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Evidence of functional duplicity of Nestin expression in the adult mouse midbrain

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ABSTRACT

Whether or not neurogenesis occurs in the adult substantia nigra pars compacta (SNc) is an important question relevant for developing better treatments for the motor symptoms of Parkinson's disease (PD). Although controversial, it is generally believed that dividing cells here remain undifferentiated or differentiate into glia, not neurons. However, there is a suggestion that Nestin-expressing neural precursor cells (NPCs) in the adult SNc have a propensity to differentiate into neurons, which we sought to confirm in the present study. Adult (>8-weeks old) transgenic NesCreER^{T2}/GtROSA or NesCreER^{T2}/R26eYFP mice were used to permanently label Nestin-expressing cells and their progeny with β-galactosidase (β-gal) or enhanced yellow fluorescent protein (eYFP), respectively. Most β-gal + or eYFP + cells were found in the ependymal lining of the midbrain aqueduct (Aq) and in the midline ventral to Aq. Smaller but significant numbers were in the periaqueductal gray (PAG), the ventral tegmental area (VTA), and in SNc. Low-level basal proliferation was evidenced by a modest increase in number of β-gal + or eYFP + cells over time, fewer β-gal + or eYFP + cells when mice were administered the anti-mitotic agent Cytarabine, and incorporation of the proliferation marker bromodeoxyuridine (BrdU) in a very small number of β-gal + cells. No evidence of migration was found, including no immunoreactivity against the migration markers doublecortin (DCX) or polysialic acid neural cell adhesion molecule (PSA-NCAM), and no dispersal of β-gal + or eYFP + cells through the midbrain parenchyma over time. However, β-gal + or eYFP + cells did increase in size and express higher levels of mature neuronal genes over time, indicating growth and neuronal differentiation. In mice whose SNc dopamine neurons had been depleted with 6-hydroxy-dopamine, a model of PD, there were ~2-fold more β-gal + cells in SNc specifically, although the proportion that were also NeuN + was not affected. Remarkably, as early as 4 days following putative Nestin-expression, many β-gal + or eYFP + cells had mature neuronal morphology and were NeuN +. Furthermore, mature neuronal β-gal + cells were immunoreactive against the self-renewal or pluripotency marker sex determining region Y-box 2 (Sox2). Overall, our data support the notion that some Nestin-expressing, presumably NPCs, have a limited capacity for proliferation, no capacity for migration, and a propensity to generate new neurons within the microenvironment of the adult midbrain. However, our data also suggest that significant numbers of extant midbrain neurons express Nestin and other classical neurogenesis markers in contexts that are presumably not neurogenic. These findings foreshadow duplicitous roles for Nestin and other molecules that are traditionally associated with neurogenesis in the adult midbrain, which should be considered in future PD research.

1. Introduction

A pathological hallmark of Parkinson's disease (PD) is degeneration of dopamine (DA) neurons in substantia nigra pars compacta (SNc) of the midbrain (Zigmond and Burke, 2002; Iversen et al., 2010). This causes the motor symptoms of PD such as bradykinesia, resting tremor, postural instability and muscle rigidity (Gelb, 1999). Systemic administration of drugs such as the DA precursor L-3,4-dihydroxyphenylalanine (levodopa or L-DOPA) or DA receptor agonists alleviate motor symptoms by restoring SNc DA neurotransmission towards normal. However, continual use of these drugs produces worsening side-effects such as dyskinesia and impulse control disorders (ICDs), psychoses and anxiety (Obeso et al., 2000; Ahlskog and Muenter, 2001; Fabbrini et al., 2007; Weintraub et al., 2010), probably due to non-physiological delivery of SNc DA and excessive activation of DA receptors in unaffected areas of brain (Weintraub, 2008). Therefore it is generally agreed that more physiological and targeted restoration of SNc DA

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neurotransmission will more effectively relieve PD motor symptoms without side-effects.

