

Interactive report

Transplantation of nasal olfactory tissue promotes partial recovery in paraplegic adult rats[☆]

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Abstract

Recent reports have highlighted the potential therapeutic role of olfactory ensheathing cells for repair of spinal cord injuries. Previously ensheathing cells collected from the olfactory bulbs within the skull were used. In humans a source of these cells for autologous therapy lies in the nasal mucosa where they accompany the axons of the olfactory neurons. The aim of the present study was to test the therapeutic potential of nasal olfactory ensheathing cells for spinal cord repair. Olfactory ensheathing cells cultured from the olfactory lamina propria or pieces of lamina propria from the olfactory mucosa were transplanted into the transected spinal cord. Three to ten weeks later these animals partially recovered movement of their hind limbs and joints which was abolished by a second spinal cord transection. Control rats, receiving collagen matrix, respiratory lamina propria or culture medium, did not recover hind limb movement. Recovery of movement was associated with recovery of spinal reflex circuitry, assessed using the rate-sensitive depression of the H-reflex from an interosseous muscle. Histological analysis of spinal cords grafted with olfactory tissue demonstrated nerve fibres passing through the transection site, serotonin-positive fibres in the spinal cord distal to the transection site, and retrograde labelling of brainstem raphe and gigantocellularis neurons from injections into the distal cord, indicating regeneration of descending pathways. Thus, olfactory lamina propria transplantation promoted partial restoration of function after relatively short recovery periods. This study is particularly significant because it suggests an accessible source of tissue for autologous grafting in human paraplegia. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Development and regeneration

Topic: Transplantation

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1. Introduction

The failure of axons to regenerate in the adult central nervous system has been attributed to a variety of factors including a non-permissive environment such as the glial scar, the presence of inhibitory factors and the lack of trophic support [29,39,54,56]. Several strategies have been

developed to rebuild the injured spinal cord in animals such as adding growth factors [21,60]; providing bridges of embryonic tissue [13,51], artificial implants [12,31] or peripheral nerves [8,11]; and transplanting Schwann cells [35,42,61] or macrophages [50].

Several recent studies have highlighted the potential therapeutic role of olfactory ensheathing cells for the repair of cord injuries [3,24,30,33,34,48,49]. In all these experiments, ensheathing cells were extracted from the olfactory bulb, often from the embryo. For human spinal therapy there are major ethical and technical problems associated with the use of embryonic tissue or tissue from the brains of adult donors. These difficulties would be avoided if the olfactory ensheathing cells could be sourced from the

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olfactory lamina propria in the nose. This tissue is accessible via a simple biopsy through the external nares [17]. Other advantages of using olfactory lamina propria are first, that it would allow autologous transplantation and second, it would provide a cellular bridge to join the gap between the cut ends of the transected spinal cord, thus avoiding the need for artificial bridge. The aim of the present study was to test whether grafts of olfactory lamina propria from the nose can promote repair of the transected adult spinal cord. We assessed the functional recovery of the animals using the well established BBB scoring system [4] and the reflex excitability of the cord below the transection, using the rate-sensitive depression of the H-reflex [55,58]. In addition we used histology, neuronal tracing and immunohistochemistry to provide anatomical evidence of regeneration across the transection site.

2. Materials and methods

Female Sprague–Dawley rats (BRC, University of New South Wales, Sydney, Australia), 250–300 g in body weight, were anaesthetised with ketamine/rompun mixture (90/10 mg/kg, intraperitoneally). Rectal temperature was maintained with a heating pad at $37 \pm 0.5^\circ\text{C}$. The skin and muscle of the back were incised to expose the T10 vertebra. A T10 laminectomy was completed using a microsurgery bone rongeur without touching the spinal cord. The spinal cord was completely transected using microscissors, causing the two stumps to separate and creating a 1–2 mm gap. The spinal cord transection was confirmed by passing a sharp scalpel through the lesion site several times, until residual fibres in the lateral recesses were surely cut and the ventral wall of the spinal canal was visible under the surgical microscope. Bupivacaine (0.5% Delta West Pty Ltd, Bentley, Western Australia) was used locally at the incision during the surgery, and Carprofen (5 mg/kg, Biopharm Australia Pty Ltd, Sydney, Australia) was given subcutaneously post-operatively. Three animals with transplants of olfactory lamina propria were retranssected 10 weeks after the original transection. The second transection was made through the scar tissue at T10, completely recutting the cord, and the animals allowed to survive a further 3 weeks. All experimental protocols and procedures for this study were approved by the Animal Ethics Committees of the University of New South Wales and Griffith University and in accordance with guidelines of the National Health and Medical Research Council of Australia.

2.1. Dissection and preparation of lamina propria

Adult Sprague–Dawley rats (Central Animal Breeding House, University of Queensland) were deeply anaesthetised with sodium pentobarbital (100 mg/kg) and killed by decapitation. The nasal septum was freed by removal of

the lower jaw, the upper teeth and the turbinates. The two olfactory mucosae lining the posterior part of the nasal septum and two similarly-sized pieces of the respiratory mucosa lining the anterior part of the septum were dissected and immediately placed in ice-cold Dulbecco's Modified Eagle Medium (DMEM, Gibco, Mulgrave, Queensland, Australia). The olfactory mucosa is easily identified in the rat by its posterior position on the nasal septum and by the yellowish appearance of the epithelial surface. Care was taken to avoid the anterior edge of the olfactory mucosa which could be contaminated with respiratory epithelium. Olfactory mucosa was always taken caudal to the bony septum (Fig. 1A) all of which is olfactory [40]. Respiratory mucosa was removed from the dorso-anterior region of the septum (Fig. 1A). The lamina propria beneath the olfactory epithelium contains olfactory nerve bundles surrounded by olfactory ensheathing cells which can be identified immunochemically with an antibody to p75^{NGFR} (low affinity nerve growth factor receptor; (Fig. 1B) [20]. The respiratory lamina propria is thinner than olfactory epithelium and devoid of olfactory ensheathing cells (Fig. 1C). The vomeronasal nerve courses ventrally in the respiratory lamina propria and was avoided in the transplanted respiratory tissue. The respiratory and the olfactory tissues were incubated separately for 45 min at 37°C in a 2.4 units/ml dispase II solution (Boehringer, Castle Hill, NSW, Australia) [15] and, in both cases, laminae propriae were carefully separated from the epithelium under the dissection microscope with a microspatula. This method separates the olfactory epithelium from the olfactory lamina propria without contamination of one with the other [15].

