derived neurospheres and E14.5 ventral mesencephalon (VM) –derived neurospheres (NS_{E11} and NSvm, respectively) are a source of factors that rescue dopaminergic neurons. First, long-term expanded NS_{E11} and NSvm rescued primary dopaminergic neurons from serum-deprivation induced apoptosis and promoted survival of dopaminergic neurons for 14 days *in vitro* and this effect was due to soluble contact-independent factor/s. Second, green fluorescent protein–expressing NS_{E11} and NSvm grafted into the midbrain of mice with unilateral 6-hydroxydopamine-induced Parkinsonism resulted in partial rescue of the nigro-striatal system and improvement of the hypo-dopaminergic behavioral deficit. Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated that intact NS_{E11} and NSvm expressed fibroblast growth factor-2, brain-derived neurotrophic factor (BDNF), pleiotrophin, neurotrophin-3, but not glial cell line–derived neurotrophic factor (GDNF). GDNF expression was also undetectable *in vivo* in grafted NS_{E11} and NSvm suggesting that NS-derived factor/s other than GDNF mediated the rescue of nigral dopaminergic neurons. Identification of NS-derived soluble factor(s) may lead to development of novel neuroprotective therapies for PD. An unexpected observation of the present study was the detection of the ectopic host-derived tyrosine hydroxylase (TH) –expressing cells in sham-grafted mice and NS_{E11}- and NSvm -grafted mice. We speculate that injury-derived signals (such as inflammatory cytokines that are commonly released during transplantation) induce TH expression in susceptible cells.

Key words: neurospheres, transplantation, substantia nigra, GFP, dopaminergic, rescue.

Parkinson's disease (PD) is a neurodegenerative condition characterized by progressive apoptosis of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) resulting in DA denervation of the striatum, the target structure for SNpc DA neurons (Hornykiewicz, 1993). Ongoing loss of nigral DA neurons is believed to underpin the progression of PD symptoms after the initial 5–7 years of the disease (Hughes et al., 1993; Lang and Lozano, 1998a,b; Hornykiewicz, 2001). PD patients typically display a loss of 50–60% of nigral DA neurons at the time of initial presentation (Hornykiewicz, 1993). Rescuing the remaining 40–50% of nigral DA neurons may be sufficient to halt clinical disease progression and maintain patients with a mild degree of disability and a sustained response to levodopa. Thus far, promising results have been obtained with glial cell line-derived neurotrophic factor (GDNF) (Barker, 2006). Despite this finding, there is an ongoing demand for molecules with neuroprotective effect on DA neurons (Rascol et al., 2002).

In this context, committed neural precursors may prove useful. While grafting of neural precursor cells (NPCs) has been extensively investigated in the context of cell-replacement therapy, recent studies have shown that undifferentiated NPCs may act via the so-called "bystander" effect (Ourednik et al., 2002; Martino and Pluchino, 2006), which refers to a therapeutic benefit not due to NPC-derived cell replacement per se, but due to NPC-derived neuromodulatory and neurotrophic signals that mitigate pivotal elements of the pathological process (Ader et al., 2001; Park et al., 2002; Pluchino et al., 2003; Martino and Pluchino, 2006).

Previous *in vivo* studies identified the "bystander" rescue effect of immortalized neural stem cell (NSC) lines on the host DA system in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) –induced Parkinsonism (Ourednik et al., 2002). A recently published study has demonstrated

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxyuridine; CDNF, conserved dopamine neurotrophic factor; CMvm, medium conditioned by NSvm; CM_{E11}, medium conditioned by NS_{E11}; CPu, caudate-putamen; DA, dopaminergic; DAT, dopamine transporter; EGF, epidermal growth factor; FGD, fluorogold; FGF-2, fibroblast growth factor -2; GDNF, glial cell line–derived neurotrophic factor; GFP, green fluorescent protein; MANF, mesencephalic astrocyte-derived neurotrophic factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF, neurofilament; NPC, neural precursor cells; NS, neurosphere; NSC, neural stem cell; NSvm, ventral mesencephalon neurospheres derived at E14.5; NS_{E11}, striatal neurospheres derived at E11; NT-3, neurotrophin-3; PBS, phosphate-buffered saline; PD, Parkinson's disease; PFA, paraformaldehyde; PLL, poly-L-lysine; PTN, pleiotrophin; RS, rotational score; RT-PCR, reverse transcription–polymerase chain reaction; SNpc, substantia nigra pars compacta; SVZ, subventricular zone; TH, tyrosine hydroxylase; VM, ventral mesencephalon; 6-OHDA, 6-hydroxydopamine.

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that simultaneous grafting of the genetically unmodified human NPC into the caudate-putamen (CPu) and midbrain improves behavioral impairment in primates with MPTP-induced Parkinsonism mainly due to "bystander" mechanism (Redmond et al., 2007). GDNF appears to be a central player that mediates the rescue effect of undifferentiated NPC on host DA neurons (Ourednik et al., 2002; Redmond et al., 2007).

Fetal- and adult-derived NPC can be maintained, propagated and expanded without genetic manipulations ex vivo using a serum-free neurosphere (NS) culture system (Reynolds and Weiss, 1992). We aimed to explore if genetically unaltered NS are a source of factors that rescue nigral DA neurons. Previous *in vitro* studies revealed enhanced survival and functional capacity of DA neurons in co-culture with expanded NS (Ostenfeld et al., 1999) and this effect could be mediated by soluble factors other than GDNF (Rafuse et al., 2005; Moses et al., 2006). Intra-striatal grafting of subventricular zone (SVZ) –derived NS progenitors alleviated behavioral parkinsonian deficits (Richardson et al., 2005); however SVZ-derived expanded NS were teratogenic *in vivo* (Rafuse et al., 2005) in contrast to fetal-derived NS that usually lack *in vivo* teratogenic potential (Uchida et al., 2000; Reynolds and Rietze, 2005).

