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Chondroitinase improves midbrain pathway reconstruction by transplanted dopamine progenitors in Parkinsonian mice

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ABSTRACT

Within the adult central nervous system the lack of guidance cues together with the presence of inhibitory molecules produces an environment that is restrictive to axonal growth following injury. Consequently, while clinical trials in Parkinson's disease (PD) patients have demonstrated the capacity of fetal-derived dopamine neurons to survive, integrate and alleviate symptoms, the non-permissive host environment has contributed to the incomplete re-innervation of the target tissue by ectopic grafts, and even more noticeable, the poor reconstruction of the midbrain dopamine pathways following homotopic midbrain grafting. One such inhibitory molecule is the chondroitin sulfate proteoglycan (CSPG), a protein that has been shown to impede axonal growth during development and after injury. Digestion of CSPGs, by delivery of the bacterial enzyme chondroitinase ABC (ChABC), can improve axonal regrowth following a number of neural injuries. Here we examined whether ChABC could similarly improve axonal growth of transplanted dopamine neurons in an animal model of PD. Acute delivery of ChABC, into the medial forebrain bundle, degraded CSPGs along the nigrostriatal pathway. Simultaneous homotopic transplantation of dopaminergic progenitors, into the ventral midbrain of ChABC treated PD mice, had no effect on graft survival but resulted in enhanced axonal growth along the nigrostriatal pathway and reinnervation of the striatum, compared to control grafted mice. This study demonstrates that removal of axonal growth inhibitory molecules could significantly enhance dopaminergic graft integration, thereby holding implications for future approaches in the development of cell replacement therapies for Parkinsonian patients.

1. Introduction

Historically the adult central nervous system (CNS) was deemed to be intrinsically incapable of undergoing regeneration. However, by the early 1980's Aguayo and colleagues radically changed this viewpoint; demonstrating that CNS neurons transplanted into the peripheral nervous system were capable of extending their axons over long distances (Aguayo et al., 1981). Added to this, transplants of fetal tissue into the neonatal brain showed increased innervation by comparison to the same grafts in the adult brain (Bentlage et al., 1999; Nikkhah et al., 1995). These findings suggested that the adult CNS microenvironment, rather than the CNS neurons per se, was non-permissive to axonal growth and regeneration. Since then, extensive research has focused on defining the factors that render the adult CNS environment restrictive to regeneration, with efforts to overcome these inhibitory cues in order to promote brain repair, including the integration of transplanted neurons (for review see Fawcett, 2009).

Cell replacement therapy is a promising treatment for a number of neurodegenerative disorders including Parkinson's disease, a disorder resulting from the progressive loss of midbrain dopamine neurons. Clinical studies have provided proof-of-principle that fetal-derived dopaminergic progenitors, ectopically transplanted into the denervated striatum, are capable of restoring dopamine neurotransmission and inducing long-term relief of motor symptoms in patients (Winkler et al., 2005). Lacking in these ectopic transplantation studies is the ability to restore normal basal ganglia circuitry. This includes the dopaminergic nigrostriatal pathway, and the reciprocal striatonigral pathway which together influence the activity of midbrain dopaminergic neurons (Winkler et al., 2000). Efforts to restore the midbrain dopamine pathways, through homotopic transplantation, have been hampered by the challenges of long distance axonal growth, including the non-permissive environment of the adult host brain. While delivery of guidance cues have been shown to improve the integration of these grafted neurons (Kauhausen et al., 2013; Thompson et al., 2009), it remains to be determined what impact removal of the hostile, inhibitory cues in host brain may have on the growth of homotopically grafted dopaminergic.

Chondroitin sulfate proteoglycans (CSPGs) are the most abundant inhibitor of axonal growth in the brain (Carulli et al., 2005; Morgenstern et al., 2002; Takeuchi et al., 2013). CSPGs are a class of extracellular matrix molecules that are comprised of a central core protein that is attached to a number of carbohydrate chains, known as glycosaminoglycans (GAGs). In addition to their structural role

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within the extracellular matrix, CSPGs have been shown to be important for correct cell migration and axon guidance during development, with migrating cells and axons actively avoiding regions that are rich in CSPG expression (Golding et al., 1999; Kubota et al., 1999). In the adult CNS these cues are down regulated, yet are re-expressed following injury — predominantly by reactive astrocytes within the glial scar. In this context, the glial scar plays an important role in cordoning off the injury, however the high expression of CSPGs within the scar also plays an influential role in the regenerative failure seen after CNS injury (Bovolenta et al., 1993; McKeon et al., 1991; Rudge and Silver, 1990; Snow et al., 1990). This has been further demonstrated in transplantation studies, including ventral midbrain fetal grafts, whereby an up-regulation of CSPGs has been observed in the glial scar surrounding the graft site where it impedes axonal growth and integration of new cells into the host tissue (Barker et al., 1996; Gates et al., 1996).

Of interest, the inhibitory effect of CSPGs on neurite extension can be reversed in vitro by treatment with the bacterial enzyme chondroitinase ABC (ChABC, an enzyme that cleaves chondroitin sulfate side chains) (Bovolenta et al., 1993; Dou and Levine, 1994; McKeon et al., 1995; Snow et al., 1990), metalloproteinases (enzymes that degrades the core proteins) (Cua et al., 2013) or inhibitors of proteoglycans synthesis (Smith-Thomas et al., 1995). Similarly, in vivo ChABC treatment at an injury site can effectively degrade the CSPGs (Lemons et al., 1999), promote axon regeneration into, and through, the scarred region (Hyatt et al., 2010; Zhao et al., 2011), and establish appropriate connections to cause a functional benefit in animal models of neural injuries (Bradbury et al., 2002; Houle et al., 2006; Moon et al., 2001). To date, the majority of studies have focused on the ability of CSPG degradation to promote endogenous regeneration of the corticospinal tract as well as exogenous repair through cell transplantation after spinal cord injury (Burnside and Bradbury, 2014). In more recent years, studies have also demonstrated the ability of ChABC to promote growth of DA axons — in vitro (Berglof et al., 2008; Mace et al., 2002), following axotomy of the nigrostriatal pathway in vivo (Li et al., 2007; Moon et al., 2002) as well as ectopic transplantation of DA progenitors into the corpus callosum (Jin et al., 2011). Here we have examined the ability of ChABC to improve the microenvironment for transplanted dopamine neurons and promote restoration of the nigrostriatal pathway following homotopic grafting into PD mice.

2. Material & methods

All methods were conducted in accordance with the Australian National Health and Medical Research Council published Code of Practice for the Use of Animals in Research and was approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee. All mice were group housed and exposed to a 12 h light/dark cycle with ad libitum access to food and water.

2.1. 6-hydroxydopamine lesioning

Thirty-five adult female Swiss mice received a partial, unilateral lesion of the substantia nigra by injection of the catecholamine selective neurotoxin 6-hydroxydopamine (6OHDA, 3 μg). Using established methods (Parish et al., 2001), this dose resulted in lesions of the midbrain dopamine neurons ranging from 60 to 80% (data not shown). In brief, all surgeries were performed under general anesthetic using 2% isoflurane inhalation (Baxter; Deerfield, IL, USA). Mice subsequently received analgesia (Meloxicam, 3 mg/mL) and were placed into a stereotaxic frame (Kopf). Using a 10 μL Hamilton syringe fitted with a fine pulled glass cannula, toxin was injected into the midbrain at the coordinates: anterior–posterior (AP): −3.2 mm and medio-lateral (ML): 1.4 mm and dorso-ventral (DV): −4.5 mm, relative to bregma and the dura of the brain.

2.2. Cell transplantation & administration of chondroitinase ABC

Mice expressing green fluorescent protein under the tyrosine hydroxylase promoter (TH-GFP, (Sawamoto et al., 2001)) were used for the isolation of ventral midbrain fetal donor tissue. Animals were time mated overnight and the presence of a vaginal plug the following morning was classified as embryonic day 0.5 (E0.5). At E12.5, pregnant females were killed by cervical dislocation, and the TH-GFP+ embryos harvested. The ventral mesencephalon was microdissected from each embryo and enzymatically dissociated into a single cell preparation using previously described methods (Dunnett and Bjorklund, 1997; Parish and Thompson, 2012). Cells were resuspended at a density of 100,000 cells/μL in magnesium and calcium-free Hank's buffered salt solution containing 0.1% DNase and stored on ice until the time of grafting. Three weeks after lesioning, host animals received a single (1 μL) intranigral injection of the E12.5 TH-GFP VM cell suspension into the same site as lesioning.

At the time of grafting animals additionally received a 1 μL injection of either high-purity, protease-free chondroitinase ABC (ChABC, from *Protus vulgaris*, Seikagaku Corporation, Tokyo, Japan; 0.05 units, pH to 8.0) or control bacterial enzyme (Penicillinase, from *Bacillus cereus*, 1 mg/mL matched for protein content, Sigma-Aldrich, USA). Enzymes were injected into the medial forebrain bundle of grafted mice at the following coordinates: AP: −2.3 mm, ML: −1.1 mm, DV: −4.6 mm. Six additional animals (three receiving ChABC and 3 administered Penicillinase) were killed at 7 days after enzyme administration to confirm activity of the enzymes. All other animals were euthanized at 12 weeks after cell grafting.

2.3. Tissue processing and immunohistochemistry

All animals received a lethal overdose of sodium pentobarbitone (100 mg/kg) and transcardially perfused with warmed (37 °C) phosphate buffered saline (PBS) followed by chilled fixative (4% paraformaldehyde (PFA) in 0.1 M phosphate buffer). The brains were removed and post-fixed for a further 2 h in 4% PFA before overnight cryoprotection in sucrose (20% *w/v* in 0.1 M phosphate buffered saline). The brains were sectioned horizontally on a freezing-stage microtome (Leica, Wetzlar, Germany), with 12 series collected at a thickness of 40 μm.

Immunohistochemical procedures were performed as previously described (Bye et al., 2012). In brief, free-floating sections were quenched (0.3% H_2O_2/methanol in PBS) for 20mins, washed (PBS) and were then incubated overnight, at room temperature, in a PBS solution containing the primary antibody diluted in 5% donkey serum and 0.3% Triton-X-100 (Ameresco, USA). For fluorescent staining, no quenching was required and sections were washed (3 × 5 min in PBS) and directly incubated in primary antibodies. The primary antibodies and dilutions were as follows: rabbit anti-green fluorescent protein (GFP, 1:20,000, Abcam), chicken anti-GFP (1:1000, Abcam), rabbit anti-tyrosine hydroxylase (TH, 1:500, PelFreez), biotinylated Wisteria Floribunda (bio-WFA, 1:200, Sigma) and mouse anti-2B6 (1:500–1:1000; Seikagaku Corporation, Tokyo, Japan).

The following day, the tissue was washed (PBS, 3 × 5 min) and incubated for 2 h in secondary antibody diluted in 2% donkey serum and 0.3% Triton-X-100. The primary–secondary complex was visualized by peroxidase driven precipitation of di-amino-benzidine (DAB). The secondary antibodies for the DAB-based detection were biotin-conjugated donkey anti-rabbit and donkey anti-mouse (1:200, Jackson ImmunoResearch). Both were then conjugated with streptavidin-HRP using the Vectastain ABC Elitekit (Vector Laboratories, USA). Secondary antibodies for direct detection were used at a dilution of 1:200—DyLight 488, 549 or 649 conjugated donkey anti-mouse, anti-chicken or anti-rabbit (Jackson ImmunoResearch) and Streptavidin-649 (1:200, Jackson ImmunoResearch). DAB-labeled slides were dehydrated in a series of alcohol baths and xylene, and were coverslipped using