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Regular Article

Meningeal cells influence midbrain development and the engraftment of dopamine progenitors in Parkinsonian mice

Fahad A. Somaa, Christopher R. Bye, Lachlan H. Thompson, Clare L. Parish *

The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria 3010, Australia

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ABSTRACT

Dopaminergic neuroblasts, isolated from ventral midbrain fetal tissue, have been shown to structurally and functionally integrate, and alleviate Parkinsonian symptoms following transplantation. The use of donor tissue isolated at an age younger than conventionally employed can result in larger grafts — a consequence of improved cell survival and neuroblast proliferation at the time of implantation. However studies have paid little attention to removal of the meninges from younger tissue, due to its age-dependent tight attachment to the underlying brain. Beyond the protection of the central nervous system, the meninges act as a signaling center, secreting a variety of trophins to influence neural development and additionally impact on neural repair. However it remains to be elucidated what influence these cells have on ventral midbrain development and grafted dopaminergic neuroblasts. Here we examined the temporal role of meningeal cells in graft integration in Parkinsonian mice and, using *in vitro* approaches, identified the mechanisms underlying the roles of meningeal cells in midbrain development. We demonstrate that young (embryonic day 10), but not older (E12), meningeal cells promote dopaminergic differentiation as well as neurite growth and guidance within grafts and during development. Furthermore we identify stromal derived factor 1 (SDF1), secreted by the meninges and acting on the CXCR4 receptor present on dopaminergic progenitors, as a contributory mediator in these effects. These findings identify new and important roles for the meningeal cells, and SDF1/CXCR4 signaling, in ventral midbrain development as well as neural repair following cell transplantation into the Parkinsonian brain.

Introduction

Parkinson's disease is a progressive neurodegenerative disorder characterized by the loss of midbrain dopamine neurons that results in disturbances in motor function. Pharmacotherapy, to restore dopamine (DA) transmission, remains the mainstay in treatment; however, it is hindered by waning efficacy and persistent side effects. In contrast, proof of principle clinical trials have demonstrated the long-term benefits of dopamine cell replacement therapy (Winkler et al., 2005). Human fetal ventral mesencephalic (VM) dopaminergic progenitors, ectopically transplanted into the striatum of Parkinsonian patients, have been shown to structurally and functionally integrate and alleviate symptoms for more than a decade (Winkler et al., 2005). In an effort to improve the survival and integration of these new neurons into the denervated striatum, studies by us and others, have demonstrated that the use of younger donor tissue results in superior outcomes (Bye et al., 2012; Freeman et al., 1995; Gates et al., 2006; Kauhausen et al., 2013; Torres et al., 2007).

To date most animal studies have isolated donor tissue at mouse embryonic 12 (E12, or the equivalent age in rats), perceived to be the peak of DA neurogenesis. However the transplantation of younger VM tissue (E10) has been shown to result in grafts containing more DA neurons, a greater proportion of A9 DA neurons (the DA subpopulation responsible for restoring motor function following transplantation (Grealish et al., 2010; Kuan et al., 2007)), increased striatal reinnervation and elevated dopamine levels (Bye et al., 2012; Torres et al., 2007). In part, these improved findings were attributed to enhanced survival and increased numbers of dividing dopaminergic neuroblasts at the time of transplantation in younger *versus* older donor grafts (Bye et al., 2012; Torres et al., 2007), as well as the observation that A9 DA neurons precede the birth of other midbrain DA neurons during development, and are thereby enriched for in younger tissue (Blaess et al., 2011; Bye et al., 2012; Hayes et al., 2011; Joksimovic et al., 2009). However it remains to be determined whether other variables may have influenced these improved outcomes.

In particular we questioned whether meningeal cells overlying the VM might have contributed to these effects. During development, meningeal cells adhere tightly to the underlying brain tissue. As development progresses this covering becomes less tightly affixed (Decimo et al., 2012; Siegenthaler and Pleasure, 2011). Consequently, while the

* Corresponding author. Fax: +61 3 9347 0446.
E-mail address: cparish@unimelb.edu.au (C.L. Parish).

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meninges can be easily removed from older donor tissue (typically E12 VM and older), at younger ages it is increasingly possible, and likely that these tightly juxtaposed cells may be incorporated into VM cell preparations.

While the meninges were previously thought of as merely protective membranes covering the brain and spinal cord – providing tight anchoring of the central nervous system (CNS) to the surrounding bone, as well as being filled with cerebrospinal fluid to provide cushioning (Decimo et al., 2012) – in more recent years the meninges have become increasingly recognized for their role in physiological and pathological events of the CNS (Decimo et al., 2012; Siegenthaler and Pleasure, 2011). The meninges are the site of origin of the brain vasculature. Consequently their strategic position within the parenchyma and connection with the vasculature enable short-range delivery of trophins (Siegenthaler and Pleasure, 2011). During development these cells have been shown to secrete a number of proteins including, but not limited to: retinoic acid, controlling both cortical and cerebellar neurogenesis and migration (Siegenthaler et al., 2009; Zhang et al., 2003); bone morphogenic protein-7, influencing callosal axonal growth and guidance (Choe et al., 2012); as well as stromal derived factor-1 (SDF1), influencing the migration of cortical progenitors and cortical layering (Borrell and Marin, 2006; Lopez-Bendito et al., 2008). The meninges also participate in the formation of the extracellular matrix, secreting collagen, fibronectin and laminin, and additionally form the basement membrane lining the brain ventricles, providing an anchor for radial processes along which neural progenitors can migrate (Montagnani et al., 2000).

In the injured adult CNS, the meninges have been shown to contribute to repair. Transplants of meningeal cells into the injured spinal cord have been shown to promote regeneration of axons by providing tropism (Franzen et al., 1999), as well as providing extracellular matrix-like support for remyelinating oligodendroglia (Lakatos et al., 2003). Added to this has been the recent observation of a potentially quiescent population of stem cells within the meninges of the spinal cord that may contribute to replacement and repair (Decimo et al., 2011).

In light of the influence of meningeal cells in development and repair elsewhere in the CNS, we examined the contribution of these cells to enhancing repair following fetal tissue transplantation in the Parkinsonian brain as well as their role in dopaminergic development.

Material & methods

All procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research, and experiments were approved by the Florey Institute of Neuroscience and Mental Health animal ethics committee. Mice were housed on a 12 h light/dark cycle with *ad libitum* access to food and water.

Isolation of ventral midbrain cells and meninges

Adult female Swiss mice were used as graft recipients while tyrosine hydroxylase-green fluorescent protein (TH-GFP) mice (Sawamoto et al., 2001) were used to generate embryos as a source of donor tissue for transplantation. The use of TH-GFP donor tissue enabled the identification of graft (GFP+) *versus* host (GFP−) derived TH+ dopamine neurons *in vivo*. Animals were time mated overnight and visualization of a vaginal plug on the following morning was taken as E0. The ventral midbrain and overlying meningeal tissue was isolated at embryonic day 10 (E10) or E12. The ventral mesencephalon (VM) was microdissected from each TH-GFP+ embryo, and dissociated into a single cell suspension as previously described (Blakely et al., 2011; Thompson and Parish, 2013). During dissection, careful attention was made to isolate and collect the meningeal tissue overlying the VM. VM or meningeal tissue was dissociated in magnesium and calcium-free Hank's buffered salt solution containing 0.25% trypsin and 0.1% DNase. For transplantation, the VM cells and meningeal cells were resuspended at 100,000 cells/μl in HBSS containing 0.1% DNase and stored on ice for the duration of the transplantation procedure. Staining for vimentin, confirmed our ability to selectively isolate meningeal cells from underlying VM tissue and 'spike' these cells back into VM preparations for co-grafting (Supplementary Fig. 1) and *in vitro* co-culturing (Supplementary Fig. 2).

6-Hydroxydopamine lesions and transplantation

All surgeries were performed under general anesthetic using 2% isoflurane inhalation (Baxter; Deerfield, IL, USA). Mice received partial lesions of the VM DA neurons by unilateral injection of selective catecholamine neurotoxin 6-hydroxydopamine (6-OHDA, 3.2 μg) into the SNpc as previously described (Parish et al., 2001). Three weeks after lesioning host animals received ectopic intrastriatal grafts of a single cell VM suspension derived from either E10 or E12 TH-GFP donor embryos with or without meningeal cells at the following coordinates relative to bregma: anterior, 1.0 mm; lateral, 2.3 mm; and 3.1 mm below dural surface. Injections were either 1 μl of VM cell suspension (100,000 cells) + 0.5 μl HBSS media or, 1 μl VM cells (100,000 cells) + 0.5 μl of meningeal cell suspension (50,000 cells). Groups were as follows: E10 VM cells (subsequently referred to as E10, n = 10); E10 VM cells + E10 meninges (E10 + M, n = 11); E12 VM cells (E12, n = 10); and E12 VM cells + E12 meninges (E12 + M, n = 9).

Ten weeks after transplantation animals received an overdose of sodium pentobarbitone (100 mg/kg) and were transcardially perfused with warmed phosphate buffered saline (PBS), followed by chilled paraformaldehyde (4% w/v in 0.1 M phosphate buffer). The brains were post-fixed for 2 h in 4% paraformaldehyde and cryo-protected overnight in sucrose (30% w/v in 0.1 M phosphate buffered saline) before being sectioned on a freezing microtome (Leica). Horizontal sections were collected in 12 series at a thickness of 40 μm.

Ventral mesencephalon primary and explant cultures

For *in vitro* culturing, cells were isolated as described above, centrifuged and resuspended in serum-free N2 medium consisting of a 1:1 mixture of F12 and MEM supplemented with 15 mM HEPES buffer, 1 mM glutamine, 6 mg/ml glucose (Sigma-Aldrich), 1 mg/ml bovine serum albumin and N2 supplement (all purchased from Invitrogen). Cells were seeded onto PDL-coated 48-well culture plates at a density of 120,000 cells per well ± meningeal cells (40,000 cells per well). Where appropriate SDF1 (20 ng/ml, R&D systems) or the CXCR4 antagonist, AMD3100 (12.5 ng/ml, Sigma-Aldrich) was added to the media at the time of plating. Cells remained *in vitro* at 37 °C and 5% CO_2 for 72 h prior to fixation with 4% paraformaldehyde.

Neuronal differentiation and neurite morphology were analyzed from 3–5 independent cultures using previously described methods (Blakely et al., 2011). In brief, TH+, TUJ+ and DAPI labeled cells were quantified from 10 sampling sites/well and 3 technical replicates (*i.e.* wells) performed per experimental condition. For the assessment of neurite morphology, the first 30 TH^+ cells (or TUJ+ cells) found to be measurable (neurites intact and distinguishable from other stained neurites) were quantified in order to avoid any potential sampling bias. In each experiment, data was compared to the mean normalized control value (set at 100%) to account for inter-experimental variation. Photomicrographs of each DA neuron (identified by TH^+) or non-dopaminergic neuron (TH−/TUJ+) were taken using a 20× objective (Olympus IX71) and the following measurements obtained using NeuronJ software (ImageJ, NeuronJ plugin, NIH): the total numbers of neurites per DA neuron, the number of neurite branches, the total length of all neurites per neuron and the length of the dominant neurite (Fuentes et al., 2008).

Explant co-cultures were performed as previously described (Blakely et al., 2011). In brief, VM explants were plated alone, or adjacent to