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DIFFERENTIATION OF NON-MESENCEPHALIC NEURAL STEM CELLS TOWARDS DOPAMINERGIC NEURONS

R. RÖßLER, E. BODDEKE AND S. COPRAY*

Department of Neuroscience, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713AV Groningen, The Netherlands

Abstract—Neural stem cells (NSCs), either isolated from fetal or adult human brain or derived from induced pluripotent stem cells, are now considered major candidates for *in vitro* generation of transplantable dopaminergic (DA) neurons and modeling of Parkinson's disease. It is generally thought that *in vitro* differentiation of neural stem cells into meso-diencephalic dopaminergic neurons, requires recapitulation of dopaminergic differentiation pathway normally occurring in the ventral mesencephalon during embryogenesis. This dopaminergic pathway is partially activated by a combination of the extracellular induction factors Sonic Hedgehog (Shh), Fibroblast Growth Factor 8 (FGF8) and Wnt1 that trigger specific intracellular transcription cascades. *In vitro* mimicking of these embryonic ventral mesencephalic conditions has been successful for dopaminergic differentiation of embryonic stem cells and ventral mesencephalic NSCs. Dopaminergic differentiation of non-mesencephalic NSCs (nmNSCs), however, is considered arduous. Here we examine whether Shh, FGF8 and Wnt1 can activate typical dopaminergic transcription factors, such as Lmx1a, Msx1 and Otx2 in nmNSCs. We found that Shh, FGF8 and Wnt1 induced the expression of Lmx1a and Otx2 in nmNSCs resulting in the differentiation of up to 39% of the nmNSCs into neurons expressing Pitx3. However, only a low number (~13%) of these cells became more DA-like neurons also expressing tyrosine hydroxylase (TH). The histone deacetylase (HDAC)-inhibitor trichostatin A combined with Shh, FGF8 and Wnt1 caused orchestrated induction of Lmx1a, Otx2, Msx1 plus the early DA transcription factor En1. Now significantly increased numbers of TH (~22%) and Pitx3 (~33%) neurons were observed. Most of these cells coexpressed the DA markers DAT and Vmat2. Taken together, we demonstrate that nmNSCs indeed can be differentiated towards DA-like neurons, but this differentiation is far from complete in comparison to ventral mesencephalic NSCs and embryonic stem cells; most likely, the nmNSCs lack the proper "primed" epigenetic state of these cells for DA differentiation facilitating the induction of DA specific transcription factors.

Key words: *in vitro* differentiation, ventralization factors, TH, Pitx3, HDAC inhibitor.

In Parkinson's disease (PD) dopamine (DA) neurons selectively and progressively undergo cell death in the substantia nigra (SN) pars compacta, resulting in the destruction of the nigrostriatal connections and subsequently in the depletion of dopamine in the striatum. Due to the specific loss of DA neurons, PD is associated with typical clinical symptoms such as rigidity, tremor, akinesia and loss of postural reflexes but also psychiatric disorders like depression or anxiety are related to this neurodegenerative disease (for review see Kempster et al., 2007 or Lees et al., 2009).

About 20 years ago, a cell graft approach to treat PD was forwarded which aimed to restore the baseline dopaminergic levels in the striatum by the striatal implantation of fetal dopaminergic neurons derived from aborted human embryos (Olanow et al., 1996; Lindvall and Hagell, 2000; Bjorklund et al., 2003). Despite clear beneficial effects in many patients, severe side effects in a number of patients were reported after some years. The occurrence of these side effects was ascribed to a number of patient-related factors (like age, stage and severity of the disease at the time of implantation) but also to the location and, in particular, the obscure, highly variable cellular composition of the graft. Therefore, practical as well as ethical reasons made this approach not suitable for clinical applications and other cell sources had to be found.

Neural stem cells, either isolated from fetal (heterologous) or adult (autologous) human brain (Westerlund et al., 2005) or derived from pluripotent stem cells, are now considered major candidates for the large scale *in vitro* generation of transplantable dopaminergic neurons. It is generally thought that *in vitro* induction of differentiation of neural stem cells into meso-diencephalic dopaminergic neurons, requires recapitulation of the dopaminergic differentiation pathway normally occurring in the neural stem cells of the ventral mesencephalon during embryogenesis. Dopaminergic differentiation in the embryonic ventral mesencephalon is regulated by a combination of extracellular induction factors that trigger specific intracellular transcription cascades. On one side, there is the secreted factor Wnt1 (and also other members of the Wnt family) which blocks the differentiation of ventral mesencephalic neural stem cells into GABAergic or serotonergic neuronal fates by repressing the transcription factor Nkx2.2 via the up-regulation of Otx2. On the other side, there is Sonic Hedgehog (Shh) and Fibroblast Growth Factor 8 (FGF8) which are considered to be essential for the specific induction of the DA neuronal lineage in the embryonic ventral mesencephalon (Ye et al., 1998) via the expression of Lmx1a and Msx1 (for review see Burbach and Smidt, 2006). These two early transcription factors, in particular Lmx1a, have been reported to be necessary and sufficient

*Corresponding author. Tel: +0031-0-50-3632758.
E-mail address: j.c.v.m.copray@med.umcg.nl (S. Copray).
Abbreviations: DA, dopamine; FGF8, fibroblast growth factors; nmNSCs, non mesencephalic neural stem cells; Shh, Sonic Hedgehog; TH, tyrosine hydroxylase; TSA, trichostatin A.

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to induce mesencephalic DA differentiation (Andersson et al., 2006a).