2.2. Culture of olfactory ensheathing cells

Olfactory laminae propriae were separated from the olfactory epithelium as described above. Pieces of olfactory lamina propria were then incubated in a 0.25 mg/ml collagenase I solution (Sigma, Castle Hill, NSW, Australia) for 10 min at 37°C , mechanically dissociated and the enzymatic activity was stopped using a 0.5 mM EDTA solution (Gibco). After centrifugation (5 min \times 300 g), the cell pellet was resuspended in DMEM-Ham culture medium containing 10% fetal calf serum and gentamicin (50 mg/ml). Cells were plated onto poly-L-lysine (50 mg/ml) (Sigma) pretreated flasks (Nunc, Underwood, QLD, Australia), at a density of 50 cells per mm^2 . Seven days after plating, cells were detached by trypsin treatment (0.25% w/v) without purification and concentrated by centrifugation just prior to grafting into spinal cord at a density of 100 000/ml.

A small aliquot of each culture was collected, replated and immunostained 2 days after with anti-GFAP and anti-p75 antibodies in order to evaluate the percentage of glial cells and with anti- β -tubulin Type III and anti-keratin to determine the degree of contamination of the cultures

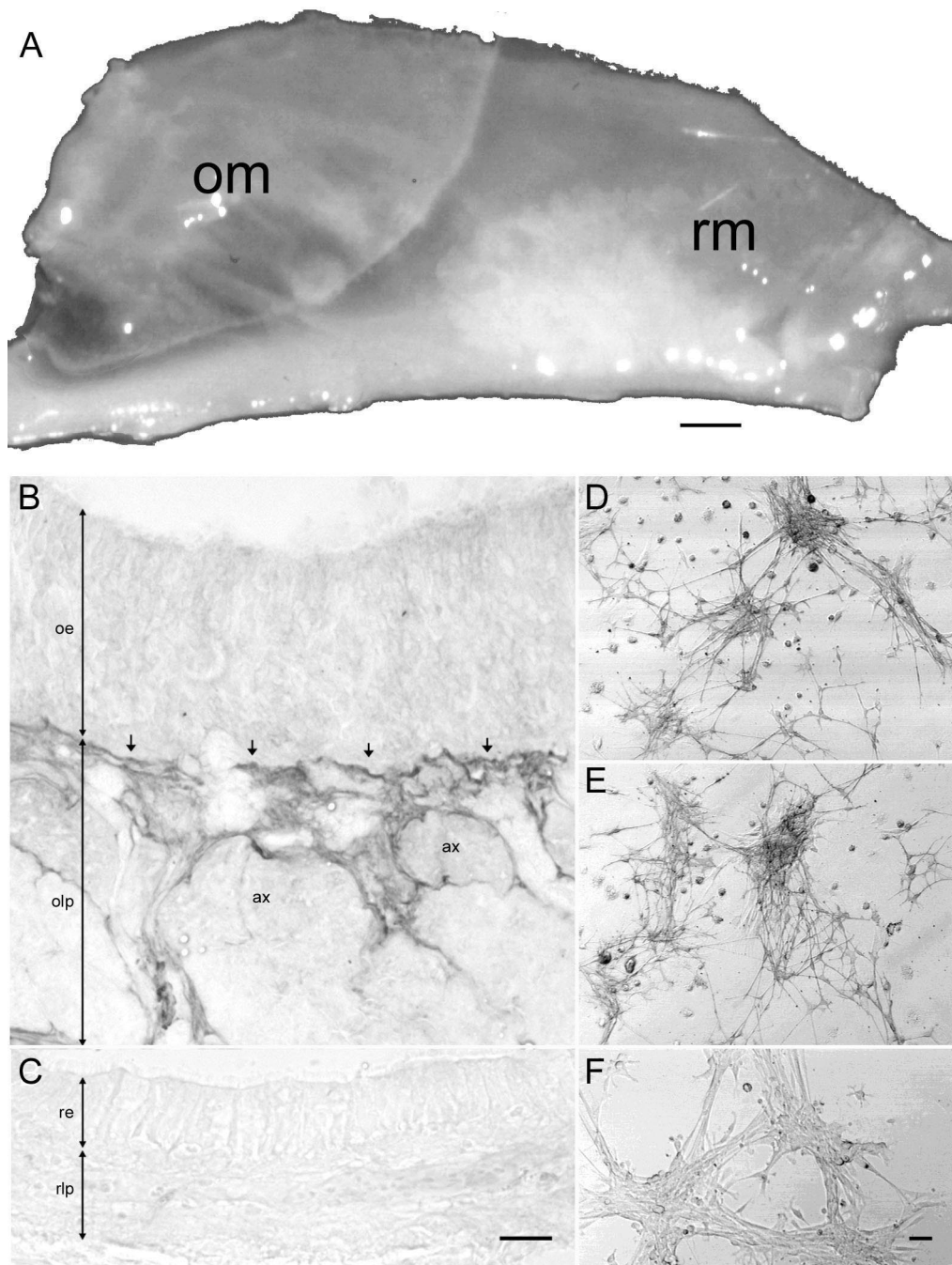


Fig. 1. Olfactory nasal transplants contain ensheathing cells. (A) A photograph of the nasal septum of a rat indicating the locations of the olfactory mucosa (om) and the respiratory mucosa (rm). Caudal is to the left, dorsal is up. Olfactory mucosa was taken from the region posterior to the semicircular line defined by the bony septum. Respiratory mucosa was taken well rostral to this line in the region indicated by the label. Scale bar=2 mm. (B) Section through the olfactory mucosa demonstrating p75^{NGFR} immunoreactivity in the lamina propria. The olfactory epithelium (oe) is indicated by the upper double arrow. The olfactory lamina propria (olp) is indicated by the lower double arrow. Anti-p75^{NGFR} immunoreactivity (dark staining) indicates the olfactory ensheathing cells which surround the bundles of olfactory sensory neuron axons (ax). The ensheathing cells associate with individual olfactory nerve fibres as they enter the lamina propria beneath the basement membrane (small arrows) (C) Respiratory lamina propria contains no p75^{NGFR} immunoreactivity. The respiratory epithelium (re) is indicated by the upper double arrow. The respiratory lamina propria (rlp) is indicated by the lower double arrow. The respiratory epithelium and lamina propria is thinner than the corresponding olfactory tissues, due in large part to the absence receptor cells and olfactory nerve bundles. Bar=20 μ m. (D and E) Photographs showing anti-GFAP (D) and anti-p75^{NGFR} (E) immunoreactivity (dark staining) indicating the olfactory ensheathing cells in a culture before transplantation. F: An immunocytochemistry control culture in which no primary antibody was added. (D), (E) and (F) are taken using Hoffman modulation contrast optics to visualise the unstained cells. Bar (in F)=50 μ m for (D), (E) and (F).

