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Efficient expansion and dopaminergic differentiation of human fetal ventral midbrain neural stem cells by midbrain morphogens ☆

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ABSTRACT

Human fetal midbrain tissue grafting has provided proof-of-concept for dopamine cell replacement therapy (CRT) in Parkinson's disease (PD). However, limited tissue availability has hindered the development and widespread use of this experimental therapy. Here we present a method for generating large numbers of midbrain dopaminergic (DA) neurons based on expanding and differentiating neural stem/progenitor cells present in the human ventral midbrain (hVM) tissue. Our results show that hVM neurospheres (hVMN) with low cell numbers, unlike their rodent counterparts, expand the total number of cells 3-fold, whilst retaining their capacity to differentiate into midbrain DA neurons. Moreover, Wnt5a promoted DA differentiation of expanded cells resulting in improved morphological maturation, midbrain DA marker expression, DA release and electrophysiological properties. This method results in cell preparations that, after expansion and differentiation, can contain 6-fold more midbrain DA neurons than the starting VM preparation. Thus, our results provide evidence that by improving expansion and differentiation of progenitors present in the hVM it is possible to greatly enrich cell preparations for DA neurons. This method could substantially reduce the amount of human fetal midbrain tissue necessary for CRT in patients with PD, which could have major implications for the widespread adoption of this approach.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by resting tremor, rigidity and slowness of movement (hypokinesia). The motor features of PD are due in part to the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), which project to the striatum and are involved in motor control. One promising therapeutic approach is cell-replacement therapy (CRT), in which DA neurons and/or precursors are grafted into the striatum in order to restore the lost nigrostriatal DA neurotransmission. Several cell types have been considered as potential sources of DA neurons, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells which, due to their capacity to expand and differentiate, can generate large numbers of DA neurons and induce behavioral improvement in animal models of PD (Barberi et al., 2003; Hargus et al., 2010; Kawasaki et al., 2000; Kim et al., 2002; Kriks et al., 2011; Lee et al., 2000; Sanchez-Pernaute et al., 2008; Wernig et al., 2008). However, the lack of purity of the differentiated cultures, the risk of excessive proliferation and teratoma formation, as well as the poor survival of human ES cell-

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derived DA cells after transplantation in animal models have so far prevented their use in clinical trials (Roy et al., 2006; Sonntag et al., 2007). In the past, human fetal ventral midbrain (VM) tissue has been used as a cell source for CRT, as these cells are correctly specified *in vivo*, pose no risk of tumor/teratoma formation and have been used in clinical trials to provide proof-of-principle that DA neurons derived from fetal human VM tissue can survive and offer significant benefits in patients (Kordower et al., 1995; Mendez et al., 2005; Piccini et al., 1999; Politis et al., 2010). However, several issues such as tissue availability, quality, standardization and the need for a high number of fetuses (6 to 7) to treat one PD patient make this strategy impractical and unlikely to become a competitive therapeutic option (Bjorklund et al., 2003; Freed et al., 2001; Olanow et al., 2003; Winkler et al., 2005). Previous studies have shown that human ventral midbrain-derived cells can be propagated with mitogens (Hovakimyan et al., 2006; Jin et al., 2005; Maciaczyk et al., 2008; Milosevic et al., 2006; Sanchez-Pernaute et al., 2001; Storch et al., 2001; Wang et al., 2004). In these studies cells were typically expanded in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor 2 (bFGF) (Hovakimyan et al., 2006; Sanchez-Pernaute et al., 2001; Storch et al., 2001) and differentiated by removal of mitogens and addition of neurotrophins such as brain derived neurotrophic factor (BDNF) (Maciaczyk et al., 2008) and glial cell-line derived neurotrophic factor (GDNF) (Jin et al., 2005; Storch et al., 2001), ascorbic acid, cyclic adenosine monophosphate (cAMP) (Sanchez-Pernaute et al., 2001) or cytokines (Jin et al., 2005; Storch et al., 2001). Immortalized human mesencephalic cell lines have also been established (Donato et al., 2007; Lotharius et al., 2002; Villa et al., 2009), however DA neurons could only be generated and maintained after stable overexpression of Bcl-X_L, an anti-apoptotic gene (Courtois et al., 2010). Surprisingly, none of the studies up to date have examined the use of region-specific developmentally appropriate morphogens for the expansion and differentiation of hVM cells.

