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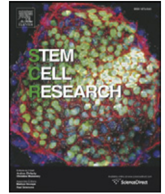

Electrophysiological and gene expression characterization of the ontogeny of nestin-expressing cells in the adult mouse midbrain

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ABSTRACT

The birth of new neurons, or neurogenesis, in the adult midbrain is important for progressing dopamine cell-replacement therapies for Parkinson's disease. Most studies suggest newborn cells remain undifferentiated or differentiate into glia within the adult midbrain. However, some studies suggest nestin + neural precursor cells (NPCs) have a propensity to generate new neurons here. We sought to confirm this by administering tamoxifen to adult *NesCreER^{T2}/R26eYFP* transgenic mice, which permanently labelled adult nestin-expressing cells and their progeny with enhanced yellow fluorescent protein (eYFP). eYFP+ midbrain cells were then characterized 1–32 weeks later in acutely prepared brain slices using whole-cell patch clamp electrophysiology combined with single-cell RT-qPCR. Most eYFP+ cells exhibited a mature neuronal phenotype with large amplitude fast action potentials (APs), spontaneous post-synaptic currents (sPSCs), and expression of 'mature' neuronal genes (*NeuN, Gad1, Gad2* and/or *VGLUT2*). This was the case even at the earliest time-point following tamoxifen (i.e. 1 week). In comparison to neighboring eYFP− (control) cells, eYFP+ cells discharged more APs per unit current injection, and had faster AP time-to-peak, hyperpolarized resting membrane potential, smaller membrane capacitance and shorter duration sPSCs. eYFP+ cells were also differentiated from eYFP− cells by increased expression of 'immature' pro-neuronal genes (*Pax6*, *Ngn2* and/or *Msx1*). However, further analyses failed to reveal evidence of a place of birth, neuronal differentiation, maturation and integration indicative of classical neurogenesis. Thus our findings do not support the notion that nestin + NPCs in the adult SNc and midbrain generate new neurons via classical neurogenesis. Rather, they raise the possibility that mature neurons express nestin under unknown circumstances, and that this is associated with altered physiology and gene expression.

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1. Introduction

The motor symptoms of Parkinson's disease (PD; e.g. tremor, bradykinesia, postural instability) are caused by loss of dopamine (DA) signaling in the caudate putamen (CPu) brought about by degeneration of DA neurons in the substantia nigra pars compacta (SNc). We know this because killing or interfering with SNc DA neurons in rodent and non-human primate models of PD produce similar motor dysfunctions (Gubellini and Kachidian, 2015) and administering drugs that elevate DA signaling or transplanting DA neurons into SNc or CPu normalizes movement in these models (Duty and Jenner, 2011; Bjorklund and Lindvall, 2017; Redmond et al., 2010). Indeed these same drugs are currently frontline treatments for PD where they effectively alleviate motor symptoms early in treatment but become less effective and produce debilitating side-effects, probably due to the unphysiological and untargeted DA signaling they induce (Barker et al., 2015). This has led many to believe the key to longer-lasting benefits with fewer side-effects is replacing SNc DA neurons, either by cell transplantation or by stimulating endogenous DA neurogenesis (Barker et al., 2015).

Both of these cell-replacement approaches would benefit from an adult midbrain microenvironment that is conducive for DA neurogenesis. Unfortunately this does not appear to be the case. In adult rodents, SNc cells rendered bromodeoxyuridine-positive (BrdU+; a marker of dividing cells) remain either undifferentiated or differentiate into glia, not neurons (Aponso et al., 2008; Chen et al., 2005; Cooper and Isacson, 2004; Lie et al., 2002; Shan et al., 2006; Klaissle et al., 2012; Worlitzer et al., 2013, but see Zhao et al., 2003).On the other hand there is evidence that some progenitor cells in the rodent midbrain have neurogenic capacity. Retinoic acid-induced differentiation of cells isolated from adult rat SNc and cultured in the presence of fibroblast growth factors (FGF2 or FGF8) generate β-tubulin III+ cells (neurons) in vitro (Lie et al., 2002). Moreover, these same cells become NeuN+ (neurons) following transplantation into the hippocampus, an established neurogenic niché, but not when transplanted back into SNc of adult rats (Lie et al., 2002). Lie et al. (Lie et al., 2002) speculated that these cells are nestin-expressing neural

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progenitor cells (NPCs) and Shan et al. (Shan et al., 2006) reported evidence that nestin + cells can indeed generate new neurons, including DA neurons, within the microenvironment of the adult mouse midbrain.

If nestin + cells do generate new neurons and DA neurons within the microenvironment of the adult midbrain, knowledge about their ontogenesis will be crucial to identify signaling mechanisms regulating neurogenesis and DA neurogenesis here, which might help progress cell-replacement therapies for PD. Hence, the aims of this study were to: (1) assess whether cells derived from nestin + cells in the adult midbrain have a neuronal phenotype as defined by electrophysiology and gene expression; and (2) if so, assess whether they achieved this via classical neurogenesis.