The leading approaches to this are DA cell transplantation, or stimulating endogenous DA neurogenesis, in either SNc or its efferent target the dorsal striatum. Despite progression of cell transplantation to clinical trials, it is still not widely available due to lack of a standardized and morally acceptable source of transplantable cells, immunological reaction, teratoma formation, and failure of transplanted cells to acquire and maintain the DA phenotype (Rao, 2001). Stimulating endogenous SNc DA neurogenesis may overcome many of these obstacles, however most studies have shown proliferating cells here either remain undifferentiated, or differentiate into glia, not neurons (Cooper and Isacson, 2004; Frielingsdorf et al., 2004; Yoshimi et al., 2005a,b; Aponso et al., 2008), but see Zhao et al. (2003). Nevertheless, the presence of cell renewal in the adult SNc engenders some hope, particularly since it was shown that new cells harvested from the adult rat SNc can generate neurons if they are provided appropriate environmental cues in vitro and in the adult hippocampus, an established neurogenic niché (Lie et al., 2002). Also, another study has reported evidence that a particular sub-class of Nestin-expressing (Nestin+) neural precursor cells (NPCs) in the normal and 1-methyl-4-phyenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated SNc, have a propensity to generate new neurons in situ (Shan et al., 2006).

It is possible that neurogenesis from Nestin+ cells was overlooked in the majority of midbrain neurogenesis studies because Nestin+ cells can be slowly or latently proliferating type-1 stem cells and therefore not incorporate much BrdU (Kempermann, 2011). Furthermore, Shan et al. (2006) could not determine the full extent of neuronal differentiation from Nestin+ cells because they could only identify Nestin-expressing cells whilst Nestin expression was occurring, i.e. they could not perform long-term lineage tracing. Given the importance for progressing DA cell replacement therapies for the motor symptoms of PD, the present study sought to confirm and extend the findings of Shan et al. (2006). An important difference between the present study and that of Shan et al. (2006) is we used transgenic mice permitting permanent reporter expression in adult Nestin+ cells and their progeny, and thus long-term lineage tracing of adult Nestin+ cells.

2. Materials & methods

All experimental procedures on animals were approved by the Howard Florey Institute Animal Ethics Committee and are in accordance with the National Health & Medical Research Council of Australia's published code of practice for the care and use of animals for scientific purposes, 7th edition, 2004.

2.1. Mice

NesCreER^{T2} (lines 5.1 & 4) C57BL/6 mice were obtained with permission from Professor Ryoichiro Kageyama and Kyoto University Institute for Virus Research (53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, JAPAN) (Imayoshi et al., 2006). These mice express an inactive form of Cre-recombinase (CreER^{T2}) under control of a 5.8 kb fragment of the promoter region and a 1.8 kb fragment containing the second intron of the *nestin* gene. These mice were crossed into two different lines of reporter mice: (1) B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J ("GtROSA") mice from Jackson Laboratories (Bar Harbor, Maine, USA); and (2) R26eYFP mice (Srinivas et al., 2001) obtained with permission from Professor Frank Costantini (Columbia University Medical Center, New York). Both reporter lines have a *loxP*-flanked DNA *STOP* sequence upstream of their reporter gene sequence [*lacZ* and enhanced yellow fluorescent protein (*eYFP*), respectively], which in both cases is driven off the ubiquitously expressed *ROSA 26* locus. Double transgenic mice (NesCreER^{T2}/GtROSA or NesCreER^{T2}/R26eYFP) permanently express the protein product [β-galactosidase (β-gal) or eYFP, respectively] of their reporter gene sequence in: (1) any cell that expresses *nestin* (a marker of NPCs) whilst tamoxifen is administered; and (2) any progeny of these cells should they divide at a later timepoint.

