

Partial recovery of proprioception in rats with dorsal root injury following human olfactory bulb cell transplantation

Andrew Collins¹, Daqing Li¹, Modinat Liadi¹, Pawel Tabakow², Wojciech Fortuna²,
Geoffrey Raisman¹, Ying Li¹

¹Spinal Repair Unit, Department of Brain Repair and Rehabilitation, UCL Institute of Neurology, Queen Square, London, UK

²Department of Neurosurgery, Wroclaw Medical University, Wroclaw, Poland.

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Daqing Li: Daqing.Li@ucl.ac.uk ; Tel 020 3448 4480

Modinat Liadi: M.Liadi@ucl.ac.uk ; Tel 020 3448 4480

Pawel Tabakow: p.tabakov@wp.pl ; Tel 48 606 137 846

Wojciech Fortuna: wfortuna@onet.pl ; Tel 48 606 137 846

Geoffrey Raisman: G.Raisman@ucl.ac.uk ; Tel 020 3448 4480

Ying Li: Ying.Li@ucl.ac.uk ; Tel 020 3448 4480

Correspondence: Dr Andrew Collins, Andrew.Collins@ucl.ac.uk, Tel: 020 3448 4480

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ABSTRACT

We transplanted human olfactory bulb OECs (hOECs) mixed with collagen into a unilateral transection of four dorsal roots (C6-T1) in a rat model. By mixing with collagen, we could maximise the limited numbers of hOEC from an olfactory bulb biopsy and optimise cavity-filling. Cyclosporine was administered daily to prevent immune rejection. Forelimb proprioception was assessed weekly in a vertical climb task. Half of the rats receiving hOEC transplants showed some functional improvement ('responders') over six weeks of the study whilst the other half did not ('non-responders') and performed similarly to 'injured only' rats.

Transplanted cells were seen at both 1 week and 6 weeks after surgery; many were concentrated within the lesion cavity but others were found with elongated processes in the overlying connective tissue. There were some fibres in the injury area associated with transplanted cells which were immunostained for neurofilament and TUJ1. We compared responder and non-responder rats with regard to microglial activation within the deep dorsal horn of cervical levels C7,C8 and also axon loss within the cuneate fasciculus at cervical level C3. We saw little difference in microglial activation or axonal loss which could account for the improved proprioception in the 'responders' group.

We believe this preliminary study is the first to transplant human olfactory bulb cells into a rat model of dorsal root injury; by refining each component part of the procedure, we can continue to maximise the repair potential of OECs in a clinical setting.

Key words: spinal roots, glia, rhizotomy, translational model

INTRODUCTION

Injury to the spinal cord can have lifelong and devastating consequences for an individual. Brachial plexus injury, which can result from a road traffic accident, disconnects the spinal roots from the spinal cord and leads to a loss of sensory and motor function and is associated with unbearable pain.¹⁻³ Damage to the spinal roots by stretching or avulsion causes a profound loss of sensation and the current clinical interventions are limited.⁴⁻⁶

Olfactory ensheathing cells (OECs) are specialised glial cells which can be obtained from either olfactory bulb or olfactory mucosa tissue.⁷ Transplantation of OECs has been shown to be effective in a number of different experimental CNS injury models.⁸⁻¹⁰ It has been shown that OECs have the potential to promote axonal regeneration, remyelination, angiogenesis, glial scar remodelling and immune system interactions.¹¹⁻¹⁵ Functional restoration of long-tract injuries to reinstate forepaw grasping, supra-spinal control of breathing or proprioceptive function on a climbing task has also been demonstrated in various rat studies.¹⁶⁻²¹ A trial in which dogs with a thoracolumbar spinal cord injury showed improved limb coordination after olfactory mucosal OEC transplantation also helped lay the foundations for their use in a clinical setting.²²

Clinical trials of transplantation of autologous OECs into spinal cord injuries using the more accessible cells obtained from the olfactory mucosa²³⁻²⁵ showed little or no neurological improvement as shown in animal studies.²⁶⁻²⁸ However, transplantation of autologous cells obtained from the olfactory bulb in a patient with complete thoracic spinal cord injury caused by a transection showed that the patient had better trunk stability, increased muscle mass in the left thigh and calf and some return of voluntary movements and sensation in the lower extremities after two years.²⁹ It should be noted that the precise mechanism underlying these improvements is hard to pinpoint exactly since the procedure also involved spinal cord resection, a dose of methylprednisolone and an extensive rehabilitation programme. The outcome of this clinical application using the cells from the olfactory bulb is consistent with the findings from the animal studies.

During the course of the surgery, Tabakow and colleagues used autologous peripheral nerve strips to span a gap of around 11 mm between the severed spinal cord stumps after injecting cells at multiple sites into the spinal cord rostral and caudal to the lesion.²⁹

In the present study we developed a method to mix the cells with a collagen gel to maximize the usage of the limited number of hOECs from the olfactory bulb biopsy. This cell-collagen scaffold would occupy a cavity and give structural integrity to support cell transplants spanning the lesion. Placing the transplant in a scaffold form would avoid time-consuming multiple injections which may cause further damage to the spinal cord. In addition, this scaffold would enable the transplanted cells to have direct contact with the nerve graft and the spinal cord. In addition, if OECs had a pre-surgery period at which to differentiate, produce neurotrophic factors and elongate their processes, they could more easily integrate within the injured host environment and optimise any potential for repair.^{30, 31}

We first determined whether the cells would survive and differentiate into their typical morphology within collagen. We then tested their function *in vivo* by transplanting the cells mixed with gel into a rat model of brachial plexus injury.

We believe that this current study is the first to examine transplantation of human olfactory bulb OECs in a rat model of brachial plexus injury. Here, we show that the cells: i) survive at the lesion site over six weeks, ii) migrate within and out of the gel, iii) interact with host tissue, iv) induce axonal regeneration and v) partially reinstate proprioception in a four root dorsal root transection injury in rats.

MATERIALS AND METHOD

Obtaining human olfactory bulb tissue

Human olfactory bulbs (OBs) were obtained from 15 patients in the period between October 2013 and March 2017 who had undergone an operation for repair of anterior skull base fracture with cerebrospinal fluid leak or an operation to remove an anterior skull case tumour. The decision for obtaining a patient's olfactory bulb was determined by the clinical and intraoperative evidence of an irreversible lesion of the olfactory bulb or olfactory tract, caused by the skull base fracture or the neoplastic infiltrative process

(Fig 1). The transcranial bifrontal microscopic endoscopy-assisted approach or the endoscopic transnasal approach were used for repair of the skull base fractures. Brain tumours were approached via craniotomy. The removal of OB tissue was approved by the Wroclaw Bioethics Committee and written consent was obtained from all of the patients. The OBs were transported on ice in a medium containing Dulbecco's Modified Eagle's Medium F/12 Nutrient Mix (DMEM/F12, Thermo Fischer) and 10% foetal bovine serum supplemented (FBS, Thermo Fischer) supplemented with 100 U/ml of penicillin and 100ug/ml of streptomycin (PenStrep, Thermo Fischer: DMEM/F). Transportation of the tissue took around 24 hours.