Indeed, using the extrinsic induction factors described above, successful *in vitro* induction protocols for dopaminergic differentiation have been developed for neural stem cells isolated from the embryonic ventral mesencephalic region (Roybon et al., 2008; Jonsson et al., 2009). However, induction of dopaminergic differentiation of non-mesencephalic neural stem cells appears to be difficult, suggesting pre-existing differences in the responsiveness to dopaminergic induction signals between mesencephalic and non-mesencephalic neural stem cells. This is of relevance since the neural stem cells presently considered for the generation of implantable dopaminergic neurons, for example those derived from induced pluripotent stem (iPS) cells, should be considered non-mesencephalic in origin. The low yield of dopaminergic neurons obtained in first differentiation experiments with iPS-derived neural stem cells seems to confirm this (Wernig et al., 2008; Soldner et al., 2009).

In the present study, we examine the potential of above mentioned extrinsic and intrinsic key players to induce dopaminergic differentiation in non-mesencephalic neural stem cells. Apart from establishing the effects of Shh and FGF8 on the expression levels of Lmx1a and Msx1, we aimed to determine whether the (forced over-) expression of these transcription factors can circumvent extrinsic induction mechanisms and can be employed to trigger pathways leading to dopaminergic differentiation of non-mesencephalic neural stem cells.

EXPERIMENTAL PROCEDURE

Animals

For all experiments C57BL/6 mice were housed under standard conditions with free access to food and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and regulations of the local Experimental Animal Committee.

Cell culture

Neural stem cells were isolated from the telencephalon of C57BL/6 mouse embryos at E14; in here they are further referred to as non-mesencephalic neural stem cells (nmNSCs). Briefly, the telencephalon was cut into small pieces at room temperature and after mechanically triturating in ice-cold phosphate buffered saline the tissue was incubated with accutase (Sigma Aldrich, Zwijndrecht, The Netherlands) for 15–20 min at 37 °C. After repeated trituration the cell suspension was passed through a cell strainer (70 μm pore size, Falcon, Franklin Lakes, NJ, USA) and seeded (1–1.5 million cells) in T25 tissue culture flasks (Nunc, Roskilde, Denmark) containing proliferation medium, which consisted of Neurobasal medium (Invitrogen, Breda, The Netherlands), supplemented with B27 (2%, Invitrogen), recombinant human epidermal growth factor (EGF) (20 ng/mL, Invitrogen), basic fibroblast growth factor (bFGF) (20 ng/mL, Invitrogen), glutamax (1%, Gibco, Invitrogen), primocin (100 ng/mL, Amaxa, VZA-1022), and heparin (5 μg/mL, Sigma Aldrich) in a humidified 5% CO_2/95% air incubator at 37 °C (standard culture conditions). Within 3–5 days the cells grew as free-floating neurospheres and were dissociated using accutase and passaged at least twice a week. To induce differentiation into neural cell types, neurospheres were treated with accutase for 5–10 min and dissociated by trituration. Subsequently these cells were plated on poly-D-lysine- and laminin-coated coverslips in 24 well plates at approximately 20.000 cells per well in Neurobasal medium supplemented with B27 alone.

Differentiation

For the differentiation induction experiments (see Fig. 1), nmNSCs (passage number 2-5) were exposed to varying concentrations of Shh (PHC2095, Invitrogen) and FGF8 (423-F8-025, R&D Systems) (10 nM or 100 nM each, adapted from Andersson et al., 2006a) and Wnt1 (50 or 100 ng/mL, PHC1804, Invitrogen; adapted from Joksimovic et al., 2009) for 72 h. After this treatment, the factors were withdrawn from the medium and the cells were put in culture for actual differentiation for 5 to 9 days (see figures/legends for exact differentiation time for each experiment). In a number of experiments, we examined the effect of the histone deacetylase inhibitor trichostatin A (TSA) on transcription factor expression before and during differentiation (see Fig. 1). To that purpose, 10 ng/mL TSA was added to the cells 24 h after beginning of the Shh/FGF8/Wnt1 treatment and remained in the medium during the first 24 h of differentiation. To analyze the effects of the induction factors on differentiation periods, cell samples were collected after different differentiation periods for RT-PCR and Western Blot analysis or were fixed for immunostaining.

Gene transfection

Overexpression of Msx1 (pCMV-SPORT6, Openbiosystems) was induced by electroporation of nmNSCs with these expression vectors. Gene transfections were performed using an electroporation-based transfection protocol (Amaxa GmbH, Cologne, Germany) specifically designed for transfection of embryonic mouse NSCs by Amaxa®. Neurospheres were dissociated, 1–3 million cells were transfected with a maximum of 10 μg DNA in total. As a control, cells have been transfected with a GFP control vector

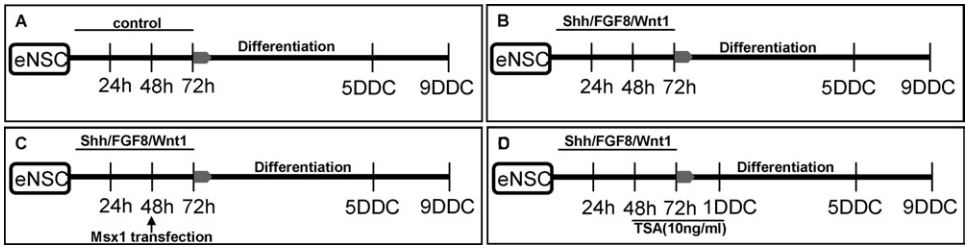

Fig. 1. Differentiation schemes. (A) Shows the control differentiation condition, no inductive factors were applied. (B) Shows the culture condition when Shh, FGF8 and Wnt1 were applied before differentiation was induced. This condition we refer to as "*in vitro* ventralization". (C) Shows ventralization condition combined with histone deacetylase inhibitor Trichostatin A. (D) Shows ventralization condition combined with transduction of Msx1.