with cells from the olfactory epithelium. Cultures were fixed in ethanol/acetic acid (5 min, -20°C) or in 4% paraformaldehyde in phosphate buffered saline (0.1 M PBS; pH, 7.4, 10 min, room temperature), depending on the antigen. Antibodies specific for epithelioid and neuronal cells were a polyclonal wide spectrum anti-keratin antibody (1:1000; Dako Z622) and a monoclonal anti- β -tubulin isotype III antibody (1:100, Sigma). Antibodies specific for glial cells were a polyclonal anti-glial fibrillary acidic protein antibody (anti-GFAP, 1:500, Dako) and a monoclonal anti-low affinity receptor for NGF (anti-p75, 1:100, Neobody, Flinders University, South Australia). Appropriate non-immune serum was used as control in place of each primary antibody. Endogenous peroxidase activity was quenched by incubating sections and cultures in 0.3% fresh H_2O_2 in PBS for 30 min. Non-specific staining was blocked by incubation for 1 h with non-immune serum, appropriate for the secondary antibody, at a dilution of 1:10 in PBS containing 2% bovine serum albumin. Each well was then incubated for 1 h at room temperature with the same blocking solution containing one of the primary antibodies, washed three times in PBS, incubated for 1 h at room temperature with an appropriate horseradish peroxidase conjugated secondary antibody (1:200, Biorad), washed three times in PBS and incubated for 10 min in Tris-HCl (0.05 M, pH 7.6) containing the chromogen diaminobenzidine (DAB, 0.05%) and H_2O_2 (0.004%).

The percentage of GFAP- or p75-positive cells ranged from 45% to 50% (Fig. 1D and E). There was no contamination by olfactory epithelial cells as judged by anti-keratin and anti-tubulin immunostaining (not shown but similar to the no-primary antibody control culture in Fig. 1F).

2.3. Transplantation of lamina propria and olfactory ensheathing cells

Before transplantation, laminae propriae were cut into pieces approximately $0.5\text{--}1\text{ mm}^2$. Four to six pieces of olfactory lamina propria and 6–8 pieces of respiratory lamina propria were gently inserted to fill the gap between the cut ends of the spinal cord. A piece of Gelfoam was applied dorsally to cover the transplants. Ten animals received transplants of olfactory lamina propria, six control animals received transplants of respiratory lamina propria and four controls received rat tail collagen matrix (Sigma, St. Louis, MO) without any cellular components. The wound was sealed by suturing the muscle and skin overlaying the exposed spine.

Nine rats with transected spinal cords were injected with the cultured olfactory ensheathing cells into three midline sites of each rostral and caudal spinal cord stump (ventral funiculus, ventral grey commissure and dorsal column). All injections were made 2 mm from the transection edges with the aid of a sterile 1 μml Hamilton syringe attached

to a micromanipulator. Each cord received approximately 100 000 cells in a total of 1 μml . A piece of Gelfoam or rat tail collagen matrix was soaked with olfactory ensheathing cell suspension and inserted into the transection site to physically bridge the gap. Nine control animals whose spinal cords were transected received the equivalent injections of medium without olfactory ensheathing cells and the gap was bridged with Gelfoam or rat tail collagen soaked in medium alone. Before grafting, laminae propriae and olfactory ensheathing cells were incubated in a 10 mM solution of Cell-Tracker Green (Molecular Probes, Eugene, OR) for 2 h at 37°C .

2.4. Postoperative care

After surgery rats were immediately placed upon a homeothermic blanket, and routinely kept under heating lamps for the first week. Urine was expressed twice daily for 10–14 days by which time the bladder was no longer distended and palpable and an autonomous bladder voidance reflex had developed. Normotonic sodium chloride (0.09%, 10 ml) was administered subcutaneously twice daily after each bladder expression on the first 3 postoperative days. To prevent urinary tract infection, prophylactic antibiotics were administered (Keflin 25 mg/kg, Eli Lilly, Sydney, Australia) twice daily for the first 2 days and then 10 mg/kg twice daily until the animal voided its own urine. Inspection for skin irritation or decubitus ulcers or evidence of autophagia, was carried out daily. Autophagia was only a problem in one animal, which was euthanised at 3 days postoperatively and not included in this study.

2.5. Behavioural assessment

The BBB scale is an operationally defined 21-point scale, designed to assess hind limb locomotor recovery after impact injury to the thoracic cord in rats [4]. This scale categorises combinations of rat hind limb joint movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position, representing sequential recovery stages that rats attain after spinal cord injury. Open-field observations were made on rats placed in a floor area of $150\times 100\text{ cm}$. All rats received bladder expression before open field testing to eliminate behaviours due to bladder fullness. All rats were measured weekly for 8–10 weeks after surgery. Rating of the behaviour was scored simultaneously and independently by two observers who were unaware of the transplantation status of the animal under observation. These two BBB scores were averaged and compared between olfactory transplanted and control groups using two-way ANOVA. For the retranssected rats, BBB scores were assessed 1 day after recutting and at weekly intervals for 3 weeks.

2.6. Physiological assessment of reflexes

Reflex excitability was tested using a modification of the method reported by Skinner et al. [55]. The H-reflex responses to repetitive stimulation at 10 Hz is normally abolished by the second and subsequent stimuli, probably through presynaptic inhibitory mechanisms. However, in transected animals, this normal inhibition is absent, and the H-reflex amplitude remains close to 100% of its control value. We assessed the H-reflex excitability in 6 transected rats 10 weeks after olfactory lamina propria transplants, 6 transected control animals transplanted with respiratory lamina propria 9–10 weeks previously ($n=4$), or with collagen matrix 2–4 weeks before ($n=2$) and 5 normal control rats. Animals were anaesthetised with Ketamine and rompun and body temperature maintained as described above. Electromyographic activity (EMG) in the fourth dorsal interosseus muscle was recorded using a bipolar tungsten electrode, in response to stimulation of the lateral plantar nerve at the ankle. The signal was amplified using a differential amplifier and recorded using the Maclab system (AD Instruments Pty Ltd., Castle Hill, NSW, Australia). Single square wave stimuli (0.5 ms, 5–15 V) were used to elicit the M-wave (direct muscle response) and H-reflex and then trains of 5 stimuli at 10 Hz were delivered at $5\times$ H-reflex threshold. The amplitude of the M-wave was monitored throughout to ensure it remained constant. H-reflex amplitude of the second response was measured from the average of 3 trials and expressed as a percentage of the first response, also averaged over 3 trials. The profiles of subsequent responses (3rd–5th) were used to assess stability of the reflex depression. H-reflex amplitudes in normal, control and olfactory lamina propria-transplanted animals were compared using ANOVA.

