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RECOVERY OF FUNCTIONAL DEFICITS FOLLOWING EARLY DONOR AGE VENTRAL MESENCEPHALIC GRAFTS IN A RAT MODEL OF PARKINSON'S DISEASE

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Abstract—It has previously been reported that dopaminergic grafts derived from early donor age, embryonic age 12-day-old (E12) rat embryos produced a fivefold greater yield of dopamine neurons than those derived from conventional E14 donors. The present study addresses whether E12 grafts are able to ameliorate lesion-induced behavioral deficits to the same extent as E14 grafts. In a unilateral rat model of Parkinson's disease, animals received grafts derived from either E12 or E14 donor embryos, dispersed at four sites in the lesioned striatum. Both E12 and E14 grafts were able to induce recovery on both amphetamine and apomorphine rotation tests, and to ameliorate deficits in the cylinder, stepping test, and corridor tests, but were unable to restore function in the paw reaching task. E12 grafts were equivalent to E14 grafts in their effects on lesion-induced deficits. However, E12 grafts resulted in cell yields greater than previously reported for untreated primary tissue, with mean TH-positive cell counts in excess of 25,000 neurons, compared with E14 TH cell counts of 4000–5000 cells, representing survival rates of 75% and 12.5%, respectively, based on the expected adult complement. The equivalence of graft induced behavioral recovery between the two graft groups is attributed to a threshold number of cells, above which no further improvement is seen. Such high dopamine cell survival rates should mean that multiple, functioning grafts can be derived from a single embryonic donor, and if similar yields could be obtained from human tissues then the goal of one embryo per patient would be achieved. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neural transplantation, dopamine grafts, donor age, functional recovery.

The poor survival of embryonic dopamine (DA) cell grafts has greatly hampered the use of transplantation as a therapy for Parkinson's disease (PD). In animal models, typically only 5–10% of implanted DA neurons survive in the host brain (Brundin et al., 2000). In human clinical trials the survival rate is similarly poor (Kordower et al., 1997; Lindvall et al., 1994; Olanow et al., 1996), such that, as many as five or six embryos per patient, per side of the brain may be required for optimal treatment. Clearly, if DA cell survival rates could be improved to 50% or more, transplants using a single embryo per patient might be possible and this would represent a considerable advance for the potential use of DA cell grafts as a therapy for PD.

In a recent study, we reported that ventral mesencephalic (VM) grafts derived from embryonic age 12-day-old (E12) rat embryos yielded fivefold greater numbers of DA cells than equivalent E14-derived grafts, and were able to produce similar amelioration of the amphetamine-induced rotational deficit (Torres et al., 2007). The question remains as to whether such grafts are capable of inducing recovery of more complex behavioral, lesion-induced deficits. In the present experiment, E12-grafted rats were compared directly to E14-grafted rats in a range of behavioral tasks. Previous experiments, primarily aimed at an assessment of cell survival, employed single striatal placements of VM tissue. However, in the present experiment, where the issue of graft function was under investigation, VM tissue was dispersed over four striatal sites, in order to obtain a more widespread reinnervation of the DA-depleted striatum.

EXPERIMENTAL PROCEDURES

Experimental animals

All experiments were conducted under license, following ethical review, and in accordance with requirements of international and the UK Animal (Scientific Procedures) Act 1986. All efforts were made to minimize the number of animals used and their suffering. Four commercially-obtained pregnant dams were used as the source of donor embryos. Pregnant dams were supplied by Charles River (Margate, Kent, UK) using a mating protocol that enabled the time of mating to be accurately established. Briefly, all animals were housed under standard conditions with free access to food and water under standard 12-h light/dark (7 a.m. to 7 p.m.) lighting. On the day of pairing, 60 to 70-day-old females were separated from the colony and paired with stud males at a male: female ratio of 1:1, between 7.00 a.m. and 9.00 a.m. The day of mating was denoted as day 0 for staging purposes. After pairing, females were checked for the presence of a vaginal plug. Plugged females were transported to the laboratory 7 days later (aged E7). Embryos were harvested 12 or 14 days after mating at 9.00 a.m. to obtain precisely aged tissue for transplantation.

Twenty-four adult female Sprague–Dawley rats weighing 200–250 g at the time of first surgery served as graft recipients, or lesion-only controls. Animals were housed under standard conditions with free access to food and water. Following completion of

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Abbreviations: ANOVA, analysis of variance; bFGF, fibroblast growth factor beta; CRL, crown to rump length; DA, dopamine; EDTA, ethylene-diamine-tetraacetic acid; E12, embryonic age 12-day-old; GDF5, growth/differentiation factor five; GDNF, glial derived neurotrophic factor; PBS, phosphate-buffered saline; PD, Parkinson's disease; Shh, sonic hedgehog; TH, tyrosine hydroxylase; TNS, Tris non-saline buffer; VM, ventral mesencephalon/mesencephalic; 6-OHDA, 6-hydroxydopamine.

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post-graft testing for drug-induced rotation, stepping and cylinder tasks, two animals, one in the E12 graft group and one in the E14 graft were below the body weight threshold for food deprivation, and so were not included in the post-graft corridor and staircase tests.

Surgery

Surgery was performed under gaseous anesthesia (2–3% isoflurane in 2:1 oxygen/nitrous oxide). Animals were placed in a stereotaxic frame and cannula placements determined using the coordinates of Paxinos and Watson (2003). All stereotaxic coordinates are given in millimeters, with anterio-posterior and lateral measured from bregma and ventral from dura. Following each surgical session, animals were given an s.c. injection of 5 ml sterile saline containing 5% glucose saline, and paracetamol analgesia and (1 mg/ml) via the water supply for 2 days post-operatively.

