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Research article

An investigation of gene expression in single cells derived from Nestin-expressing cells in the adult mouse midbrain *in vivo*

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HIGHLIGHTS

- Many large and mature midbrain neurons express Nestin in some as yet unknown context.
- 'Immature' pro-neuronal genes are more prominent in eYFP+ than eYFP− midbrain cells.
- Nestin-expressing cells arise anywhere in the adult midbrain and mature locally.

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ABSTRACT

Generation of new dopamine (DA) neurons in the adult midbrain is a controversial issue in development of better treatments for Parkinson's disease (PD). Previous research suggests Nestin-expressing neural precursor cells (NPCs) have a propensity to differentiate into neurons here, including DA neurons. In the present study we sought confirmation of this by studying gene expression in single Nestin-expressing cells and their progeny/ontogeny within the adult mouse midbrain. Cells were identified by administering a pulse of Tamoxifen to adult Nestin-CreER^{T2} × R26eYFP transgenic mice. Samples of cytoplasm were harvested 4 days to 8 months later from individual eYFP+ cells in acutely prepared midbrain slices and analysed by RT-qPCR for gene expression. Remarkably, most eYFP+ cells co-expressed genes associated with mature (including DA) neurons (*i.e.* NeuN, Gad1, Gad2, vGlut2, TH and/or D2R) and neurogenesis (*i.e.* Ki67, Dcx, Ncam, Pax6, Ngn2 and/or Msx1), and this was true at all time-points following Tamoxifen. Indeed, cell proliferation genes (Nestin, Ki67) were exclusively expressed by eYFP+ cells with mature neuronal morphology and gene expression, and only at early time-points after Tamoxifen. Expression of proneuronal genes (Pax6, Msx1, Ngn2) was, however, higher in eYFP+ cells with immature morphology compared with mature morphology. Gene expression bore no relationship to cell location indicating that, in contrast to development, Nestin-expressing cells arise throughout the midbrain parenchyma and do not migrate long distances. On the other hand, gene expression did change with time after Tamoxifen, although not in a way consistent with neurogenesis. Overall, our results suggest that Nestin expression in the adult midbrain occurs in mature neurons, casting doubt on the premise of neurogenesis from Nestin+ NPCs here.

1. Introduction

The motor symptoms of Parkinson's disease (PD) (tremor, slowness of movement, postural instability) are caused by degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) of the midbrain. PD motor symptoms can be effectively treated in the early stages of disease by drugs that increase brain DA. However, these produce motor complications that may require advanced therapies such as deep brain stimulation.

Many in the field believe the key to long-term effective treatment of PD motor symptoms is providing targeted and physiological DA delivery. While the most common approach is to use pump delivery of therapies DA cell replacement therapies (CRTs) including DA cell transplantation are being actively studied as alternatives. Although several obstacles prevent DA cell transplants for PD from being translated into human therapies, if these are resolved, DA cell transplantation should be effective in PD patients. These obstacles include: (1) survival of cells and their integration into the adult midbrain; and (2) acquisition and maintenance of

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the DA phenotype. Solutions to these problems may be revealed by learning how new DA neurons are physiologically generated in the adult midbrain.

Previous studies of neurogenesis and DA neurogenesis in the adult mammalian midbrain have provided conflicting results. Most studies used Bromodeoxyuridine (BrdU) to label proliferating cells and found that most or all remain undifferentiated or differentiate into glia, not neurons [1–5]. Nevertheless, one study suggested a propensity for Nestin-expressing neural precursor cells (NPCs) in the adult midbrain to differentiate into neurons, including DA neurons [6]. Because Nestin+ NPCs in the adult brain divide relatively slowly [6,7], those studies that administered BrdU over a few days [1,2,8] may have been overlooked these more slowly proliferating cells.

This study has addressed the question of whether Nestin+ NPCs do form neurones in the adult midbrain. We used transgenic (Nestin-CreER^{T2} × R26eYFP) mice in which any cell that expresses Nestin during a period of Tamoxifen administration is permanently labelled with enhanced yellow fluorescent protein (eYFP). In addition, because eYFP expression is induced in these cells by a gene recombination event, any progeny of eYFP+ cells will also be eYFP+. We administered a 'pulse' of Tamoxifen once/day over 3–4 successive days to these mice when they were older than 8-weeks (*i.e.* were sexually mature or adult) and examined *in situ* gene expression in eYFP+ cells at the single-cell level 4-days to 8-months later.