2.7. Retrograde labeling of axons crossing the transplantation site

After a survival period of 8–10 week, rats were anaesthetised as described above and the spinal cord was exposed below the lesion at the T11 level. Fluororuby (10% of dextran tetramethylrhodamine; 10 000 M_w ; Molecular Probes Inc.) was injected into the cord at the T11 level, using a Hamilton syringe. Three syringe placements were made, at the midline and 1 mm lateral on each side, to penetrate the dorsal columns and corticospinal tract, and the ventrolateral and dorsolateral funiculi. For each placement, 3 pressure injections of Fluororuby (0.05 μ l each at 1.5 mm, 1 mm and 0.5 mm deep) were made over a period of 3 min. Following a post-injection survival period of 2 to 4 days the rats were anaesthetised as described above and intracardially perfused with heparinised physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4). The spinal cord extending from 5 mm rostral to 5 mm distal to the transection site, together with the brainstem, was removed, post-fixed for 2 h in the

same fixative, cryoprotected in 30% sucrose overnight and prepared for cryo-sectioning. The spinal cord was sectioned longitudinally and the brainstem coronally at 50–100 μ m. Fluorescent tissues were observed with confocal laser microscopy.

2.8. Immunohistochemistry of spinal cords

Following incubation with 5% bovine serum albumin in phosphate buffered saline (PBS) for 30 min, monoclonal antibody to neurofilament 200 kDa (NF, Sigma Co., St. Louis, MO, diluted 1:400 in 0.1 M phosphate-buffered saline, PBS, pH 7.4) was used as a primary antiserum to detect nerve fibres at the lesion site. After 4 h of incubation at room temperature, sections were washed and incubated in secondary antibody (biotinylated horse anti-mouse, Vector Laboratories Inc., diluted 1:200 with PBS plus 0.5% Triton X-100, PBST) for 1 h followed by the Vector ABC procedure for peroxidase staining and visualisation with 3,3'-diaminobenzidine (DAB). The specificity of the immunostaining for neurofilament was verified by omission of primary antibody.

Selected sections were processed for serotonin immunostaining of fibres in the grafting site and the adjacent cord. After the blocking step in 5% normal goat serum, the sections were incubated in primary antibody at 4°C overnight (rabbit, DiaSorin Inc; diluted 1:1000 in PBS). The following day, sections were washed with PBS and incubated with the secondary antibody (biotinylated goat-anti-rabbit IgG, Sigma Co.; diluted 1:200 in PBST) for 1 h. The sections were then reacted with ABC reagent with DAB as chromogen to visualise the 5-HT positive axons. Rat brainstem raphe neurons were used in staining as positive controls for the specificity of the anti-serotonin antibody, and first antibody was omitted for negative controls.

3. Results

3.1. Recovery of locomotor behaviour

Significant functional recovery in hind limb usage occurred in olfactory lamina propria-transplanted animals and olfactory ensheathing cell transplanted animals compared with controls, transplanted with respiratory lamina propria or collagen matrix or with culture medium alone (Fig. 2). Olfactory lamina propria-treated rats developed the ability to sweep with the hind limb, in a motion that involved all three joints. By 10 weeks post-surgery 6 out of 10 animals grafted with olfactory lamina propria achieved a BBB score of 6–8 in one or both legs, with ankle, knee and hip movement and dorsiflexion of the foot (Fig. 2A–E). None of the animals showed coordinated fore and hind limb movements or the ability to bear weight on the hind limbs. The maximal hind limb movement of controls

after 10 weeks was limited to ankle or slight knee movement, with the foot plantar-flexed and dragged behind (BBB score, 0–2; scores in the control animals with respiratory lamina propria or collagen matrix were similar so results from both these groups were pooled).

Transplantation of cultured olfactory ensheathing cells also resulted in significant functional recovery (Fig. 2E). The BBB score of these animals was tested weekly until 8 weeks post-transection by which time 6 out of 9 animals transplanted with olfactory ensheathing cells achieved a BBB score of 6–8 in at least one leg. By contrast, the control animals injected with culture medium alone achieved maximum scores of only 2. The mean BBB score for the best leg was similar in both olfactory transplant groups (lamina propria and ensheathing cells, Fig. 2E). The two control groups were also similar (respiratory lamina propria/rat tail collagen and medium, Fig. 2E). Comparing individuals, the recovery was better than all the controls in 15 of 19 olfactory tissue transplanted animals (Fig. 2E). The mean best BBB scores of the olfactory transplant animals were significantly better than the controls ($F_{1,34}=45.76$, $P<0.001$). Among the olfactory transplant animals (lamina propria and ensheathing cells), improvements could occur in one or both hind limbs, with either side showing movements. When asymmetrical recovery occurred it was not obviously associated with asymmetrical reflex modulation or histological repair (see below), but was generally linked to an asymmetrical posture; most animals lay on one side with the recovered leg uppermost.

The recovery of hind limb movement began to be measurable by 4 weeks in both olfactory transplant groups (Fig. 2F), with continued divergence of the mean BBB scores until the end of testing at 8 weeks (ensheathing cell transplants) and 10 weeks (lamina propria transplants). By week 5 the ensheathing cell transplanted animals showed a higher mean BBB score than the lamina propria transplanted animals and the mean score at 8 weeks was better than for lamina propria transplants at 10 weeks ($F_{1,136}=4.282$, $P=0.04$).

In order to test whether the functional recovery of olfactory transplant animals was dependent on an intact spinal cord, three olfactory lamina propria transplant animals with BBB scores of 4–6 were re-transected at 10 weeks and their functional recovery was assessed. One day after the re-transection neither leg showed any movement (Fig. 2F). Over the subsequent 2 weeks the BBB scores increased to 1–2 then remained stable at this level for a 3rd week.

3.2. Recovery of spinal reflex inhibition

Examples of EMG activity in the 4th dorsal interosseous muscle following stimulation of the lateral plantar nerve stimulation are shown in Fig. 3. In each case the response consists of the M-wave, the EMG elicited by direct stimulation of motor axons, followed by the H-reflex, the

EMG elicited indirectly by stimulation of the sensory axons. In normal animals, stimulation at 10 Hz resulted in a marked reduction in the H-reflex amplitude for the 2nd and subsequent stimuli ($17\pm 6\%$, normalised to 1st response, Fig. 3), as has been noted before [55]. This rate-sensitive depression is absent in transected animals and was not seen here in rats transplanted with respiratory lamina propria ($83\pm 8\%$). However, olfactory lamina propria-transplanted animals showed an intermediate level of reflex depression ($59\pm 20\%$). While there was considerable variability in individual animals, the mean value was significantly different from both normal ($P<0.01$) and transected control rats ($P<0.05$).

3.3. Motor axon regeneration

The olfactory transplants integrated very well into the damaged spinal cord (Fig. 4A). Axons penetrating the graft were identified using anti-neurofilament immunoreactivity and many were seen clearly within the centre of the graft (Fig. 4B) and entering the rostral and caudal spinal cord. Olfactory grafts prelabelled with Cell Tracker Green showed graft cells penetrating into the rostral and caudal spinal cord stumps for up to 3.5 mm away from the site of transplantation and many were still present within the graft 10 weeks after transplantation. Fig. 4C shows Cell Tracker Green labelled cells located 2 mm caudal from the injection site after transplantation of cultured olfactory ensheathing cells. The grafted cells give the appearance of radiating out along defined pathways, perhaps along nerve fibre tracts. Injections of Fluororuby were made into the spinal cord caudal to the graft site. Fluororuby labelled axons could be seen penetrating and crossing the graft site (Fig. 4D).

Fluororuby was retrogradely transported to brainstem motor nuclei across the spinal cord transections after injections into the spinal cord caudal to the olfactory tissue transplant. After olfactory lamina propria grafts labelled cell bodies were observed in the nucleus raphe magnus (Fig. 4E). After cultured olfactory ensheathing cell grafts labelled cell bodies were observed in the nucleus raphe magnus and the nucleus gigantocellularis (Fig. 4F). There were no other labelled cells observed in the rest of the brain at this period after transplantation.

In control spinal cords with grafts of either respiratory lamina propria, collagen matrix or culture medium, there were no neurofilament-positive axons in the graft. Fluororuby labelled axons extended up to the distal edge of the graft but were never observed to penetrate the graft site. No Fluororuby labelled cell bodies were observed in the brainstem. The two animals with olfactory lamina propria transplants which showed no behavioural recovery (BBB score 2) also showed no histological evidence of axonal regeneration.

Serotonergic fibres in the spinal cord arise from brainstem nuclei including raphe nuclei and nucleus gigantocellularis [41,59]. As expected numerous serotonin-immuno-

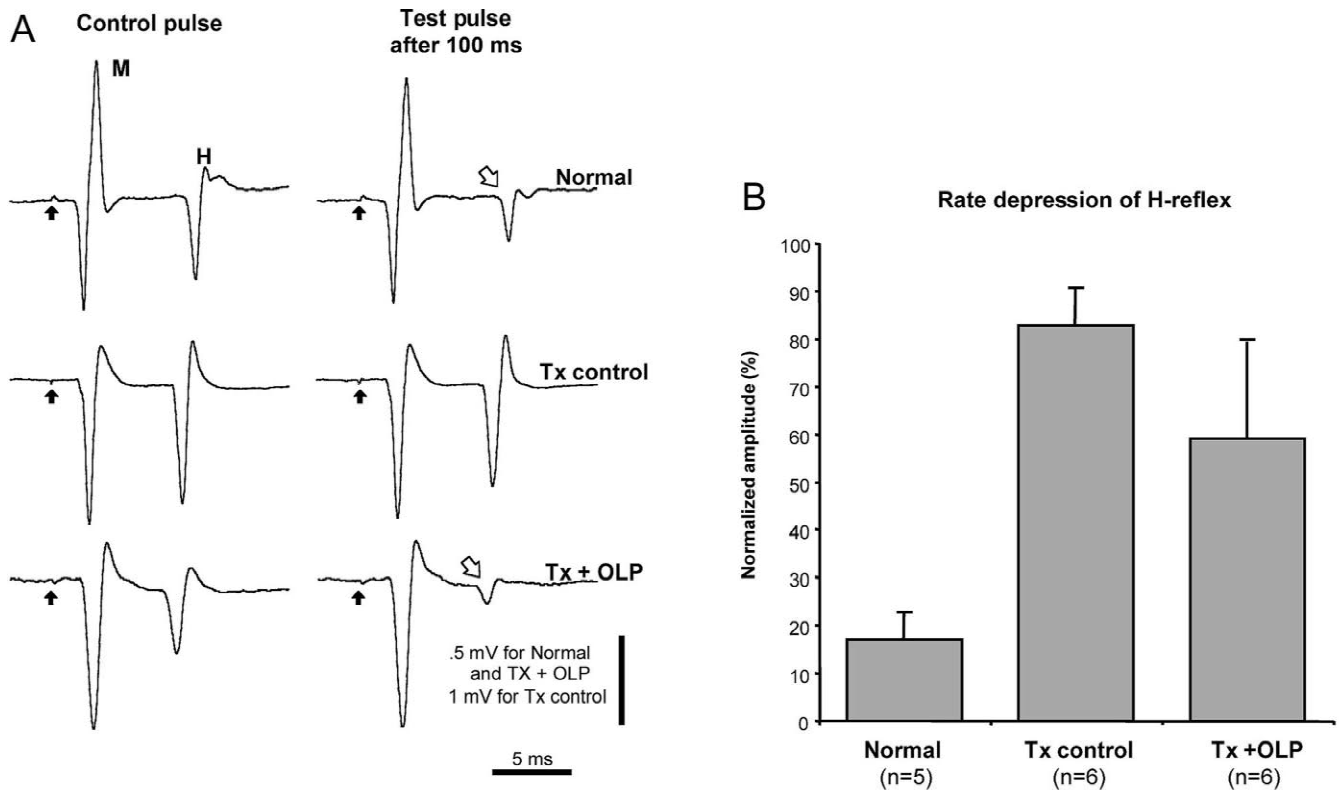


Fig. 3. Functional recovery of descending suppression of spinal reflexes. (A) Traces of single EMG waves recorded from the 4th dorsal interosseous muscle in response to stimulation of the lateral plantar nerve. Upper pair, Normal rat; middle pair from a transected rat transplanted with respiratory lamina propria 10 weeks previously (Tx control); lower pair from a transected rat with an olfactory lamina propria transplant 10 weeks previously (Tx + OLP). The traces on the left are the responses to the first stimulus (Control pulse) and on the right to the second of a train of stimuli at 10 Hz (Test pulse after 100 ms interval). The black arrows indicates the position of the stimulus artefact and in each trace the M-wave (EMG response to stimulation of motor axons) is followed by an H-reflex (reflex response to stimulation of sensory axons). The H-reflex amplitude to the 2nd stimulus is depressed in normal and olfactory lamina propria-transplanted animals (white arrows). (B) Histogram showing the H-reflex amplitude of the 2nd response (mean and S.D., expressed as a percentage of the 1st response amplitude) for normal animals, animals transected with respiratory lamina propria grafts (Tx control) and animals transected with olfactory lamina propria transplants (Tx + OLP). Each group is significantly different from the other 2 groups (normal versus both transected groups, $P < 0.01$; transected control versus olfactory lamina propria-transplant animals, $P < 0.05$).

reactive fibres were observed in the grey and white matter of the spinal cord rostral to both olfactory lamina propria grafts and respiratory lamina propria grafts (Fig. 5A and C). However, only after olfactory transplants were serotonergic fibres seen within the transplant site and within the spinal cord caudal to the graft (Fig. 5D); these fibres were not present in control animals (Fig. 5B). Essentially similar findings were made in the animals transplanted with olfactory lamina propria and with cultured olfactory ensheathing cells. In these animals, serotonergic axons were observed at least 6 mm caudal to the graft. They were mostly present in the grey matter of the ventral cord, and along the border zone between the grey and white matter, but a few were also present within the white matter.