Lesion surgery

DA lesions were carried out by unilateral injection of 6-hydroxydopamine (6-OHDA), hydroxydopamine hydrobromide (Sigma, Poole, UK) into the median forebrain bundle using a 30-gauge cannula, connected via fine polyethylene tubing to a 10 μl Hamilton syringe, positioned in a microdrive pump set to deliver at 1 μl/min. The toxin was used at a concentration of 3 μg/μl (calculated as the free base weight) dissolved in a solution of 0.2 mg/ml ascorbic acid in 0.9% sterile saline. The stereotaxic coordinates used for injection were: A=−4.4, L=−1.0, V=−7.8 (below dura), with the nose bar set at −2.3 mm below the interaural line. Injections were carried out over 3 min with 3 min allowed for diffusion of the toxin into the surrounding striatum, before withdrawal of the cannula, cleaning and suturing of the wound.

Dopaminergic grafts

DA cell grafting was carried out 8 weeks post-lesion, following completion of rotational testing. To control potential variability between suspension preparations, grafts in each donor age group were derived from two cell suspensions prepared from two separate litters. VM grafts were prepared from either E12 or E14 embryonic donors and prepared as a cell suspension according to a standard protocol (Dunnett and Bjorklund, 1997). The final cell suspensions were diluted to one VM/4 μl (groups 1 and 2), and aliquoted into Eppendorf tubes, each containing 4 μl and kept on ice prior to use. This technique avoids the repeated re-suspension of the cells each time the injection syringe is loaded, a method which we have show previously has a beneficial effect in extending the viability of the cells (Dunnett et al., 2006). Grafted animals received 4 μl of cell suspension delivered via four 30-gauge cannulae attached to 10 μl Hamilton syringes, driven by an electronic syringe pump. The cannulae were 1 mm apart in a purpose made holder. Holes were drilled in the skull using a 0.5 mm diameter drill bit at the following coordinates: AP +1.3, ML −2.7; AP +0.4, ML −3.1; AP −0.4, ML −4.3; AP −1.3, ML −4.7. The cannulae were then lowered into the brain to a depth of −5.0 mm below dura. One microliter of cell suspension was dispensed from each cannula at a rate of 1 μl per minute, with 0.5 μl deposited at each of two depths: V=−4.5, and V=−4.0. After injection of the graft material, the cannulae were left in place for 3 min to allow the diffusion of injected material, prior to careful removal of the cannulae from the brain.

Behavioral testing

All behavioral testing was conducted blind to the treatment of the rats.

Drug-induced rotation

Rotational testing under the influence of the DA agonist methamphetamine was carried out 2 weeks and 4 weeks after lesioning to obtain an estimate of the extent of DA depletion in each animal. Rotation was assessed using an automated rotometer system based on the design of Ungerstedt and Arbuthnott (1970). Rotation test scores were accumulated over a 90-min test session following 2.5 mg/kg i.p. injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline, Sigma). The collected data are reported as net ipsilateral scores (i.e. ipsilateral minus contralateral counts) over each test session. Only rats with post-lesion net rotation scores of ≥600 turns per session were used in the experiment. Grafted rats were tested 4 weeks and 6 weeks post-implantation using the same method.

As an index of the hypersensitivity of the lesioned striatum, apomorphine-induced rotation tests were carried out in the same apparatus 5 weeks after lesioning and again 12 weeks post-grafting. Rotation counts were collected over 60 min following injection of 0.05 mg/kg apomorphine hydrobromide s.c. in the neck. The collected data are reported as net contralateral scores (contralateral minus ipsilateral counts) over each session.

Stepping test

Forelimb akinesia was assessed using the stepping test (Olsson et al., 1995; Schallert et al., 1992). This involved restraining one forelimb and counting the number of adjusting steps made by the unrestrained forelimb when a rat was moved sideways along a table surface for 90 cm in either a medial or lateral direction with respect to the unrestrained paw. Stepping data were collected in a single post-lesion and a single post-graft session. The data presented are the number of adjusting steps made with the contralateral paw.

Cylinder test

Forelimb asymmetry was measured using the cylinder test (Schallert and Tillerson, 2000). The rat was placed into a clear cylinder for 5 min and the number of times it touched the side walls with either its ipsilateral or contralateral paw was counted. Cylinder data were collected in a single post-lesion and a single post-graft session. Data are expressed in terms of bias toward the contralateral paw calculated as the number of contralateral touches, expressed as a percentage of the total touches made using both paws.

Corridor task

Lateralized response selection was tested using the corridor task which has been described in detail elsewhere (Dowd et al., 2005). Rats were food restricted to 90% of free feeding body weight for testing. For the version used in this study, containers for sugar pellets (45 mg) were arranged as 14 adjacent pairs along the floor of a long narrow corridor. Rats were placed individually at one end of the corridor, and allowed to move freely along its length. The number of pellet retrievals the rat made from its ipsilateral and contralateral sides was counted. Trials were terminated when the rat made 20 retrievals or when the maximum trial time of 5 min had elapsed. Corridor data were collected in a single post-lesion and a single post-graft session. Data were expressed as percent ipsilateral bias, i.e. the number of retrievals the rat made from its ipsilateral side expressed as a percentage of total retrievals made.

Staircase task

Lateralized paw dexterity was measured in the staircase test. Rats were food restricted to 90% of free-feeding body weight for testing. For this version of the task, the seven steps on either side were each baited with two sugar pellets (45 mg), and the rats placed in the box for 5 min. The number of successful retrievals was re-

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