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Functional determination of the differentiation potential of ventral mesencephalic neural precursor cells during dopaminergic neurogenesis

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ABSTRACT

The ventral mesencephalic neural precursor cells (vmNPCs) that give rise to dopaminergic (DA) neurons have been identified by the expression of distinct genes (e.g., *Lmx1a*, *Foxa2*, *Msx1/2*). However, the commitment of these NPCs to the mesencephalic DA neuronal fate has not been functionally determined. Evaluation of the plasticity of vmNPCs suggests that their commitment occurs after E10.5. Here we show that E9.5 vmNPCs implanted in an ectopic area of E10.5 mesencephalic explants, retained their specification marker Lmx1a and efficiently differentiated into neurons but did not express the gene encoding tyrosine hydroxylase (Th), the limiting enzyme for dopamine synthesis. A proportion of E10.5-E11.5 implanted vmNPCs behaved as committed, deriving into Th^+ neurons in ectopic sites. Interestingly, implanted cells from E12.5 embryos were unable to give rise to a significant number of Th^+ neurons. Concomitantly, differentiation assays in culture and in mesencephalic explants treated with Fgf2+LIF detected vmNPCs with astrogenic potential since E11.5. Despite this, a full suspension of E12.5 vmNPCs give rise to DA neurons in a similar proportion as those of E10.5 when they were transplanted into adult brain, but astrocytes were only detected with the former population. These data suggest that the subventricular postmitotic progenitors present in E12.5 ventral mesencephalon are unable to implant in embryonic explants and are the source of DA neurons in the transplanted adult brain. Based on our findings we propose that during DA differentiation committed vmNPCs emerge at E10.5 and they exhaust their neurogenic capacity with the rise of NPCs with astrogenic potential.

1. Introduction

The mammalian central nervous system (CNS) develops from neural precursor cells (NPCs) that respond to spatial and temporal cues in order to differentiate into specific neuron types. Astrocytes and oligodendrocytes also derive from these NPCs but they might significantly change their differentiation potential to commit into these fates (Hirabayashi and Gotoh, 2010; Rowitch and Kriegstein, 2010). During differentiation, NPCs go through distinct phases of commitment such that their natural neuronal multipotentiality (i.e., the ability to differentiate into different neuron types) detected in early developing CNS is lost at later stages. Despite the significant relevance of knowing the differentiation potential of developing NPCs, the actual plasticity of these cells has been scarcely studied.

The specification of developing cells establishes the stage at which cells have a defined fate but, yet, is dependent on their allocation in the embryo; this means that cell fate can be modified by changing the adjacent cells or growth factors (e.g., by transplantation). On the other hand, determination defines the stage at which cells differentiate according with their natural fate without the influence of the surrounding environment. Although NPC specification and commitment can be estimated by a code of gene expression (Bang and Goulding, 1996), a definitive evaluation can come only from a functional assay. Theoretically, a specified cell would continue its differentiation in the absence of external factors however, experimentally, this evaluation is limited by the survival of tissues or cells in the absence of growth factors. Therefore, fate switch evaluation of presumably specified cells (e.g., expressing a set of specification markers) after ectopic transplan-

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tation is a preferred assay for NPC commitment.

The plasticity of NPCs has been estimated within transplanted tissue fragments, a strategy particularly amenable for easily accessible embryos (Alvarado-Mallart et al., 1990; Grapin-Botton et al., 1997; Itasaki et al., 1996). Relevant to mention is that NPCs in this condition are still exposed to components of their niche and this may prevent revealing their actual differentiation potential. In mice, determination of plasticity of dissociated NPCs from different regions and developmental stages has been evaluated in few instances after reintroduction into the embryonic nervous system by ventricular or ultrasound-guided parenchymal injection (Campbell et al., 1995; Fishell, 1995; Olsson et al., 1998, 1997). These studies show low site-specific integration and few integrated NPCs. With the aim of increasing site-specific graft efficiency of NPCs, we developed the transplant-to-explant protocol. Using this experimental system, we were able to determine the differentiation potential of fresh and cultured NPC populations (Baizabal et al., 2012, 2010; Baizabal and Covarrubias, 2009).

The time at which ventral mesencephalic NPCs (vmNPCs) become committed to differentiate into midbrain dopaminergic (mDA) neurons has not been determined. Previous studies have shown that midbrain tissue of HH stage 10 chicken embryos (around developmental stage E9 in mouse) implanted in forebrain regions maintained their regional fate (i.e., *En1* expression) (Alvarado-Mallart et al., 1990; Gardner and Barald, 1991). However, dissociated mesencephalic cells of E10.5 mouse embryos re-specify in the ventral telencephalon, while those of E13.5 embryos retain their original identity (Campbell et al., 1995; Olsson et al., 1997). In contrast, E12.5 vmNPCs lose their identity after few cell divisions in culture and poorly differentiate into DA neurons (Bang et al., 2015; Chung et al., 2006; Meyer et al., 2012; Yan et al., 2001).