4. Discussion

The present study shows that olfactory nasal cell grafts can promote functional recovery after complete transection of the spinal cord in the adult rat both when transplanted as

whole pieces of olfactory lamina propria and when transplanted as 50% pure cultures of olfactory ensheathing cells. Animals receiving olfactory grafts recovered hind limb movement and spinal reflex inhibition. Histological investigation indicated obvious growth of axons across the transection, through the graft and into the distal cord stump and regrowth of brainstem serotonergic fibres into the distal cord. Re-section of the cord after 10 weeks confirmed that locomotor recovery was dependent on regeneration through the transplant site.

It is most likely that the cells responsible for promoting recovery in our experiments are the olfactory ensheathing cells present in the nasal cavity. First, these cells are present in the lamina propria grafts from olfactory mucosa but not respiratory mucosa. Second, transplantation of cultured nasal olfactory ensheathing cells also promoted functional recovery and re-growth of brainstem axons across the transection. Even at the short survival periods examined here, the rate of functional recovery after ensheathing cell transplantation was better than after lamina propria tissue transplants, further supporting the role of the ensheathing cells in the recovery process. Third,

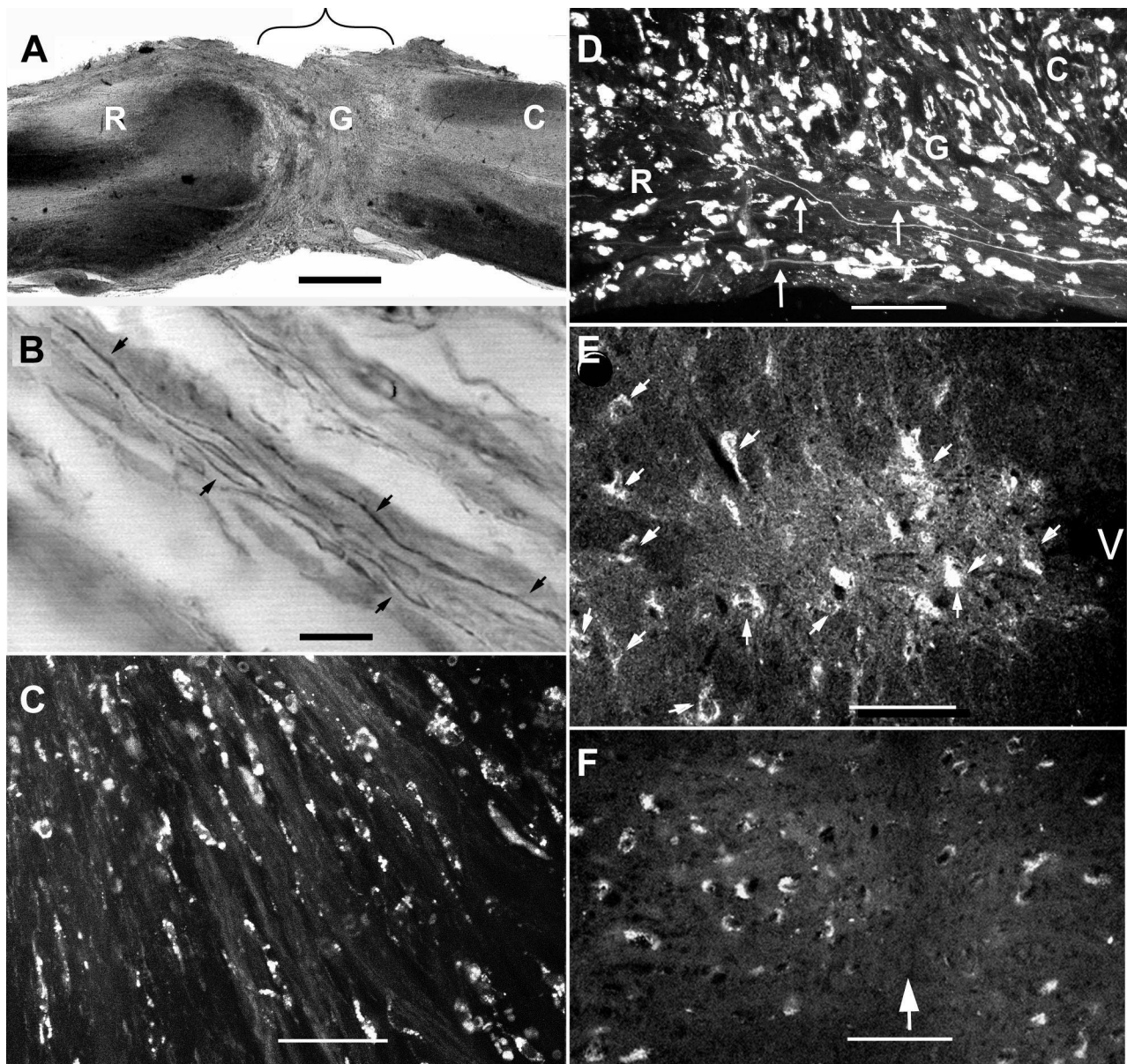


Fig. 4. Regeneration of axons was promoted by olfactory lamina propria tissue and cell grafts. (A) Horizontal section through the graft site in an olfactory lamina propria-transplanted animal. The graft (G) integrated well with the rostral (R) and caudal (C) cord. The region of the grafted tissue is shown by the bracket. (B) High-power view within the centre of the olfactory lamina propria graft showing neurofilament immunoreactivity. At this focal plane many neurofilament-positive axons can be observed (arrows). (C) Cultured olfactory ensheathing cells pre-labelled with Cell Tracker Green labelled located in the spinal cord 2 mm caudal to the injection site, 8 weeks after transplantation. (D) The graft site of an animal transplanted with olfactory lamina propria prelabelled with Cell Tracker Green after injection of Fluororuby into the spinal cord caudal to the graft site. Green fluorescent cell bodies are distributed in the spinal cord rostral spinal cord (R), the graft at the transection site (G) and in the caudal spinal cord (C). The fluorescence filter was adjusted to visualise simultaneously axons labelled with Fluororuby (arrows). Axons were seen traversing the graft and entering the rostral spinal cord. (E) Cell bodies in the nucleus raphe magnus were labelled retrogradely after injection of Fluororuby in the spinal cord caudal to the olfactory lamina propria graft. V marks the ventral edge of the medulla and the small arrows indicate labelled cell bodies. No cells were labelled after injections of Fluororuby caudal to respiratory lamina propria grafts. (F) Cell bodies in the nucleus gigantocellularis were labelled retrogradely after injection of Fluororuby in the spinal cord caudal to the olfactory ensheathing cell graft. The arrow indicates the midline demonstrating cells on both sides. Dorsal is up. No labelled cells were observed in nucleus gigantocellularis in control animals Scale bars: (A) 1 mm; (B–F) 100 μ m.

functional recovery previously reported was achieved with either semi-purified [33] or 98% pure [48] cultures of ensheathing cells from olfactory bulb. A major finding from the present study is that a pure population of ensheathing cells was not necessary for repair of the spinal

cord. This has implications for therapeutic use of these cells in human spinal cord injury because autologous olfactory lamina propria transplantation could take place quickly without a period of ensheathing cell culture and purification.

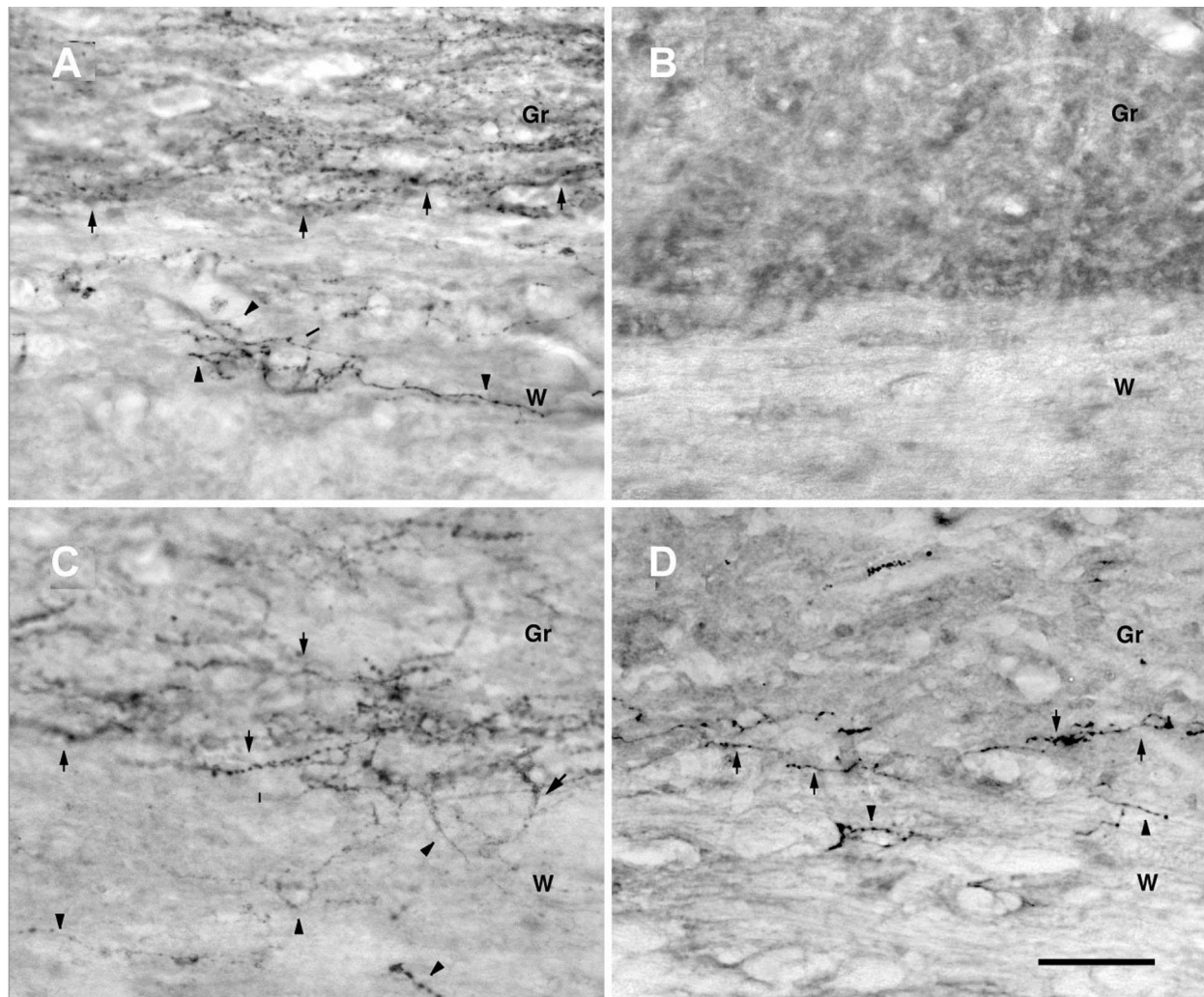


Fig. 5. Serotonergic fibres were present caudal to the olfactory lamina propria graft. (A, C) Horizontal sections through the spinal cord rostral to the graft site after respiratory lamina propria grafts (A) and after olfactory lamina propria grafts (C). Serotonergic axons are evident throughout the grey matter (Gr, arrows) and within the white matter (W, arrowheads). (B, D) Horizontal sections through the spinal cord 3 mm caudal to the graft site after respiratory lamina propria grafts (B) and after olfactory lamina propria transplantation (D) at the border between the grey matter (arrows) and within the white matter (arrowheads). Serotonergic axons were similarly observed in caudal spinal cord after olfactory ensheathing cell transplantation. Scale bar: 50 μm .

The lack of recovery after respiratory lamina propria transplantation rules out other cell types being responsible for the recovery after olfactory lamina propria transplantation. For example, Schwann cells and macrophages can assist spinal cord repair [50,62] but are present in low numbers in both olfactory and respiratory laminae propriae. Fibroblasts and endothelium are also present in both tissues but endothelium is not reported to assist spinal cord regeneration and although genetically engineered fibroblasts can assist regeneration, unmodified fibroblasts do not [37]. Finally, neither the lamina propria transplants nor the cultured olfactory ensheathing cells were contaminated with olfactory epithelium. Lamina propria prepared in this way does not contain olfactory epithelium [15] and the olfactory ensheathing cultures were shown here to contain neither β -tubulin III-positive nor keratin-positive cells,

both of which are found in cultures of olfactory epithelium [15].

4.1. Olfactory ensheathing cells and spinal cord regeneration

The present results confirm and extend recent reports demonstrating that olfactory ensheathing cell transplants can promote spinal cord regeneration in adult rats after complete spinal cord transection [48,49]. We demonstrate here improvement over 19 control animals of locomotor behaviour in 15/19 transplanted animals (79%) after relatively short recovery periods (8–10 weeks). For the first time we demonstrate that behavioural recovery after olfactory ensheathing cell transplantation is abolished by re-transection of the spinal cord. For the first

time we demonstrate that olfactory ensheathing cell transplants promote recovery of descending spinal reflex inhibition. For the first time we identify brainstem motor neurons whose axons regenerated after olfactory ensheathing cell transplants into the transected spinal cord. In support of these observations we demonstrate regeneration of serotonergic axons in the spinal cord caudal to the transection after olfactory ensheathing cell transplantation.

Our observations are based on olfactory ensheathing cells obtained from the nasal olfactory mucosa. Previous studies have used ensheathing cells from the olfactory bulb to promote spinal cord repair after localised cord injury [24,33,34] and after complete spinal transection [48,49]. Ensheathing cells from the human olfactory bulb were shown to assist re-myelination of the rat spinal cord [3,23].