2. Materials and 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. Transgenic mice

NestinCreER^{T2} (lines 5.1 & 4) C57BL/6 mice were obtained with permission from Professor Ryoichiro Kageyama and Kyoto University Institute for Virus Research [9]. These mice express an inactive form of Cre-recombinase (CreER^{T2}) under the control of the Nestin promoter and enhancer. NestinCreER^{T2} mice were crossed with R26eYFP reporter mice obtained with permission from Professor Frank Costantini [10]. R26eYFP mice have a loxP-flanked DNA STOP sequence upstream of their reporter sequence (enhanced yellow fluorescent protein or eYFP), which is driven off the constitutively and ubiquitously active ROSA 26 gene locus. Adult (≥8-weeks old) double transgenic (NestinCreER^{T2}/R26eYFP) mice were administered the estrogen receptor agonist/antagonist Tamoxifen (10 mg/day in 0.5 ml corn oil *via* oral gavage) for 3–4 consecutive days to activate any CreER^{T2} present in cells. In this way, any cells that expressed Nestin during the period of Tamoxifen administration would permanently express eYFP. Mice were killed at different times (4-days to 8-months) following Tamoxifen administration and living slices of midbrain were prepared as described next.

2.2. Single-cell qPCR

2.2.1. Cell samples

Mice were anesthetized 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, 1.25 mM $NaH_2PO_4{\cdot}H_2O$, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 25 mM glucose, bubbled with 95% O_2 and 5% CO_2, pH 7.4. Slices (300 μm thick) were cut with a vibratome through the midbrain and transferred into 35 °C artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.25 mM $NaH_2PO_4{\cdot}H_2O$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, bubbled with 95% O_2 and 5% CO_2, pH 7.4.

A slice was transferred into a bath perfused with 30 °C ACSF. eYFP+ cells were viewed at high (63× objective) power using fluorescence and infrared differential interference contrast (IR-DIC) microscopy. A glass micropipette, (~1 μm tip diameter, ~6–10 MΩ resistance) containing 144 mM K-gluconate, 3 mM $MgCl_2{\cdot}6H_2O$, 10 mM HEPES, 0.5 mM EGTA, pH 7.2, osmolarity 290, was then advanced with a microdrive onto the cell's membrane and a tight seal (measured as GΩ electrical resistance) was formed between the micropipette tip and the membrane by applying a small amount of negative pressure through the pipette. The membrane patch inside the pipette tip was then broken by applying a short pulse of high negative pressure through the pipette to obtain access to the inside of the cell. A sample of the cell's cytoplasm was aspirated into the tip of the pipette and the pipette was rapidly removed from the slice. The broken micropipette tip and ~6 μl of internal solution were collected in a 200 μl eppendorf tube placed immediately at −80 °C for later reverse transcription, pre-amplification and qPCR.

Cell-negative controls were collected by pushing a micropipette tip through a slice to near a cell, leaving it there for a time, then withdrawing it, almost exactly as if we were characterizing the cell. The only difference was positive pressure was maintained through the pipette tip the entire time so that no physical contact was made with the cell; nothing was aspirated into the micropipette tip. Cell samples collected on days where cell-negative controls returned positive gene expression readings were excluded from analyses. qPCR-negative and qPCR-positive controls were performed on aliquots of RT buffer only samples and RNA harvested from tissue dissected from developing mouse midbrain, respectively.

2.2.2. Reverse transcription

To perform first-strand cDNA synthesis, the eppendorf tube containing an aspirated cell was thawed, briefly centrifuged and kept on ice until the reverse transcription (RT) reaction (<half an hour). First, the volume of the aspirate was measured and made up to 7.5 μl with 1.3333× First-Strand RT buffer (66.67 mM Tris–HCl, 100 mM KCl, 4 mM $MgCl_2$, pH 8.3). Using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen™, catalog #18080-051), 0.5 μl of random hexamers (50 ng/μl) was added and incubated 5 min at 65 °C then 1 min on ice. Next, 2 μl of RT mix [0.5 μl RNaseOUT (40 U/μl), 0.5 μl of 10 mM dNTP mix, 0.5 μl of 0.1 M DTT and 0.5 μl of superscript III RT (200 U/μl)] was added and the total 10 μl reaction volume was incubated for 10 min at 25 °C, 60 min at 42 °C and 5 min at 85 °C.

2.2.3. Pre-amplification

We used Single Cell-to-CT Kit (Ambion®, catalog #4458236). First, 0.2× pooled TaqMan Gene Expression Assays for our target genes (Supplemental Table 1) were prepared using 1× TE buffer, pH 8.0. Next, 3.22 μl of PreAmp Mix and 3.87 μl of 0.2× pooled TaqMan Gene Expression Assays were added to each RT sample then placed in a thermal cycler where it was held at 95 °C for 10 min, cycled (20 cycles) at 95 °C for 15 s then 60 °C for 4 min, and finally held at 99 °C for 10 min.

2.2.4. qPCR

Single Cell-to-CT Kit was again used. First a 1:20 dilution of pre-amplified products was prepared using 1xTE buffer, pH 8.0. Next, 50 μl of mix (25 μl of 2× TaqMan Gene Expression Master Mix, 2.5 μl of 20xTaqMan Gene Expression Assay, 12.5 μl of Nuclease-free water, and 10 μl of 1:20 diluted pre-amplified products) was loaded into a Micro Amp Fast Optical 96-well (0.1 ml) Reaction Plate with Barcode and placed into a ViiA7 PCR machine (Applied Biosys-