In this study we examined the capacity of fetal-derived NS to rescue mesencephalic DA neurons under conditions of serum deprivation–induced apoptosis. For that purpose we derived and expanded NS from the developing ventral mesencephalon (VM), a brain region that contains DA neuron cell bodies, and the developing striatum, the innervation target of DA neurons. We further explored the efficacy of NS-derived factors in an *in vivo* "rescue" paradigm using the 6-hydroxydopamine (6-OHDA) rodent model of hemi-Parkinsonism and delayed engraftment of NS into the area of lesioned SN. The "rescue" paradigm is clinically relevant since it resembles the symptomatic stage of PD, characterized by established degeneration of DA neurons (Bowenkamp et al., 1996; Bjorklund et al., 1997; Kirik et al., 2001; Wang et al., 2002). Intra-striatal grafting may not be the optimal method for that purpose as 6-OHDA lesions cause retraction of nigrostriatal projections (Rosenblad et al., 2000) essentially preventing the retrograde axonal transport of intra-striatally delivered trophic factors to the cell bodies of nigral DA neurons (Tomac et al., 1995; Rosenblad et al., 1998; Kirik et al., 2001), whereas grafting into the lesioned midbrain may facilitate access of NS-derived factors to nigral DA neurons.

Earlier studies investigated grafting of single cell suspension into the midbrain, but were met with unsatisfactory graft survival (Bjorklund et al., 1983; Dunnett and Björklund, 1992; Freeman et al., 1995). In the present study, NPCs were engrafted as intact NS because we anticipated that this approach could confer an *in vivo* survival advantage to grafted NS as the complex three-dimensional organization of intact NS comprising biologically active factors, such as extracellular matrix, growth factors and cytokines (Benoit et al., 2001; Campos, 2004; Deleyrolle et al., 2006; Martino and Pluchino, 2006) would not be disrupted.

We report that expanded fetal-derived NS produce soluble factors that support long-term survival of DA neurons *in vitro*. *In vivo*, NS-derived activity increased the number of surviving DA neurons in the SN resulting in DA re-innervation of the ipsilateral striatum and partial restoration of the hypo-DA behavioral deficit. Grafts of NSvm did not differentiate into DA neurons, confirming that the rescue effect was on the host DA system rather than mediated by graft derived cell-replacement.

EXPERIMENTAL PROCEDURES

All methods conformed to the Australian National Health and Medical Research Council published code of practice for the use of animals in research and approved by the Howard Florey Institute animal ethics committee. All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Generation and long-term expansion of NS

NS were derived from E11 or E14.5 embryos of time-pregnant C57BL/6J mice. Matings are set up and mice examined for the presence of the vaginal plug at 08:00 h, the date the vaginal plug is first identified is designated as E0. Alternatively, fetal NS were derived from transgenic mice expressing enhanced green fluorescent protein (GFP+) under the control of a chicken beta-actin promoter and cytomegalovirus enhancer (Jackson Laboratories, Bar Harbor, ME, USA) (Okabe et al., 1997). It is important to note that GFP+ mice were also on a C57BL/6J background. Previous studies demonstrated NSCs derived from this GFP mouse strain maintain GFP expression upon terminal differentiation (Mizumoto et al., 2003; Jensen et al., 2004).

Time-pregnant mice were killed by cervical dislocation and embryonic sacs were dissected and collected in ice-cold hibernation medium (phosphate-buffered saline (PBS) supplemented with Hepes 10 mM (Gibco, Invitrogen, Carlsbad, CA, USA), sodium pyruvate 1 mM (Sigma Aldrich Corp., St. Louis, MO, USA), 0.6% D-glucose and penicillin/streptomycin (Gibco, 50 IU/50-μg per ml). For derivation of E11 striatal NS, whole brain was extracted and forebrain was separated from the mesencephalon followed by isolation of the ventral forebrain using a dissection microscope. For generation of NS derived from VM, the mesencephalic flexure of the E14.5 embryonic brain was identified and dissected, and the VM was separated from the dorsal mesencephalon along the line of the presumptive sulcus limitans. Harvested tissue was pooled in hibernation medium and enzymatically dissociated using serum-free conditions (Drago et al., 1991; Moses et al., 2006). Initially, 5×10^4 cells/ml were plated in uncoated 25 cm^2 flasks (BD Falcon, Heidelberg, Germany) at a final volume of 5 ml of NS medium and incubated at 37 °C in humidified 5% CO_2/95% atmosphere air incubator. The NS medium was composed of DMEM/F12/2.5 mM glutamine (Gibco, cat. No.: 11320–033), 1% N2 supplement (Gibco), 0.3% D-glucose, penicillin/streptomycin, 10 ng/ml of epidermal growth factor (EGF, Peprotech) and 10 ng/ml of fibroblast growth factor-2 (FGF-2, Peprotech, Rocky Hill, NJ, USA). Cultures were supplemented with 20% of the fresh NS medium and 100% FGF-2/EGF every 48 h. Under these conditions primary NS were formed within 7–9 days. Primary and all subsequent NS were propagated by enzymatic dissociation with trypsin and seeded at a density 5×10^4 cells/ml in the presence of FGF-2/EGF resulting in formation of the subsequent generation of NS within 6–7 days. Intact NS were plated onto poly-L-lysine (PLL, Sigma Aldrich Corp.) –coated chamber-slides for assessment of tripotentiality as previously described (Moses et al., 2006).