Morphogens are secreted factors that form gradients and play a prominent role in development by providing regional and cellular identity to cells in the neural tube. The most important morphogens for midbrain development are Sonic Hedgehog (Shh, a ventralizing signal), Fibroblast growth factor 8 (Fgf8, that provides the midbrain–hindbrain identity), and Wnt5a (which regulates cell orientation and differentiation, reviewed in Inestrosa and Arenas, 2010). Interestingly, we have previously shown that mouse and rat VM neural stem cells can be propagated as neurospheres (VMN) and differentiated in the presence of Shh, FGF8 and Wnt5a. In these conditions, VMNs gave rise to a large number of functional DA neurons that were able to survive, integrate and induce functional recovery after transplantation in animal models of Parkinson's disease (Parish et al., 2008). Much less is known about the capacity of human endogenous VM progenitors to respond to morphogens and give rise to human DA neurons *in vitro*. Here, we examined whether VM morphogens, such as Shh, FGF8 and Wnt5a, can be applied to human VM neural stem cells, in order to improve the yield of DA neurons derived from human fetal midbrain tissue. Our results show that hVMN can be cultured as spheres and that the total number of cells can be expanded 3 fold over a 2–3 week period. Furthermore these VMN-expanded cells maintain the same DA differentiation potential after each passage and addition of Wnt5a increases the number of DA neurons per field by 3.3-fold, compared to controls. These increases together (total cells and TH cell density) resulted in a 6-fold increase in the $\%TH^+$ cells/Hoechst and total TH^+ cells obtained from one VM. Moreover, morphogen treatment maintained or increased the expression of typical midbrain markers such as TH, Nurr1, Foxa2, Lmx1a and Lmx1b, compared to non-expanded hVM tissue. In particular, we found that Wnt5a increased the morphological and molecular differentiation of VMN cells into midbrain DA neurons, as well as their functionality, as assessed by DA release and electrophysiological properties. In summary, our results show that morphogen-treated human neural stem/progenitor cells give rise to a 6-fold increase in the number of DA neurons compared to the starting VM preparation. Hence, the use of developmentally relevant midbrain factors for the expansion and differentiation of hVM cells may significantly reduce the need for multiple donor fetuses for grafting individual PD patients, thereby increasing the feasibility and accessibility of this technology in the future development of CRT for Parkinson's disease.

Materials and methods

hVM tissue

Human fetal ventral mesencephalic tissue was collected and experiments performed at the Centre for Brain Repair (University of Cambridge), Freiburg University Medical Center and Wallenberg Neuroscience Center (Lund University). All fetuses were collected from routine termination of pregnancies under full ethical approval in line with the United Kingdom's Department of Health guidelines and local ethical approval (Local Research Ethics Committee, reference no. 96/085), the local ethical committee of the University of Freiburg meeting the regulations of the German law (research project no. 96/03) and the Swedish National Board of Health and Welfare (Socialstyrelsen, Dnr. 23 11667/2008 and Dnr. 23 2981/2009).

hVMN cultures

The VM was dissected from fetuses at post-conception (PC) weeks 6–11. VM tissues were dissociated in serum-free N2 medium supplemented with BDNF (30 ng/ml, R&D) using collagenase/dispase (700 μg/ml; Roche) with agitation on an orbital mixer incubator (80 rpm, 30 min) followed by mechanical dissociation by gently pipetting through 200 μl pipettes or flame-narrowed Pasteur pipettes. For proliferation, 2/3 of the cells from the dissociated tissue were plated at a final density of 150,000 cells/cm^2 on non-coated or non-adherent tissue culture plates in serum-free N2 medium (1:1 mixture of F12 and MEM, 15 mM HEPES buffer, 1 mM glutamine, 6 mg/ml glucose (Sigma-Aldrich), 3 mg/ml Albumax, and N2 supplement, all purchased from Invitrogen) supplemented with Shh (100 ng/ml), FGF8 (100 ng/ml), BDNF (30 ng/ml) and bFGF (20 ng/ml), all purchased from R&D. These cultures were referred to as passage 0 (P0). For differentiation, 1/3 of the cells were plated onto poly-ornithine (15 μg/ml, Sigma-Aldrich) and laminin-coated 48 well plates (10 μg/ml, Trevigen) and left to differentiate for 4–14 days in N2 medium supplemented with BDNF (20 ng/ml, R&D), GDNF (10 ng/ml, R&D), TGFβ3 (1 ng/ml, R&D), ascorbic acid (2 mM, Sigma-Aldrich) and dibutyryl cAMP (1 mM, Sigma-Aldrich) and Wnt5a (100 ng/ml, R&D) or an equivalent volume of 0.1% bovine serum albumin (BSA) as a control for Wnt5a treatment. These cultures were referred to as differentiation 0 (D0). Cells in proliferation grew as spheres and were supplemented with fresh medium every 3 days. After 7 days of proliferation, half of the spheres were passaged using collagenase/dispase and half were plated for differentiation using the same conditions as described above; these cultures were referred to as passage 1 (P1) and differentiation 1 (D1), respectively. After 14 days of proliferation, the remaining spheres (referred to as passage 2, P2) were plated for differentiation (referred to as differentiation 2, D2).

Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde for 20 min, washed three times with PBS and pre-incubated for 1 h in blocking solution (PBS, 0.25% Triton-X 100 and 5% normal goat serum) followed by incubation at 4 °C overnight with one or more of the following primary antibodies diluted in blocking solution: rabbit anti-tyrosine hydroxylase (TH, 1:300, Pel-Freeze), mouse anti-βIII tubulin (Tuj1;1:1000, Promega, Abcam), mouse anti-TH (1:200 ImmunoStar), goat anti-Lmx1a (1:200 Santa Cruz), goat anti-Nurr1 (1:200, R&D Systems). After washing,