In the present study, the spinal cord of adult rats was completely transected at the T10 level. Although this method of spinal damage is not comparable to most human spinal injury it was chosen as a repeatable and standardised lesion, which allows comparisons with other rat studies [48–50]. Also for comparison with previous work we used the BBB score for measuring hind limb movement after spinal injury [4]. The BBB score has been used before in transection injuries to the thoracic cord of adult rats [50]. The locomotor recovery we observed after nasal olfactory ensheathing cell transplantation is comparable to that obtained 20 weeks after transplantation of activated macrophages [50] and similar to the hind limb movement described by Ramon-Cueto et al. [48] 2 months after transplantation of olfactory bulb ensheathing cells. In the latter study, longer survival times were associated with further improvements and might similarly have been expected here, but were not tested because of restraints imposed by the University animal ethics committees.

The nasal olfactory ensheathing cell transplants integrated into the host spinal cord and prelabelled olfactory cells were still evident around the graft site 10 weeks after transplantation. The ability of transplanted ensheathing cells to migrate into the host spinal cord and myelinate host axons has been noted before [3,23,24,34,49] and is presumably an important component of their therapeutic success. This tendency of olfactory ensheathing cells to migrate towards the central nervous system is illustrated during development [36] and after damage to the olfactory sensory neurons [10]. Similarly, as indicated above, cells migrated from the olfactory transplants to incorporate into the spinal cord both rostral and caudal to the site of the graft.

In the use of olfactory lamina propria one aim was to test whether ‘native’ ensheathing cells could assist recovery from spinal cord injury when transplanted in their normal cellular and extracellular environment, in order to avoid any alteration in phenotype which could result from dissociation, purification and culture *in vitro* before transplantation [49]. Additionally, we did not want to inadvertently select for any specific olfactory ensheathing cell type

which could be present and biased in a cultured sample. For example, olfactory bulb ensheathing cells can be divided into at least two types, with different antigenic and morphological properties [18,46]. The present results suggest that the spinal regenerative properties of olfactory lamina propria ensheathing cells are present before and after culture. Furthermore, there does not appear to be any obvious differences in the ability of these cells despite differences in pre-treatment. The more rapid recovery after transplantation of cultured ensheathing cells is probably due to the higher relative numbers of cells transplanted. The robustness of these properties is important when considering the possibility of using these cells for human transplantation therapy — differences in cell preparation methods may not affect the outcome of transplantation. Thus nasal olfactory ensheathing cells appear to provide an environment which is as permissive for axon regrowth as ensheathing cells isolated from the olfactory bulb.

4.2. Recovery of descending motor pathways

The recovery of movement in the present study is consistent with improvements in reflex function of the distal cord but there was no evidence of recovery of coordinated fore and hind limb movements or voluntary control. Nevertheless, the recovery of hind limb movement seen here was abolished when the spinal cord was re-transected at the site of the transplantation. This demonstrates that locomotor recovery was dependent on descending motor control pathways and not simply due to functional rearrangements in the distal cord. This was also demonstrated after transplantation of activated macrophages [50] but has not been tested before with olfactory ensheathing cells. Other evidence for descending motor pathways was sought functionally, via observation of modulation of spinal reflex pathways, and structurally, via retrograde labelling of descending pathways and via serotonergic reinnervation of the distal cord.

Hyperreflexia is a well known consequence of spinal injury in both animals and humans [6,58]. For example, after contusion injuries, rats showed reduced reflex thresholds, decreased recruitment, decreased post-tetanic potentiation and diminished rate-sensitive depression [57,58]. The reduction in rate-sensitive depression persisted for at least 2 months and was considered to be particularly useful for evaluating reflex excitability. Rate-sensitive depression in the normal cord primarily reflects presynaptic inhibitory mechanisms [38]. Such inhibition is known to be under the influence of descending controls from the cortex and brainstem [1,28,52] and the reduction of rate-sensitive depression after cord injuries is considered to reflect the loss of these descending influences [57,58]. Skinner et al. [55] used this technique to monitor reflex excitability after cord transections and fetal tissue implants. They showed reestablishment of normal levels of rate-sensitive depression 3 months after transection. Similarly, the present

study has shown significant restoration of reflex depression towards normal levels after olfactory lamina propria transplants, indicating partial recovery of descending control mechanisms. The high variability is consistent with the variable functional recovery seen in this group, although restoration of reflex depression did not correlate with BBB scores in an individual animal. It is important to note that the reestablishment of reflex inhibition may reflect reduced spasticity and thus may be clinically, useful even if full functional recovery is not achieved.

It has long been known that the descending serotonergic pathway is involved in the spinal modulation of sensory processing, autonomic function, and motor activity [2,41,53]. Serotonergic spinal projections arise primarily from raphe magnus, obscurus and pallidus and from nucleus gigantocellularis and travel in the dorsolateral, intermediate and ventral funiculi to terminate widely throughout the grey matter [59]. Besides their role in pain transmission, descending serotonergic pathways modulate spinal reflexes, and have been suggested to play a role in locomotor patterns [26,27,41,45]. It is known that complete spinal transection eliminates all serotonergic axons caudal to the transection site [9,43,44]. The findings here of serotonergic axons in both the grey and white matter of the distal cord and of retrogradely labelled raphe magnus and gigantocellularis neurons indicate regeneration within this pathway. Regeneration of serotonergic axons is also seen after transplantation of olfactory bulb ensheathing cells into the transected spinal cord [48,49] and other models of spinal cord repair [5,8,25].

Several workers have implicated serotonergic regeneration in functional locomotor recovery [22,48]. The results here on modulation of rate-sensitive reflex depression indicate descending motor pathways were partially restored. Serotonergic activation modulated group Ia afferents involved in the H-reflex [7,47] as well as group II inputs [45]. However, other pathways may also contribute to the locomotor recovery. For example, activation of the locus coeruleus is known to modulate cord reflexes [27,45] and Ramon-Cueto et al. [48] reported regeneration of corticospinal and coeruleospinal pathways after olfactory ensheathing cell transplants. Thus the identity of the pathway causing rate-sensitive changes in the H-reflex is still uncertain.

4.3. A source of cells for autologous transplantation

In recent years, several researchers have indicated the advantages of olfactory glia over Schwann cells for nerve repair [14,19,39]: They combine properties of peripheral Schwann cells, promoting axon growth, with properties of central glia, being able to live and migrate within the spinal cord. We show here another major advantage of olfactory ensheathing cells, they have the potential to be used in autologous grafts, because they can be removed from the nose and placed into the damaged spinal cord,

with or without culture or purification. The olfactory biopsy procedure is simple and does not affect the sense of smell [16,32]. If applied to human spinal therapy, autologous grafting of olfactory lamina propria could occur at the time of spinal surgery without the need for pre-harvesting and culture of the ensheathing cells.

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