Cell culture

Upon arrival, images of the OBs were taken and their weights recorded. All dissecting and culture protocols were carried out under sterile conditions. After peeling off the adherent meningeal membranes and removing a small piece for histological examination, the OBs were cut open, the outer nerve and glomerular layers of around 1 mm thickness were dissected out and cut into 2 mm² fragments which were then incubated in 1% trypsin in Hank's balanced salt solution (HBSS) at 37°C for 15 min. After the enzymatic reaction was stopped by addition of DMEM/F and then spun down at 250g by centrifugation, the supernatant was discarded and the precipitated cell pellet was re-suspended into DMEM/F. Cells were stained with trypan blue, counted on a cell counter (Countess, Invitrogen) and seeded on poly-D-lysine (PDL, Sigma-Aldrich) coated 35 mm plastic dishes at a density of 1.5-2 x 10⁴ viable cells/cm². The cultures were maintained in a humidified incubator enriched with 5% CO₂ at 37 °C. Culture medium was first replaced on the 5th day and then subsequently changed every 3 days.

Preparation of collagen gel containing hOECs

After 10 days in culture, the cells were transduced with LV-GFP as described previously.³² They were then washed three times with DMED/F12 and incubated in trypsin/EDTA (TE) for 5 min to lift the cells from the surface of the culture dish. After TE activity was inactivated by adding DMEM/F, the cells were collected and triturated into suspension.

The hOEC/collagen gel was made by mixing the cell suspension with collagen, NaOH (Sigma) and modified Eagle's medium (MEM, 10x; Sigma).

We refer to the cultured human olfactory bulb cells within the gel as hOECs throughout this text. This includes fibroblasts and other cell types such as mesenchymal cells that are found in the olfactory biopsies but here we simplify the nomenclature to 'hOEC'.

[Insert Fig 1 around here]

Microsurgery

Dorsal Root Injury Model

Twenty four male Sprague Dawley rats (200-220g at surgery) received a unilateral four root dorsal root transection injury. Under isoflurane anaesthesia, dorsal roots C6, C7, C8 and T1 were exposed as previously described.³³ Briefly, in a posterior approach, a skin incision was made through the dorsal midline, the paraspinal muscles were separated and the prominent T2 process located. Hemi-laminectomies were carried out from C4 to T2 and the dura was incised with microscissors to reveal the dorsal roots. The four dorsal roots of C6, C7, C8 and T1 were then transected with microscissors as close as possible to the spinal cord in a plane approximately perpendicular to their entry into the cord. After haemostasis was achieved, 19 rats received human olfactory bulb cultured cells ('hOECs') within a collagen scaffold. In all cases, the transplant was positioned between the central and peripheral stumps of the cut dorsal roots and held in place with fibrin glue (Tisseel Kit, Baxter, Thetford, UK). Immediately after transplantation, cyclosporine (Novartis, Surrey, UK) made up at 15 mg/ml in 20% Tween 80 solution (Sigma-Aldrich) at a dosage of 6 mg/Kg with 100% ethanol (VWR, Fontenay Sous Bois, France) was delivered intraperitoneally (IP) via Microfine insulin syringes (BD Ltd, Dun Laoghaire, Ireland) and repeated daily.

The remaining 5 rats received the same surgery but no cells were transplanted; cut roots were reapposed and held in place with fibrin glue. With all animals, muscles were repaired in layers, the skin sutured, and the rats were given postoperative pain relief (0.05 mg per kg buprenorphine, s.c. daily for 3 days).

In terms of housing conditions, room temperature was maintained between 20°C and 22°C under standard lighting conditions (12:12 light-dark cycle) with food and water available ad libitum. Animals were handled according to the UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1986 with the ethical approval of the University College London (UCL) Institute of Neurology.

Vertical Climbing Test

On arrival, the rats weighed 150-175g and were acclimated to housing conditions for one week. The following week, they were handled each day and placed on a 1 m near vertical grid (15° inclination) to climb up the rungs to the top. They did this naturally without any need for a reward. Starting one week before surgery and for up to six weeks post-surgery, rats were video-recorded climbing the vertical cage weekly.

In the subsequent slow motion analysis, the forepaw on the injured side was scored for degree of accuracy in locating and grasping the grid bars. As described previously,³³ a grasp was recorded as successful when there was purposeful movement ending in functional full flexion of the digits to grasp the bars. Unsuccessful grasps were graded from 1-4 in increasing order of severity depending on how far the limb protruded through the grid (1, grid level but no grasping; 2, paw protrudes through grid as far as the wrist; 3, as far as the elbow; 4, as far as the axilla.)

After behaviour analysis, rats which received hOEC transplants were separated into non-responder and responder groups based on vertical climbing performance at 2 weeks post-injury - if their % error score was above or below 85%, respectively. This was based on previous studies with rat bulb OEC transplants, where animals which failed to improve by 2 weeks post-surgery did not show any signs of recovery in the rest of the study (Collins A and Li Y, unpublished observations). One rat (which received an hOEC transplant) had to be culled at 3 weeks post-surgery because there was bleeding under the nails of the injured forepaw due to autotomy; spinal cord sections from this animal were not used for immunostaining.

Tissue Processing and Immunohistochemistry

The rats were terminated under deep carbon dioxide anaesthesia and transcardially perfused with 50ml 0.1 M phosphate buffer solution followed by 400ml phosphate buffered with 4% paraformaldehyde for 30min. The vertebral columns were dissected out from the cranio-cervical junction to the upper thoracic level and left to harden in the same fixative for 48 hours. The spinal cord and associated roots were carefully dissected out of the bony skeleton under a dissecting microscope to preserve the continuity across the avulsed dorsal roots and transplants to the spinal cord. The dissected tissues were placed into 10 and 20% sucrose solution until sunk, frozen with cryospray (Cell Path, UK) and 16µm sections were cut on a cryostat (Leica CM3050). Tissue from 23 rats was sectioned at cervical levels C6-T1 (n=15 in the transverse plane; n=8 in the horizontal plane) to check for the presence of transplanted cells and for immunostaining. Of these, 6 rat spinal cords were also sectioned at cervical level C3 for quantification of axon loss. For immunostaining, the sections were incubated in 2% milk (Oxoid Limited, Basingstoke, Hampshire, UK) containing primary antibodies outlined in Table 1 and incubated overnight at 4°C. Secondary antibodies are also detailed in Table 1 (all Molecular Probes, Invitrogen) and were incubated for 2 h in the dark at room temperature. Some sections were counterstained with the nucleus stain, Sytox (1/1000; Thermofisher, UK). Control sections were performed with primary antibodies and the omission of secondary antibodies.

[Insert Table 1 around here]

Quantification of NF+ axons within cuneate fasciculus at cervical level C3

To assess the degree of axon loss within the ascending dorsal columns, we quantified neurofilament-positive axon bundles in a region of interest (ROI1) within the cuneate fasciculus of transverse sections at cervical level C3. Specifically, we wanted to compare axon bundles at ROI1 between 'responder' rats which showed some return of proprioceptive function with 'non-responders' who did not show any improvement.

Three 'responder' rats and three 'non-responder' rats which had 6 week survival times were chosen at random from their respective groups. For each animal, three cryostat sections at cervical level C3 were immunostained as described below for NF (light and

heavy). For ROI1, a square area (of size 600 μ m by 600 μ m) was imaged immediately dorsal to the medial part of the substantia gelatinosa (see Fig 6A). For each section, images were taken at ROI1 on both the injured and the non-injured sides of the spinal cord section.

To quantify the number of axon bundles, imageJ (National Institutes of Health, Bethesda, Maryland) was first used to manually threshold the ROI1 images from injured and non-injured sides of a tissue section. Using the 'Particle Analysis' function, two distinct size ranges of axon bundles were counted: small axon bundles had a diameter of 2-4 μ m, large bundles were 4-10 μ m. This analysis was done by someone blind to whether the tissue came from Responder or Non-Responder groups.

Quantification of Iba1+ within the deep dorsal horn at cervical level C7/C8

To compare the extent of dorsal horn microglia activation in responder and non-responder rats, we first selected three animals at random from these groups, all with a survival time of 6 weeks. For ROI2, as shown in Fig 7A, a square area (1200 μ m x 1200 μ m) was imaged adjacent to the central canal within Rexed laminae V and VI. This was done for both the injured and non-injured sides of the spinal cord, for three separate tissue sections in each animal, all at cervical level C7-C8.

To count the number of cells positively stained for Iba1, imageJ was used to manually threshold the images before the number of Iba1+ cells in ROI2 was found using the 'Particle Analysis' function. Images from injured and non-injured sides of the tissue were analysed under the same conditions, by a blinded assessor. We distinguished small (400-2000 μ m²) microglia from larger, activated microglia (>2000 μ m²) because recent evidence has suggested that changes in microglial size might underlie behavioural changes such as after exposure to chronic stress.³⁴

Statistical analysis

Results are expressed as means \pm SEM, with statistical comparison between groups made using a one-way analysis of variance (ANOVA), to determine F-ratio significance. Post hoc analysis was with Bonferroni multiple comparisons and IBM SPSS Statistics 22.0 software was used. Details of animal numbers are given below each graph.

RESULTS

Climbing Performance

Before surgery, rats climbed the vertical cage with baseline error scores of only around 5% (see Fig 2, BL). One week following C6-T1 dorsal roots transection, however, both the control rats (injury alone) and those receiving hOEC transplants had an error score of over 95% - virtually every attempt to grasp a vertical bar with the injured paw resulted in failure.

[Insert Fig 2 around here]

The hOEC non-responder group were rats which received transplants but had no noticeable recovery. Video recording analysis showed no sign of improvement in proprioception over the 6 week course of the study; their mean error score deteriorated and was close to 97% in the final week.

In the hOEC responder group, the rats received transplants and also had some functional recovery. This group of rats decreased their error score by a noticeable 20% between 1 week post-surgery ($93.2\% \pm 2.8$) and 2 weeks post-surgery ($72.5\% \pm 4.2$). Their performance improved gradually each week until at 6 weeks post-surgery where they accurately grasped close to 36% of cage bars (corresponding to $63.7\% \pm 2.0$ error) on the surgery side in each upward movement.

In comparison, control rats (injury alone) performed similarly to the non-responder group. They had a high error score of 100% one week after surgery and then continued to perform poorly (89-99% range) over the course of the six week study. At 6 weeks post-surgery, the control rats made around 30% more errors than rats from the hOEC responder group (94.5% versus 63.7%).

Foot Fault Score

To examine forepaw performance more closely and determine whether 'non-responder' rats had any subtle improvements in proprioception, we assessed the magnitude of error for each of the misplaced grasps. Misplaced grasps were graded from '0' to '4', with '4'

representing the worst level of fault in which the forelimb slid straight through to the axilla.

Prior to injury, rats had a foot fault score very close to 0 (0-0.4), where almost all grasps successfully located the vertical bars of the cage during upward movements. At one week after surgery, injury alone control rats and those in the hOEC non-responder group both had similar scores of 3.3 (\pm 0.2). This means that the average misplacement of the forearm was to where it slipped through to just past the elbow.

[Insert Fig 3 around here]

At the same time point, the hOEC responder group had a slightly lower fault score of 2.9 (\pm 0.3). These rats then showed a noticeable reduction in foot fault score between 1- and 2 weeks post-surgery, down from 2.9 to 1.3, indicating that the forearm slipped less far through, to an area between the digits and the wrist. In contrast, at two weeks post-injury, control and hOEC non-responder rats had foot fault scores of 3.0 (\pm 0.2) and 2.5 (\pm 0.3) respectively.

Between four and six weeks post-surgery, control and hOEC non-responder rats showed similar foot fault scores of around 2.4-2.8. Rats from the hOEC responder group gradually decreased their foot fault score for the remainder of the study, with a 6 week post-surgery score of 0.9 (\pm 0.1), indicating a misplacement slippage only to the palmar area of the forepaw.

Immunohistochemistry

Analysis of OEC-collagen gel:

Eight intact olfactory bulbs were used from 15 biopsy samples. Following removal of meningeal membrane, a portion of tissue for histology and olfactory tract tissue, the average tissue mass used for OEC culture weighed 84.18 ± 3.91 mg (mean \pm SE). On the 3rd day of culture a small number of cells started to attach to the bottom of the culture dish and extend processes. By the fifth day, a large number of cells had attached to the dish and differentiated into two major morphology types: i) small, 5 μ m cell bodies with a low ratio of cytoplasm/nucleus in a spindle shape with long, wire-like processes or ii) large cell

bodies (10-20 μm) with a high ratio of cytoplasm/nucleus with a rounded or square shape and short, thick processes. Immunostaining showed that the majority of cells with a spindle shape with long processes were positively stained for p75 (OECs) and the larger cells with short processes which stained for fibronectin were olfactory nerve fibroblasts (ONFs).

On day 10 the culture was 80% confluent and ready for transferring to gel. At this stage, immunostaining showed that the ratio of OECs to ONFs was 1:2. After 4 days in collagen, the cells had extended their processes to form a continuous meshwork (Fig 4C-D). Almost 90% of the cells were positively stained for p75 and very few for fibronectin. We found that 4.8 mg/ml collagen with a cell density of $1 \times 10^6/\text{ml}$ was appropriate to give a gel which had enough cells to form a continuous cellular meshwork (Fig 4D) and at the same time was easy to handle and manipulate for transplantation. Each collagen gel was made up of 250 μl collagen and cells and was around 10 mm in diameter and had a thickness of 200 μm .

Cell Survival & Distribution

In vitro: Prior to transplantation, epifluorescent microscopy revealed densely packed hOECs labelled with GFP within the collagen gel (see Fig 4C,D). The hOECs were distributed evenly within the collagen gel to form a cellular meshwork. Cell processes were of varying lengths, from between 30 μm to 130 μm , and extended in all directions within the gel.

The gel itself was pliable and so could be cut into thin strips which facilitated its transplantation into the injury site (Fig 4B). These strips were easy to manipulate, and could be further broken down and tailored to suit the actual size and contour of the junction between the cut ends of the dorsal root and the spinal cord. For example, the first thoracic dorsal root (T1) tended to be narrower and enter the spinal cord at a more oblique angle than dorsal roots C7 and C8.³³ More pieces were thus required for the cervical roots and ad hoc manipulations of the gel were simple to perform at the time of transplantation.

[Insert Fig 4 around here]

In vivo: Transplanted hOECs were identified at both early and late stages after surgery by using three separate approaches: a lentiviral mediated GFP tag, anti-human mitochondria immunostaining and an antibody against GFP to amplify the fluorescent signal.

Transplanted hOECs were seen close to the dorsal root entry zone at one week and also at six weeks post-surgery. Fluorescent cells tended to be seen either individually or as part of a larger, concentrated group at the injured site (see Fig 4E). The morphology of transplanted hOECs remained similar to those seen prior to transplantation. Where hOECs were found in a large cluster, it was apparent that they remained within the collagen gel. However, individual hOECs which were found in the surrounding connective tissues had elongated processes which suggests they migrated from the gel.

[Insert Fig 5 around here]

To account for potential fading of the GFP signal within the gel over the course of the study, immunostaining against human mitochondria and against GFP was employed, as shown in Fig 4 parts E-I. The scattered distribution of human-mitochondria+ cells within the dorsal horn contrasted with the concentrated aggregation of cells which was often seen (arrows to these cells in Fig 4E & Fig 4G) in the centre of the cavity and also adhering to its contours (arrowheads in Fig 4H). The cavity was enclosed dorsally by connective tissue which presumably helped to retain the transplanted cells at the injured site.

Astrocyte Responses & Axonal Outgrowth

The horizontal sections show that tissue from six weeks post-injury rats revealed outgrowth from the spinal cord in the form of GFAP+ immunostaining. In horizontal sections, multiple strands of filamentous GFAP+ axons were seen to extend for up to around 200 μm (Fig 5A) at the same angle towards the transplanted cells as we have seen before in previous studies. Two adjacent sets of serial sections with immunostaining against neurofilament and TUJ1 antibodies showed numerous axonal outgrowths (NF+ in Fig 5B) at the transplant site in the same region where GFAP+ processes were extended and where TUJ1+ axons were found within the vicinity of the injured area in Fig 5C.

Transverse sections also showed neurofilament+ axons (red in Fig 5D,E) in the damaged dorsal horn, distributed amongst the vacuole-like spaces which were formed after injury. This damage observed to the dorsal horn (dh in Fig5D,E) is typical of a dorsal root transection injury and was most apparent between the injured segments, from the 6th cervical level to thoracic level T1. A number of TUJ1-positive fibres (red in Fig 5F) were found to closely associate with GFP-positive hOECs (green in Fig 5F) at the transplant site, with a number apparently showing ensheathment of the newly grown axons by the transplanted hOECs.

Quantification of axon numbers and activated microglia: Responders v Non Responders

We examined sections of the spinal cord with adjacent tissue from both responder and non-responder rats at 6 weeks post-surgery to find any differences which could account for their disparity in proprioceptive performance. We did not see any difference in cell survival between responder and non-responder rats; tissue sections showed multiple portions of collagen gel at the injury site, where some hOECs remained within the gel and others had migrated into the spinal cord. We quantified axon numbers within the cuneate fasciculus at cervical level C3 and also the activation of microglia within the deep dorsal horn at cervical level C7,C8.

Counting axon bundles from NF immunostaining:

The cuneate fasciculus tract has axons concerned with fine touch, fine pressure and importantly, proprioception, which synapse at the cuneate nucleus in the brainstem. At 6 weeks post-surgery, there was an obvious loss of axon bundles within the cuneate fasciculus sample area at level C3 (see Fig 6A), three spinal cord segments rostral to the injury.

Counting revealed a marked reduction in small (2-4 μm) axon bundles from 201 (\pm 11) per sample area (ROI1) on the normal side to 75 (\pm 12) on the surgery side. There was also a reduction in large (4-10 μm) axon bundles, from 44 (\pm 9) on the normal side to 25 (\pm 13) after surgery. This corresponds to a 63% and a 43% reduction in the small and large axon bundles in the hOEC Responder group. The extent and pattern of loss of axons in the same

region of non-responder hOEC rats was similar; 68% and 65% loss of small and large axon bundles respectively.

[Insert Fig 6 around here]

Examining the activation of microglia with Iba1+ immunostaining:

To determine whether altered immune responses could account for differences in proprioception on the vertical climbing task, we examined microglial activation (by Iba1+ immunostaining) within the deep dorsal horn at cervical levels C7,C8 at 6 weeks post-surgery. Both responder and non-responder groups showed similar levels of Iba+ staining on their normal side (Fig 7B,D). There was a substantial increase in the number of cells positively stained for Iba1, from 49 to 112 on the surgery side (Fig 7C) of responder rats. There was a similar although slightly attenuated increase in Iba1+ staining in the non-responder group, from 46 cells to 94 cells after injury. In terms of morphology, it was apparent that after surgery, microglia had enlarged processes and took up a larger area than those at baseline (refer to Fig 7D). Preliminary studies showed CD68-positive stained macrophages at the injured dorsal horn 6 weeks post-surgery; there was no detectable difference in terms of the distribution or quantity of macrophages between responder or non-responder rats (unpublished data).

[Insert Fig 7 around here]

DISCUSSION

For the first time, in this study, OECs from human olfactory bulb tissue were mixed with collagen and transplanted into a rat model of spinal roots injury. This builds upon a number of preclinical animal studies which found benefits from OEC transplantation and has culminated in their safety being confirmed in recent clinical trials.^{25, 26, 28} We obtained and cultured cells from human olfactory bulb samples and mixed these hOECs with collagen gel. The cells were transplanted into a four dorsal root transection injury at the cervical level in rats. Half of the rats receiving hOEC transplants showed an improvement in proprioception as assessed on a vertical climbing task.

Optimising hOEC transplants for cavity filling

Damage to the spinal cord often involves the progressive loss of tissue over time which can result in cavitation at the injury site.³⁵ The present protocol of hOEC cell preparation cannot produce the quantity or composition of cells to adequately fill a lesion cavity on their own. In a clinical trial, Tabakow and colleagues injected hOECs into the patient's sectioned spinal cord stumps rostral and caudal to a lesion cavity.²⁹ The 11 mm gap between the injured ends was filled with four autologous peripheral nerve grafts. The limited mass of biopsy tissue yields far lower cell numbers than what is needed for repair, so the use of collagen may allow hOECs to distribute within a much larger volume. In this current study, mixing hOECs with collagen enabled cell expansion prior to transplantation where they distributed evenly in the gel and many had formed elongated processes.

This method was suitable because hOECs were optimally positioned within the gel prior to transplantation, with early process elongation potentially enhancing their interaction with host astrocytes. For example, it has been shown that aligned poly-lactic acid microfibers provided a better scaffold for regenerating axons and astrocytes than randomly assorted fibers after a transection injury in rats.³⁶

The further benefit of using a gel is that it could avoid the numerous time-consuming cell injections which can damage the spinal cord. Although it has been shown in clinical studies in patients with complete spinal cord injuries that multiple spinal cord OEC-microinjections were safe and did not cause deterioration of the patient's neurological state,^{25, 27-29} a delivery of OECs embedded within scaffolds and not as single cell suspension microinjections seems to be less invasive both for the spinal cord tissue and transplanted cells.

Transplanted hOECs were predominantly located as a concentrated aggregate at the lesion site, while a few other individual cells with elongated processes had apparently migrated away. These findings are similar to a study by Deng and colleagues where they injected human OECs (from the nasal lamina propria) into a rat spinal cord after a hemisection injury.³⁷ In our current study, some small diameter axons were found alongside outgrowths of spinal cord astrocytic processes which have both been seen previously with

rat OEC transplants;^{16, 38} this suggests the human OECs have a similar effect, even if the host species is different.

Climbing performance

The vertical climbing test is an objective and reliable measure of forelimb proprioception; our previous work determined that transection of dorsal roots C6, C7, C8 and T1 led to simple, consistent and permanent functional deficits of the upper limb.³³ In this and in previous studies, animals which failed to show any improvement by around 2 weeks post-surgery tended to perform poorly for the remainder of the experiment (Collins A & Li Y unpublished observations). For this reason, after analysis, we assigned rats as either 'responder' or 'non-responder' based on their early post-injury climbing performance.