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. 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 a tamoxifen-inducible form of Cre-recombinase (CreER^{T2}) under the control of a 5.8-kb fragment of the promoter region and a 1.8-kb fragment of the second intron of the rat nestin gene. This second intron fragment contains a neural stem cell/progenitor-specific enhancer (Mignone et al., 2004; Zimmerman et al., 1994). Activation of CreER^{T2} is achieved by administering tamoxifen, an estrogen receptor agonist/antagonist, to the mice. *NesCreER^{T2}* mice were crossed with *R26eYFP* C57BL/6 reporter mice obtained with permission from Professor Frank Costantini (Columbia University Medical Center, New York) (Srinivas et al., 2001). *R26eYFP* reporter mice have a *LoxP*-flanked *DNA STOP* codon upstream of their reporter sequence (*eYFP*), which prevents reporter expression in the absence of active Cre-recombinase (i.e. absence of nestin promoter/enhancer activity and absence of tamoxifen). If on the other hand the *DNA STOP* codon is removed by active Cre-recombinase (i.e. presence of nestin promoter/enhancer activity and presence of tamoxifen) *eYFP* expression is driven by the constitutively and ubiquitously active *ROSA26* gene locus. All experimental *NesCreER^{T2}/R26eYFP* mice used were F1 generation obtained via *NesCreER^{T2}* homozygous (male or female) and *R26eYFP* homozygous (male or female) crosses. The majority (85%) of cells included in the analyses were harvested from Line 5.1 *NesCreER^{T2}/R26eYFP* mice. Line 5.1 is more specific (i.e. less tamoxifen-independent recombination CNS-wide) but less efficient at labelling nestin + cells in the adult hippocampal SGZ than line 4 (Imayoshi et al., 2006). Thus line 5.1 is the better to avoid false-positive labelling.

Thus, to identify and lineage trace nestin + cells in the adult midbrain, adult (≥8-weeks old) *NesCreER^{T2}/R26eYFP* male and female mice were administered a 'pulse' of tamoxifen (10 mg/day in 0.5 ml corn oil via oral gavage) for 3–4 consecutive days, which permanently labels cells with concurrent nestin promoter/enhancer activity (i.e. *Nes* gene expression) with the protein product of *eYFP* (i.e. enhanced yellow fluorescent protein or eYFP). Note that this tamoxifen dose is similar to that used in other studies on these mice (Sun et al., 2014; Sakamoto et al., 2011). Importantly, because the *eYFP* transgene is constitutively induced by a gene recombination event, eYFP protein will also be permanently expressed in any progeny of cells rendered eYFP + at the time of tamoxifen administration (i.e. eYFP + cells that divide any time after tamoxifen).

2.2. Electrophysiology

At different times (1–32 weeks) following administration of the tamoxifen 'pulse' *NesCreER^{T2}/R26eYFP* 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 in the coronal plane 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 and individual eYFP + and neighboring eYFP − (control) cells were viewed at high power (×63 objective) using fluorescence and infrared differential interference contrast (IR-DIC) microscopy. Whole-cell recordings were made with glass micropipettes (~1 μm tip diameter, ~6–10 MΩ resistance) containing ~6 μl 144 mM K-Gluconate, 3 mM $MgCl_2.6H_2O$, 10 mM HEPES, 0.5 mM EGTA, pH 7.2, osmolarity 290. Membrane current and voltage were recorded and controlled using an Axoclamp 2B amplifier (3 kHz bandwidth) and Clampex 9.0 software (Molecular Devices LLC, CA, USA). Data were digitized at 10 kHz using an Axon Digidata 1550 (Molecular Devices LLC, CA, USA).

2.3. Single-cell RT-qPCR

2.3.1. Cell samples

Following electrophysiological characterization and with the glass micropipette still tightly attached (i.e. GΩ resistance) to the cell's membrane, a <1 femtolitre sample of its cytoplasm was aspirated into the tip of the pipette using negative pressure and under visual guidance. The pipette was removed from the slice and its outer surface cleared of any cellular debris by rapid removal from the bath solution (the tip was re-examined under the microscope following this to ensure no cellular debris remained). The broken micropipette tip and its entire contents (~6 μl of internal pipette solution plus cell sample) were collected in a 200 μl eppendorf tube and immediately placed at −80 °C for later RT, pre-amplification and qPCR.

Care was taken to avoid contamination of cell samples with extraneous RNA and DNA. The capillary glass used to pull micropipettes was autoclaved; the micropipette holder and wire were sterilized under ultraviolet light and bleach solution, respectively; the internal pipette solution was autoclaved; the syringe used to fill the pipette was sterilized under ultraviolet light; the eppendorf tube into which the aspirate was placed was sterile; and the electrophysiologist wore gloves and used sterile technique throughout the cell-sampling procedures.

Cell-negative controls were collected in exactly the same way as described above except the micropipette tip was not sealed onto the cell membrane, no whole-cell electrophysiology was performed, and no cell cytoplasm was aspirated. Rather, the micropipette tip was placed near a cell and left there for the same amount of time it takes to seal, record and aspirate (approximately 10 min) before being withdrawn. All cell samples collected on days where cell-negative controls returned positive gene expression readings were excluded from analyses. RT-qPCR-negative and RT-qPCR-positive controls were performed on aliquots of RT buffer-only samples and RNA harvested from tissue dissected from developing mouse midbrain, respectively.

2.3.2. Reverse transcription

To perform first-strand cDNA synthesis the eppendorf tube containing a cell sample was thawed, briefly centrifuged and kept on ice until the RT reaction (~30 min.). First, the volume of the aspirate was measured and made up to 7.5 μl with 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

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