Currently, the commitment stage of dopaminergic NPCs has been molecularly estimated by the expression of genes throughout the differentiation process. For example, $Lmx1a^{+}Foxa2^{+}$ cells identify specified mDA NPCs (Andersson et al., 2006a, 2006; Ferri et al., 2007) which appear to become committed once gain *Neurog2* expression, mostly present in postmitotic progenitors (Lacomme et al., 2012; Thompson et al., 2006). However, it is not clear whether the emergence of *Msx1/2* expression in dividing ventricular NPCs (Trujillo-Paredes et al., 2016) marked the commitment for differentiation into DA neurons. In the present work, we functionally tested the DA differentiation potential of vmNPCs at different developmental stages. In addition, we examined the gain of astrogenic potential in these cells during mDA neuronal differentiation. We found that mDA NPC commitment establishes around E10.5 and markedly decays at E12.5 when NPCs start to display astrogenic potential.

2. Materials and methods

2.1. Animals and tissue preparation

In this study, CD1 and EGFP mouse strains were used. Transgenic male mice that constitutively express Enhanced Green Fluorescent Protein (EGFP; gift from Andras Nagy) were crossed with wild type CD1 females in order to obtain EGFP heterozygous embryos. The day of detection of vaginal plug was considered as E0.5. Pregnant female mice were sacrificed by cervical dislocation. To analyze dividing cells and their fate, incorporation of 5-Bromo-2-Deoxyuridine (BrdU, Sigma) was determined at 2, 12, 24 and 48 h after a single BrdU intraperitoneal injection (80–250 mg/kg). All the procedures were approved by the Bioethical Committee of our Institution.

2.2. Collagen explant culture

Mesencephalons were obtained from E9.5 to E11.5 from CD1 mouse embryos and cultivated in collagen matrix (Baizabal and Covarrubias, 2009). E10.5 explants were used to assess the role Shh on implant differentiation. In this case, explants cultures were treated with the Smoothened agonist SAG (400 nM, Santa Cruz) which was added at the beginning of the culture and at day 2. For some experiments explants were treated with Fibroblast Growth Factor 2 (Fgf2; 20 ng/mL, Peprotech), Leukemia Inhibitory Factor (LIF; 80 μL/mL, Millipore) or both Fgf2 and LIF (Fgf2 during day 1 and both after day 2) and cultivated for 7 days (E9.5 and E10.5 explants) and during 6 days for E11.5 explants. Explants for cell transplantation were prepared from E10.5 embryos and cultivated for 7 days.

2.3. Transplant-explant protocol

The ventral region of 10–15 mesencephalons (from the cephalic flexure to the isthmus) from EGFP embryos was dissected and pooled together. Cell suspension was obtained by mechanical dissociation in DMEM-F12 (Gibco) media. For transplantation, a flame-stretched Pasteur pipette coupled to a hose with a mouthpiece was used to deposit donor cells over the ventricular surface of collagen-embedded explants, as previously described in Baizabal and Covarrubias (2009). For implantation of vmNPCs, the exposed ventricular surface of mesencephalons was gently aspired using a thin glass capillary and cells were deposited over the explants.

2.4. Cell aggregation

E9.5 and E12.5 ventral mesencephalons were obtained as described above (E9.5 embryos were EGFP). Equal number of ventral midbrains (n = 8) were mixed and cell suspension obtained. Five μL of cell suspension were mixed with collagen and then allowed to polymerize in a single well (48-well plates were used) before adding explant media. After two days of culture cells were fixed inside collagen and immunofluorescence staining were made.

2.5. Transplantation into adult rats

Male Wistar rats of ~ 250 g were used for transplantation procedures as reported in Maya-Espinosa et al. (2015). Animals were transplanted under ketamine-xylazine anesthesia (10 and 1 mg/kg, respectively) by intracranial injection of 1–2 μL of cell suspension (approx. 150,000 cells prepared as described below) using a 5 mL Hamilton syringe. After 6 days, rats were deeply anesthetized with pentobarbital and perfused intracardially with 250 mL of 0.1 M phosphate buffered saline (PBS) followed by 250 mL of 4% paraformaldehyde (PFA).

2.6. Monolayer culture of mesencephalic NPCs

Cell suspension from ventral midbrains was prepared as described above. Cells were seeded at a density of 1×10^5 cells in 24-well dishes pre-coated with poly-D-ornithine (0.0025%; Sigma)/laminin (10 mg/mL; Invitrogen) in a supplemented serum-free media [DMEM-F12 (Invitrogen), Neurobasal (Invitrogen), N2 (1% v/v), Insulin (10 μg/mL; Sigma), Glutamine (0.5 mM; Gibco), β-Mercaptoethanol (0.1 mM; Gibco)] supplemented with 10 ng/mL Fgf2. Cells were incubated to allow their attachment during 24 h and then media was changed and the different factors were added; cells were cultured for 48 h.

2.7. Tissue processing and Immunofluorescence staining

Embryonic brain tissue and midbrain explants sections were fixed with 4% Paraformaldehyde (PFA, Sigma) for 2 h at 4 °C, washed with PBS and dehydrated overnight with 30% Sucrose (J.T. Baker)/PBS. Tissues were frozen and sliced in coronal sections of 10 μm thickness using a cryostat (Microm HM550, Thermo Scientific). Adult rat brains were post-fixed in 4% PFA and dehydrated by sequential 24 h incubations in 10%, 20%, and 30% sucrose/PBS. Afterward, brains were sliced