At the end of the 6 weeks study, half of the rats which received hOEC transplants (i.e. 9 responders) had scored 30% higher in terms of climbing performance. They also had a foot fault score around 2 points lower than the control rats (injury alone). By assessing the magnitude of error of misplaced grasps, i.e. the foot fault score, we could detect subtle changes in proprioception rather than simply whether a paw grasp was successful or not. In our previous electrophysiological study we examined transplants of rat olfactory bulb OECs in the same four dorsal root transection model, we found that 70% of rats showed some recovery of proprioception whilst the remaining 30% lacked any recovery (non-responders).¹⁶ Given the preliminary nature of this study, it is unsurprising that as many as half of the injured rats which received hOECs failed to show signs of recovery; potential reasons for this are discussed below.

Source of OECs

The anatomical source of OECs is a crucial factor in functional repair. We have shown previously that transplantation of cells cultured from rat olfactory bulb but not olfactory mucosa could reinstate proprioception in a climbing task.³⁹ In a subsequent clinical case study, a patient showed some functional improvement following autologous transplantation of olfactory bulb OECs to treat a thoracic level injury.²⁹ The observed clinical, neurophysiological and radiological evidence of regeneration of descending and ascending axons from a central origin throughout the area of spinal cord reconstruction

contrasted with clinical studies performed with olfactory mucosal OECs that showed either minimal²⁵ or no recovery from the transplantation.^{26, 28} Here, we have transplanted human olfactory bulb OECs into another species, the rat, to see for the first time whether olfactory bulb cells from one species can repair dorsal root injury in another.

Few studies have investigated transplants of human OECs in rat models of spinal cord injury. Olfactory bulb tissue from non-human primates has been used to show behavioural recovery after a T9/T10 level spinal transection in immunodeficient rats.⁴⁰ Other groups have used human OECs but not from the olfactory bulb; Gorrie et al transplanted human mucosa OECs into a rat spinal cord contusion model and Kato et al transplanted human olfactory nerves into a rat model of demyelination.^{41, 42} There was some functional recovery after the contusion injury and evidence of remyelination of rat axons in the other study.

Limiting factors which may account for partial recovery

A number of factors could explain why only half of rats (nine out of eighteen) which received hOEC transplants in this current study showed recovery and why the best performance in the vertical climbing test at the end of the trial still resulted in 60% of grasps being misplaced.

First, although hOECs have been shown to have similar repair properties to rat OECs in terms of remyelination; their heterogeneity, morphological diversity and lack of cell specific markers makes it difficult to fully compare OECs from these two different species.⁴³⁻⁴⁵

Second, the yield of hOECs in this study is lower than what would be obtained from a rat. This reduction is due to the long time (24 hours) taken for tissue transportation (unpublished data). It should also be noted that these olfactory bulbs may have had pathomorphological changes prior to being obtained such as intraparenchymal haemorrhages that were visible in some cases of OB microscopic dissection. This could have been caused by contusion injury to the frontal lobe and the olfactory bulb accompanying the skull base fracture or the presence of neoplastic infiltration in other

cases. Also, interpersonal variability in the anatomy of the human olfactory bulb or prior exposure to toxic agents or infections could alter the condition of bulbar cells.⁴⁶⁻⁴⁸

Third, the proportions of rat OECs and ONFs used in our previous studies were around 50% respectively. In our cellular scaffold, the proportion of p75+ OECs was close to 90% and fibronectin deposition was low. This may have an effect on their repair function.

Fourth, this is the first study in which we have mixed OECs with a collagen gel – we assume this would not negatively impact on their repair properties but as yet this has not been fully explored. We do not know if hOECs which remain within the gel and those that migrate out have similar repair properties. We need to explore how to optimise the transplant biomaterial for repair. In particular, we must consider what proportion of hOECs, olfactory fibroblasts and collagen gel characteristics are best for promoting repair.

Fifth, there are a number of technical aspects of the surgery procedure which may have led to differential functional recovery in the rats. For example, although care was taken to transplant a similar number of cells to each injury site, it is possible that some cells may not have remained at the injury site, or may not have formed a complete bridge between the cut ends of the dorsal roots and the spinal cord or that fibrin glue may not have completely covered each portion of collagen gel. Examining tissue at the end of the study, we saw no apparent difference in cell survival within the responder or non-responder groups of rats.

Responders v Non-Responders: Quantification of axon loss & microglial activation

Rats which recovered proprioception (responders) and those which did not (non-responders) showed similar grooming behaviour in their home cage and had no locomotor deficits when exploring their cage (Collins, A & Li Y, unpublished observations). The two groups could only be distinguished when their forelimb grasping was assessed on a vertical cage climbing test. Histological examination could not uncover any difference in terms of location or survival of hOECs which could explain the disparity in climbing performance. In both groups, we saw the collagen gel maintain its structure at the injury site, with hOECs remaining in the gel and others in a cluster within the spinal cord; the elongated spindle-like morphology of these cells was also similar in responder and non-responder rats. Very

few OEC processes were seen crossing the injury site into the spinal cord; in the case of those that did, the axons travelled with them at the entry zone.

We know from our previous electrophysiological study which showed that responder rats which had received bulb OECs had partial recovery of an afferent volley through the transplanted dorsal root entry zone which ascended rostrally up the dorsal columns to the cuneate nucleus.¹⁶ The transplants may have better preserved or limited the secondary damage to axons of the ascending dorsal column to enable this attenuated afferent transmission. In the present study, we therefore compared responder and non-responder rats in terms of axon loss within the cuneate fasciculus of cervical level C3.

Griffin and colleagues have described the proximo-distal spread of axonal degeneration which is known to begin around one day after dorsal root rhizotomy and proceed as quickly as 3 mm per hour.⁴⁹ Axons within the cervical cuneate fasciculus are known to transmit, amongst other afferents, skin and tactile proprioception from the upper half of the body, which is relevant given the nature of the vertical climb test used in the present study.⁵⁰ Although we could not identify any quantifiable difference in axon loss between responder and non-responder groups, this method is likely not sensitive enough to detect the relatively few repaired axons it would require for transmission through to the cuneate nucleus.

Early, neuroprotective actions of OECs have been suggested to account for recovery of appropriate forelimb responses to thermal stimuli in a recent dorsal root injury study.⁵¹ Although short, CGRP-positive fibres were observed at the injured dorsal root entry zone of rats which received OEC transplants, there was no accompanying increase in sensitivity to heat or cold stimuli. We did not examine responses to painful stimuli in this current study but future work should investigate whether hOEC transplants in a rat model of dorsal root avulsion injury can prevent abnormal sensory responses in the same way as rat OEC transplants.⁵¹ This would have obvious implications for their use in a clinical setting.

The interaction between the immune system and the damaged spinal cord is now viewed as highly relevant.^{52, 53} Microglia have been heavily implicated in generating and maintaining pain states following spinal cord injuries, as well as modulating levels of

inflammation.^{54, 55} By choosing to examine microglial activation at the deep dorsal horn, we were able to see whether improved proprioception was associated with altered immune system responses. In this case there was no obvious association: both responder and non-responder groups of animals showed a very similar activation of microglia at 6 weeks post-surgery. We cannot rule out the possibility that administering cyclosporine daily throughout the study may have obscured or modified any hOEC transplant related effects on immune system function.

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Table 1. Antibodies used in immunohistochemistry

Primary Antibody	Host	Dilution	Company	Secondary Antibody	Reactivity
GFAP	ms	1/500	Millipore	Alexa gt anti ms 1/1000	reactive astrocytes
Neurofilament 68kd (light)	rb	1/500	Abcam	Alexa gt anti rb 1/1000	small diameter axons
Neurofilament 200kd (heavy)	rb	1/500	Abcam	Alexa gt anti rb 1/1000	large diameter axons
Anti-GFP	rb	1/1000	Abcam	Alexa gt anti rb 1/1000	GFP
Tuj1	ms	1/500	Sigma	Alexa dk anti ms 1/1000	immature neurons
Hu Mitochondria	ms	1/100	Millipore	Alexa gt anti ms 1/1000	human mitochondria
Iba1	goat	1/250	Abcam	Alexa dky anti gt 1/1000	microglia
p75	ms	1/500	Millipore	Alexa gt anti ms 1/500	nerve growth factor receptor
Fibronectin	rb	1/1000	Dako	Alexa gt anti rb 1/500	glycoprotein/ECM

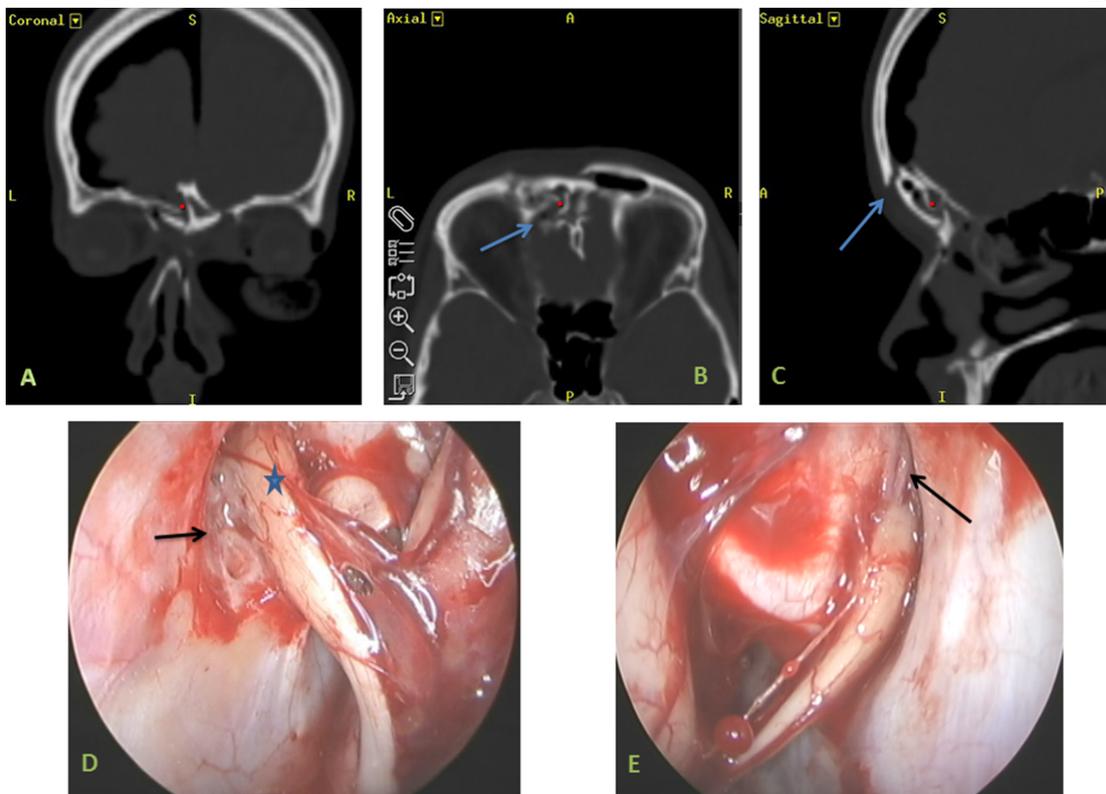


Fig. 1 A case of a 35-year –old woman sustaining severe anterior skull base fracture with cerebrospinal fluid leak and pneumocephalus. A. Computed tomography (CT) coronal scan showing intracranial air accumulation predominantly in the left frontal subdural area, arising from fracture of both walls of the frontal sinus and fracture of the crista galli and left cribriform plate of the ethmoid bone (red point). B. Axial CT scan showing fracture of the frontal and ethmoid bone with invagination of the bones towards the intracranial cavity with compression of the left olfactory bulb (blue arrow). C. Sagittal CT scan. Blue arrow is pointing the area of frontal sinus wall fracture. D. Intraoperative view obtained with a 30-degree angled neuroendoscope showing the left olfactory bulb (asterisk) being detached from the filia olfactoria of the cribriform plate (black arrow). E. Endoscopic view of the olfactory cleft of the right side showing the anatomically intact right olfactory bulb covering the whole right cribriform plate. Olfactory filia are shown attached to the bulb (black arrow). In this case the left olfactory bulb was obtained, before the performance of the skull base fracture repair.

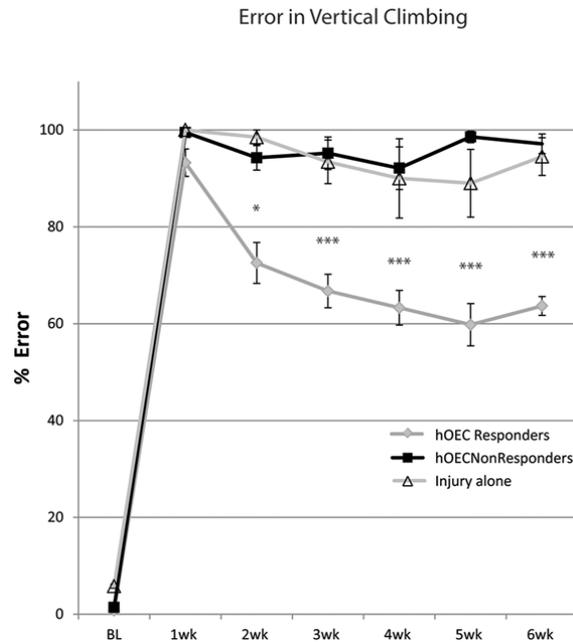


Fig 2. Error on a vertical climbing task.

Half of the rats which received hOEC transplants showed some recovery (grey circles) after surgery; the other half did not (black squares) and performed no differently from rats which had the injury alone (open triangles).

Error bars: mean \pm s.e.m. One Way ANOVA $F(20,126)=88.763$, $p<0.05$ (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ Post hoc Bonferroni)

hOEC Responders: $n=9$, hOEC Non-Responders: $n=9$, Injury alone: $n=5$

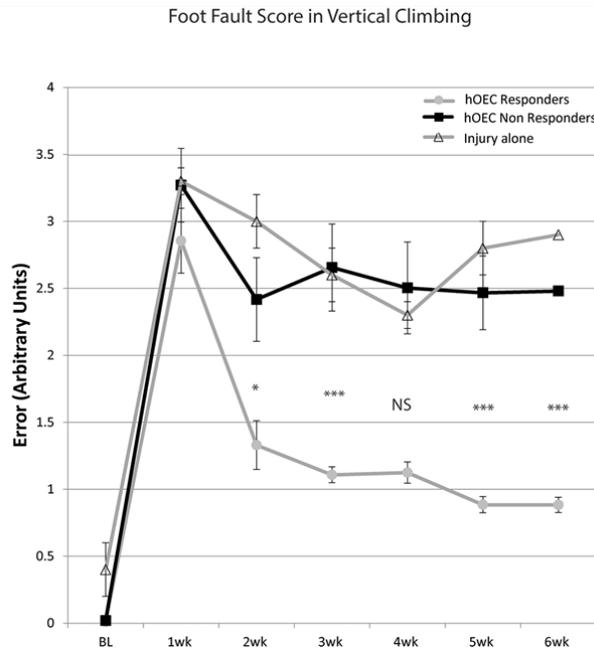


Fig 3. Foot fault score on vertical climbing task – magnitude of error in misplaced grasps. Half of the rats which received hOEC transplants had a reduction in the magnitude of grasp misplacement error following surgery (grey circles); the other half (black squares) showed a similar grasping error misplacement as injury alone controls (open triangles).

Error bars: mean ± s.e.m. One Way ANOVA $F(20,124)=23.475$, $p<0.001$

(NS $p>0.05$, * $p<0.05$, *** $p<0.001$ Post hoc Bonferroni)

hOEC Responders: $n=9$, hOEC Non-Responders: $n=9$, Injury only: $n=5$

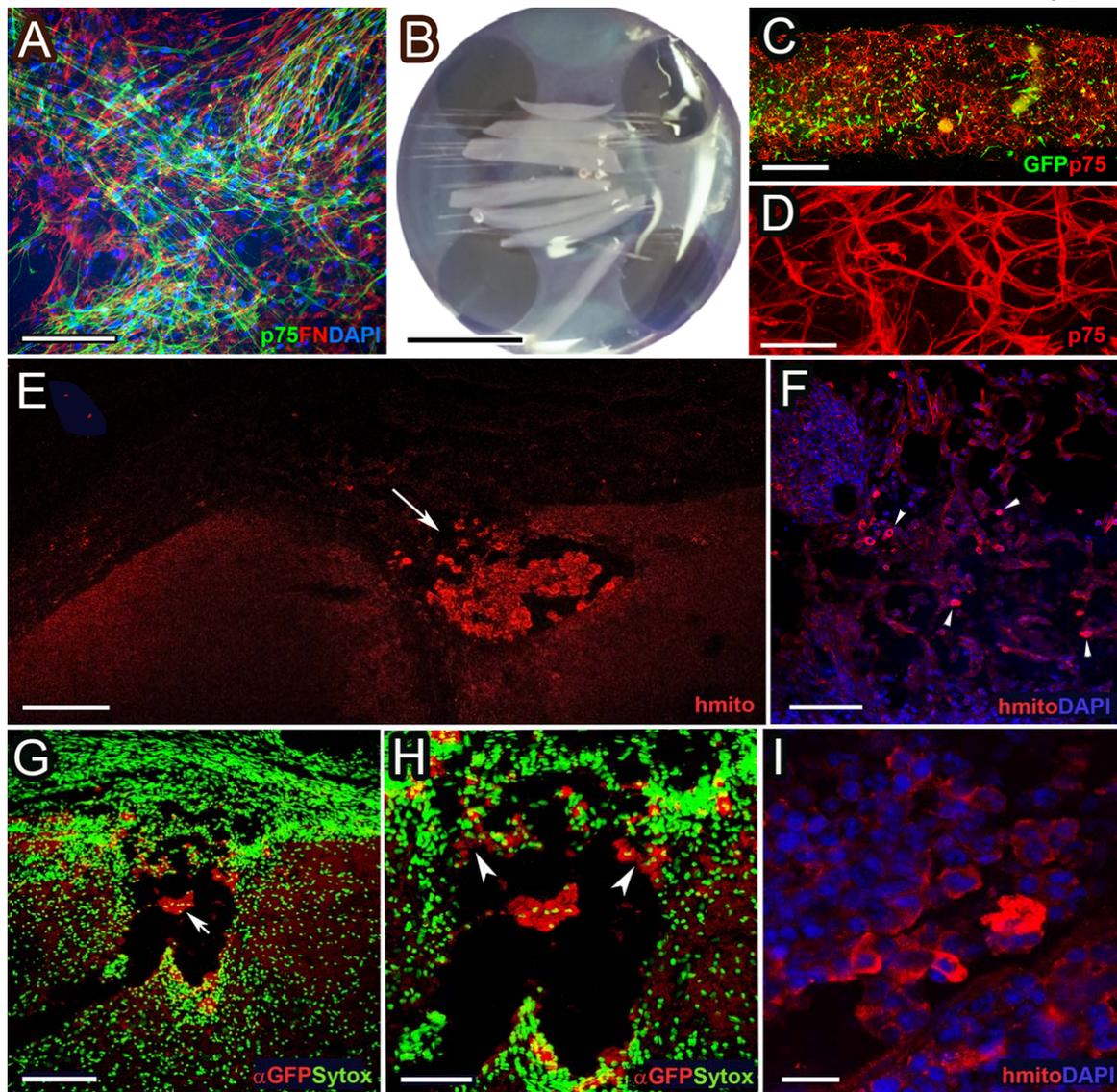


Fig 4. Cultured human olfactory bulb cells labelled with lentiviral GFP shown prior to and after transplantation with various immunostaining.

Cultured human olfactory bulb cells immunostained for anti-p75 and anti-fibronectin (FN) along with a DAPI nuclear stain (A), strips of collagen prior to transplantation (B), lentiviral GFP labelled hOECs with anti-p75 immunostaining (C) and the meshwork of processes shown in high power with staining against p75 (D).

hOECs positively stained for anti-human mitochondria, concentrated within the lesion cavity (arrow) (E) and scattered around the vicinity (arrowheads) in both low power (F) and high power (I).

A small group of cells positive for anti-GFP staining (α GFP) within the lesion cavity showing the overlying connective tissue (G,H) and a high power view from G to show other hOECs around the outside of the cavity (arrowheads in (H)). Survival time, A,C,D 14 days; E-I, 6 weeks, scale bars, A: 200 μ m, B: 10 mm, C: 1mm, D: 100 μ m, E,G: 200 μ m; F,H: 100 μ m; I: 25 μ m.

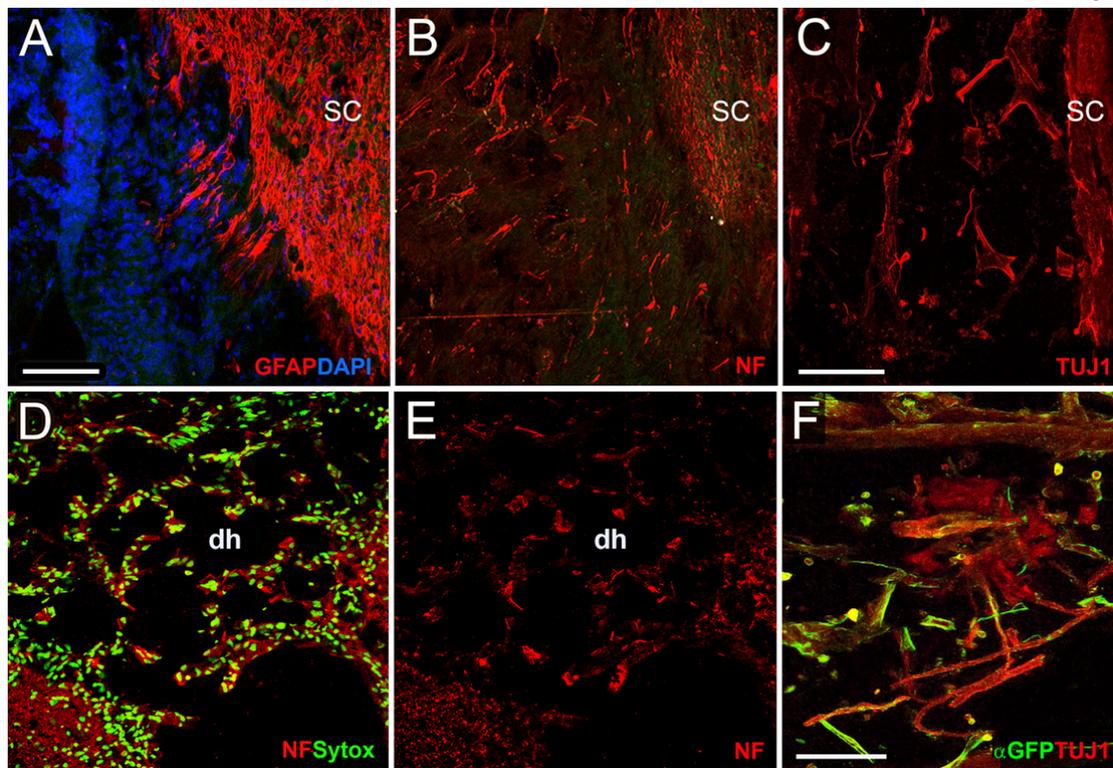


Fig. 5 Host spinal cord tissue and axons responded to the hOEC transplants following dorsal root transection.