The general experimental protocol was to administer tamoxifen (10 mg/day in corn oil via oral gavage) to adult (≥8-weeks old) mice, then later kill the mice for study of β-gal immunoreactive (β-gal+) or eYFP immunoreactive (eYFP+) cells in sections processed histologically, or innate eYFP+ cells in acutely prepared living midbrain slices. These are putative adult Nestin+ cells and any progeny thereof.

2.2. Immunohistochemistry

Mice were given an overdose of anesthetic (100 mg/kg i.p. sodium pentobarbitone) and intracardially perfused with heparinized phosphate buffered saline (PBS) at 37 °C followed by 4% paraformaldehyde in PBS (1.15 ml/g body weight). The brain was removed and placed in PBS containing 30% sucrose for 2–3 days. Coronal sections were cut through the brain on a cryostat at 40 μm thick, collected free-floating in cryoprotectant, and stored at −20 °C.

Note that in both the BrdU and the 6-OHDA experiments coronal sections were cut at 16 μm thick, mounted directly onto gelatinized microscope slides, and stored at −20 °C.

Sections were washed twice in PBS (10 min each) and incubated in 5% normal goat serum (NGS), 0.3% triton X-100 in PBS for 30 min. They were then immunoreacted with primary antibodies in NGS and triton X-100 for 72 h at 4 °C. Primary antibodies used were rabbit anti-β-gal (1:2000, Cappel/ICN Pharmaceuticals cat#55976), chicken anti-β-gal (1:500, ABCAM cat#ab9361), chicken anti-GFP (1:10,000, ABCAM cat#ab13970), rat anti-BrdU (1:200, Accurate Chemical cat #OBT0030), mouse anti-Nestin (1:100, Chemicon), rabbit anti-GFAP (1:400, DAKO), rabbit anti-Olig2 (1:500, Millipore), mouse anti-NeuN (1:2000, Santa-Cruz), rabbit anti-TH (1:400, Chemicon), sheep anti-TH (1:800, Pel-Freez®), goat anti-Sox2 (1:200, R&D Systems cat#AF2018), rabbit anti-Pax6 1:1000, MBL, polyclonal antibody, Code No PD022. Sections were then washed three times in PBS and incubated in secondary antibodies in PBS and triton X-100 overnight at 4 °C. Secondary antibodies used were appropriate combinations of Alexa-fluor 594 and Alexa-fluor 488 (Invitrogen) at concentrations of 1:100–200. Sections were washed three times in PBS and in some experiments they were incubated in Hoechst (1:1000 in PBS) for 5 min and washed again three times in PBS. Free-floating sections were then mounted on gelatinized microscope slides and air dried. All sections were coverslipped using fluorescence mounting medium (DAKO).

For the BrdU and β-gal double-labelling, sections on slides were first incubated with the β-gal primary antibody (as above). They were then post-fixed in 4% paraformaldehyde for 30 min, washed three times in PBS, incubated in a 1:1 solution of formamide in PBS, pH 7.4, for 2 h at 65 °C, rinsed three times in PBS, incubated in 2 M HCl at 37 °C for 30 min for antigen retrieval, washed three times in PBS, incubated in 0.1 M borate buffer for 15 min at room temperature, washed three times in PBS, then processed with the BrdU primary antibody (above). Sections were then processed with appropriate secondary antibodies (as above).

In some experiments (e.g. Fig. 1) β-gal immunoreactivity was detected using a polyclonal goat anti-rabbit biotinylated secondary antibody (1:1000, DAKO, 2 h) followed by avidin-peroxidase (1:500, 1 h), cobalt- and nickel-intensified diamino-benzidine (0.5 mg/ml, 15 min.), and hydrogen peroxide (0.01%, 3-5 min). Diamino-benzidine reacted sections were dehydrated in alcohol, cleared (X-3B) and coverslipped.

2.3. Single-cell qPCR

2.3.1. Brain slice preparation

Mice were anaesthetized with isofluorane in air then decapitated. The brain was rapidly (<1 min) removed and placed in ice–cold (0 °C) "cutting mix" containing 125 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl,