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Ventral midbrain neural stem cells have delayed neurogenic potential *in vitro*

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HIGHLIGHTS

- VM NSCs from older embryos and later passages are more gliogenic (after 7DD).
- E14 VM NSC cultures have significant increases in neurons at 14DD and 21DD.
- E14 VM NSC cultures require 3 weeks to complete their differentiation.
- Neurons at 7DD in E14 VM NSC cultures are not NSC-derived (BrdU-negative).
- GFAP-positive cells at 7DD may be both neurogenic and gliogenic.

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ABSTRACT

Neural stem cells (NSCs) have been the focus of an intensive effort to direct their differentiation *in vitro* towards desired neuronal phenotypes for cell replacement therapies. It is thought that NSCs derived from older embryos have limited neurogenic capacity and are restricted towards an astroglial fate. This idea is largely based on studies that typically analysed NSC-derived progeny following one week of *in vitro* differentiation. In this report, the neurogenic capacity of older ventral midbrain (VM) NSCs was assessed. When the older NSCs were differentiated for three weeks, there were significant increases in the numbers of newly born neurons at 14 and 21 days, as assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation. Therefore this study demonstrates that older NSCs retain significantly more neurogenic potential than was previously thought. These data have implications for NSC preparatory protocols and the choice of donor age for cell transplantation studies, and contributes to the understanding of NSC behaviour *in vitro*.

1. Introduction

In recent years, NSCs and other stem cell types have been the focus of much research aimed at directing their differentiation *in vitro*, firstly into neurons and secondly into a committed VM dopaminergic (DA) phenotype, for use in transplantation approaches in Parkinson's disease [3,6,20]. The most relevant source of NSCs for the generation of VM DA neurons are those isolated from the VM during the period of DA neurogenesis, which occurs between embryonic day (E) 11 and E14 in the developing rat VM *in vivo* [1,9,16]. Understanding the *in vitro* development of these NSCs is crucial for the choice of donor ages from which to culture VM NSCs. This study thus focused on E12 and E14 rat VM NSCs.

NSCs can be isolated from multiple regions of the embryonic brain, and their numbers expanded *in vitro* as free-floating aggregates termed "neurospheres" when grown in the presence of the mitogens, epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) [7,14,22,23]. The proliferating NSCs then spontaneously differentiate into neurons and glia upon mitogen withdrawal [7,22,23]. It has been suggested that the age of the donor embryo from which NSCs are initially isolated is a critical determinant of subsequent neuronal differentiation *in vitro*, as NSCs derived from younger donors gave rise to more neurons than those derived from older donors [9,19]. These studies have suggested that NSCs from older donor embryos are more restricted towards an astroglial fate.

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CNS, central nervous system; DA, dopaminergic; DD, days of differentiation; DIV, days *in vitro*; E, embryonic day; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NP(s), neural progenitor(s)/precursor(s); NSC(s), neuroepithelial/neural stem cell(s); PBS (-T), phosphate buffered saline (-Triton X); VM, ventral midbrain/mesencephalon; VZ, ventricular zone.

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Neurosphere studies typically determine their NSC-derived progeny by assessing the numbers of neurons and glia generated following differentiation for one week *in vitro* [12,19,21,24,26]. In this report, by assessing the neuronal progeny for longer differentiation periods, we show that older NSCs retain significantly more neurogenic potential than previously thought, and suggest that care should be taken when interpreting *in vitro* studies that use glial fibrillary acidic protein (GFAP) as a marker of "differentiated" astrocytes.

2. Materials and methods

2.1. Preparation of embryonic rat VM NSCs cultures

Cultures of E12/E14 Sprague-Dawley rat VM NPCs were prepared as previously described (O'Keeffe and Sullivan, 2005). 2×10^6 cells were grown in T-25 culture flasks in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12, 100 nM L-Glutamine, 6 mg/ml D-Glucose, 100 U/ml Penicillin, 10 μg/ml Streptomycin (Sigma), 2% B27 (Invitrogen), 20 ng/ml EGF (Sigma) and 20 ng/ml of FGF2 (Millipore) for 7 days *in vitro* (DIV). Neurospheres were enzymatically dissociated [19] and reseeded in flask for subsequent expansion. Also 5×10^4 cells per well in poly-D-lysine-coated 24-well tissue culture plate were allowed to differentiate for 7, 14 or 21 DIV in the medium above minus EGF/FGF, with the addition of 1% FCS. 0.2 μM of 5-bromo-2′-deoxyuridine (BrdU) (Sigma B5002) was added during expansion from 5 DIV, and supplemented every 3 DIV from the time of plating.

2.2. Immunocytochemistry

Cultures were fixed in ice-cold methanol for 10 min, washed in 10 mM phosphate buffered saline (PBS) containing 0.02% Triton X-100 (PBS-T), and incubated in blocking solution (5% bovine serum albumin (Sigma), 0.2% Triton X-100 in 10 mM PBS) for 1 h at room temperature. Cultures were incubated in the following antibodies: mouse anti-βIII-tubulin (1:300; Medical Supply), rabbit anti-βIII-tubulin (1:300; Millipore), mouse anti-nestin (1:400; Millipore), mouse anti-GFAP (1:300; Sigma), and mouse anti-BrdU (1:4; Millipore) diluted in 1% bovine serum albumin in 10 mM PBS at 4 °C overnight. Following washes in PBS-T, cells were incubated in the appropriate Alexa Fluor 488 and/or 594-conjugated secondary antibodies (1:500; Invitrogen) diluted in 1% bovine serum albumin in PBS, at room temperature for 2 h. Cultures were counterstained with DAPI or Sytox (1:1000; Invitrogen) and imaged using Olympus IX70 inverted microscope. The total number of cells (assessed by DAPI staining) and the numbers of each cell type were counted in each individual image [19].

2.3. Statistical analysis

Unpaired Student's *t*-test or one-way ANOVA with a *post hoc* Tukey's test were performed as appropriate to determine statistical significance. Results were expressed as means with SEM and considered to be significant when $p < 0.05$.

3. Results

3.1. Effect of gestational age and passage number on VM-derived NSCs in vitro

Firstly, E12 and E14 rat VM NSCs which had been expanded for 7 DIV (passage 1), 14 DIV (passage 2) or 21 DIV (passage 3) as free-floating neurospheres were allowed to differentiate after mitogen withdrawal for 7 days of differentiation (DD) (Fig. 1a), before being immunocytochemically stained for βIII-tubulin (neurons) or GFAP (astrocytes) (Fig. 1b–e). Later passages were not characterised due to a marked increase in cell death and lack of neurosphere formation following 28 DIV (data not shown). Passage 1 VM NSCs, isolated at E12 or E14, generated a significantly higher percentage of βIII tubulin-positive neurons than at passage 2 or 3 (Fig. 1b). Passage 2 or 3 VM NSCs, isolated at E12 or E14, generate a significantly higher percentage of GFAP-positive astrocytes in comparison to passage 1 VM NSCs (Fig. 1d). Therefore, VM NSCs of early passages are more neurogenic, and less gliogenic, than those of older passages, irrespective of the age of the donor embryo.

In all passages examined, the 7DD progeny of VM NSCs isolated at E12 generated a significantly higher percentage of βIII

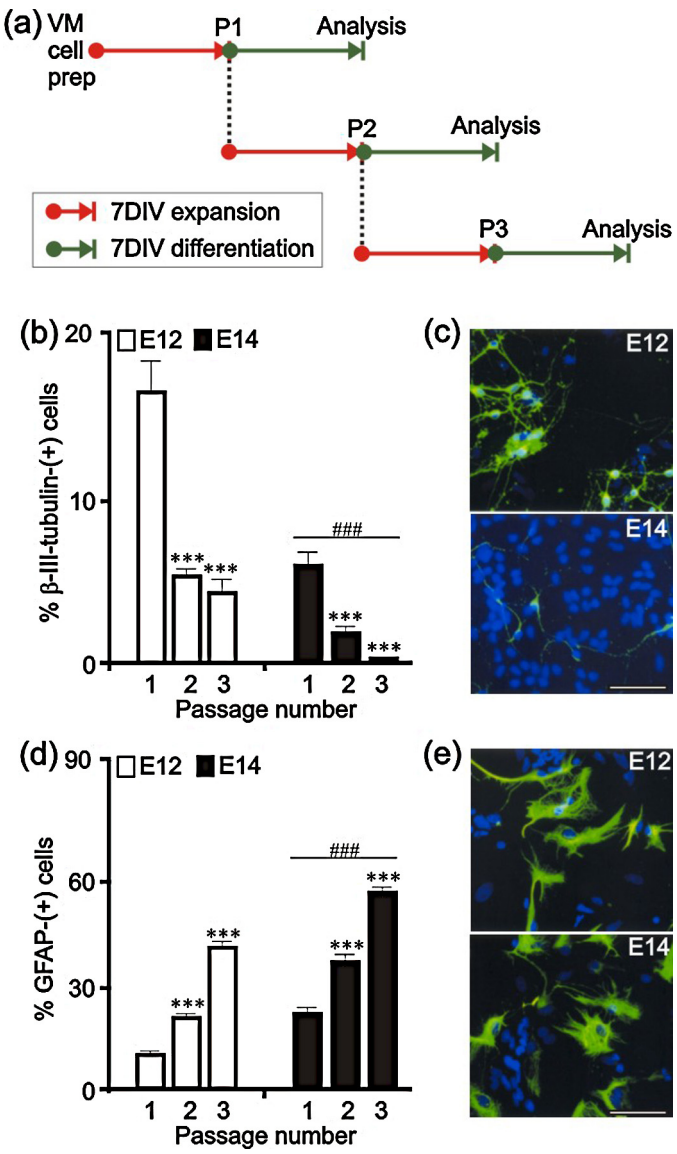

Fig. 1. Characterisation of neurogenesis and gliogenesis in cultures of VM NSCs of various gestational ages and passage numbers. (a) Schematic representation of the passaging protocol for E12 and E14 VM NSC neurosphere cultures. Graphical representation of the mean numbers (expressed as a percentage of total cells) of (b) βIII-tubulin-positive cells and (d) GFAP-positive cells following 7 DD, in neurosphere cultures of E12 or E14 VM NSCs which were passaged once, twice or three times before differentiation, as indicated ($^{***}p < 0.001$ *vs* passage 1; $^{\#\#\#}p < 0.001$ *vs* E12, ANOVA with *post hoc* Tukey's test). Data are expressed as mean ± SEM, $n = 60$ fields. Representative photomicrographs of cultures of passage 1 VM NSCs isolated at E12 or E14, differentiated for 7 DIV and immunocytochemically stained for and (c) βIII-tubulin or (e) GFAP, and counterstained with DAPI. Scale bar = 100 μm.