The horizontal plane of sections (A-C) show outgrowth of astrocytic processes (red in A) from the spinal cord (SC) in the transplanted area. Adjacent two sets of sections showing neurofilament (red in B) and TUJ1-positive fibers (red in C) in the same area.

The transverse sections showing the cavity at the dorsal horn (dh), with neurofilament positive fibres (red in D,E); anti-GFP staining (green) alongside TUJ1-positive fibres (red) including the ensheathment of some TUJ1+ fibers by transplanted hOECs in F. Survival times A-H, 6 weeks. Scale bars 100 μm .

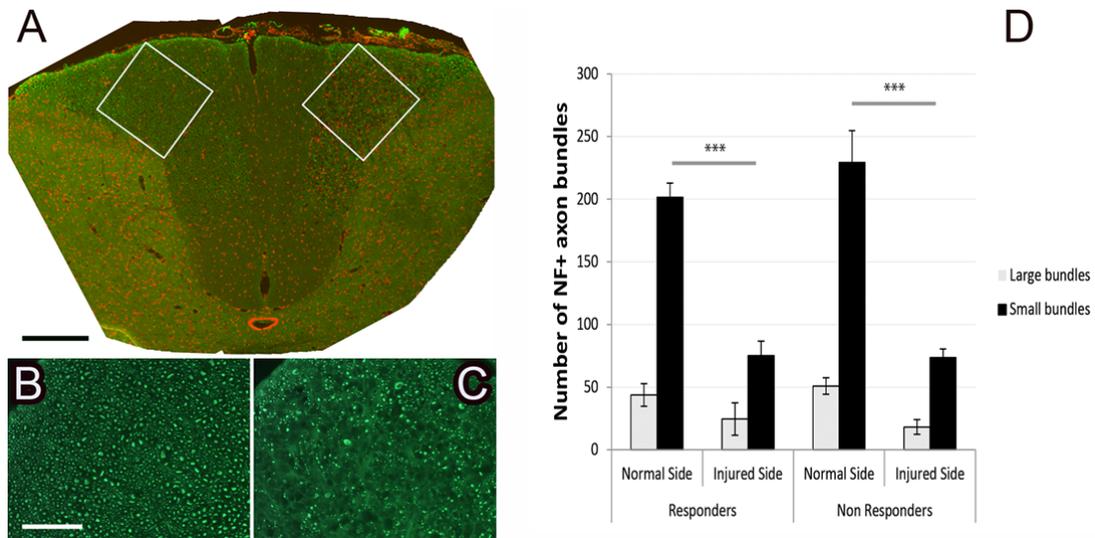


Fig 6. Quantification of axonal loss within cuneate fasciculus at cervical level C3 in rats which recovered proprioception (responders) and those which did not (non-responders).

Low power image showing the region of interest (ROI1) in (A). High power to show the normal side (B) and injured side (C) of the spinal cord with transverse sections. Axon loss (C) was clearly seen 6 weeks after injury with neurofilament immunostaining in C; responders and non-responders had similar levels of axon loss within the cuneate fasciculus from the counting. Counts were made of small (2-4 μm ; black bars) and large NF+ axon bundles (4-10 μm ; white bars) of axons, where there was a reduction in both responders and non-responders after surgery (D). NF staining, green; survival time, 6 weeks; scale bars, 500 μm in A and 200 μm in B,C.

Error bars: mean \pm s.e.m. One Way ANOVA $F(7,16)=40.497$, $p<0.001$

(*** $p<0.001$ Post hoc Bonferroni) Responders: $n=3$, Non-Responders: $n=3$

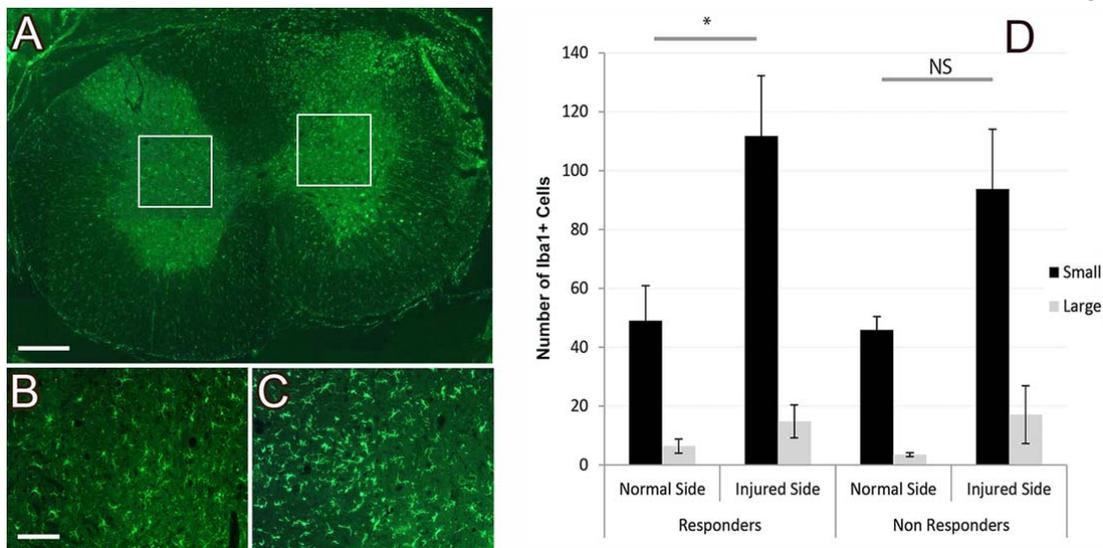


Fig 7. Quantification of microglial activation within the deep dorsal horn at cervical levels C7-C8 in rats which recovered proprioception (responders) and those which did not (non-responders).

Low power image showing the region of interest (ROI2) in (A). High power to show the normal side (B) and injured side (C) of the spinal cord with transverse sections. Microglial activation was clearly seen 6 weeks after injury with Iba1+ immunostaining in C; responders and non-responders had similar levels of microglial activation within the deep dorsal horn from the counting. Small ($400\text{-}2000\ \mu\text{m}^2$) and large ($>2000\ \mu\text{m}^2$) Iba1+ cells were counted within the deep dorsal horn; there was an increase after surgery but no difference between responders and non-responders (D). Iba1 staining, green; survival time, 6 weeks; scale bars, $500\ \mu\text{m}$ in A and $200\ \mu\text{m}$ in B,C.

Error bars: mean \pm s.e.m. One Way ANOVA $F(7,16)=11.923$ $p<0.001$

(*** $p<0.001$ Post hoc Bonferroni) Responders: $n=3$, Non-Responders: $n=3$