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Restoration of hand function in a rat model of repair of brachial plexus injury

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The incurability of spinal cord injury and subcortical strokes is due to the inability of nerve fibres to regenerate. One of the clearest clinical situations where failure of regeneration leads to a permanent functional deficit is avulsion of the brachial plexus. In current practice, surgical re-implantation of avulsed spinal roots provides a degree of motor recovery, but the patients neither recover sensation nor the use of the hand. In the present rat study, we show that transplantation of cultured adult olfactory ensheathing cells restores the sensory input needed for a complex, goal-directed fore-paw function and re-establishes synaptic transmission to the spinal grey matter and cuneate nucleus by providing a bridge for regeneration of severed dorsal root fibres into the spinal cord. Success in a first application of human olfactory ensheathing cells in clinical brachial plexus injury would open the way to the wider field of brain and spinal cord injuries.

Keywords: dorsal horn; spinal cord; transplantation

Abbreviations: BD = biotin dextran; DR = dorsal root; GFP = green fluorescent protein; OEC = olfactory ensheathing cell

Introduction

Transplantation of olfactory ensheathing cells (OECs) has been reported to promote repair of long tract injuries in the spinal cord (Li *et al*., 1997; Ramón-Cueto *et al*., 2000; Lu *et al*., 2002; Steward *et al*., 2006). Varying results have been reported in attempts to use OECs to repair dorsal root (DR) lesions (Ramón-Cueto and Nieto-Sampedro, 1994; Gomez *et al*., 2003; Riddell *et al*., 2004). In our laboratory (Li *et al*., 2004), we have observed that transplantation of OECs at the site of re-apposition of a severed lumbar DR induces the formation of a tissue bridge over which the cut axons regenerate into the spinal cord. In a previous paper (Ibrahim *et al*., 2009), we showed that unilateral section of the C6 to T1 DRs of the brachial plexus abolishes ipsilateral fore-paw grasping in a climbing test. In the present study, we took advantage of our previous endogenous matrix method of transplantation (Li *et al*., 2003) to test the ability of cultured bulbar OECs to ameliorate this functional deficit.

Methods

Ethical approval

The procedures were approved by the Ethical Review Process of the Institute of Neurology and were in accordance with UK legislation [Animals (Scientific Procedures) Act 1986]. Adequate measures were taken to minimize pain and discomfort.

Cell culture

The detailed protocol for cell culture is given in (Li *et al*., 2003). Briefly, under aseptic conditions the nerve and glomerular layers of

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the olfactory bulbs from female adult rats of an inbred Albino Swiss strain were dissected out, dissociated using 0.1% trypsin and cultured in both DMEM/F-12 medium and 10% foetal serum. The cultures were monitored until confluent (generally reaching around 1.5×10^6 cells/35 mm dish at around 14–16 days), at which time the OECs were accompanied by approximately equal numbers of olfactory nerve fibroblasts and were embedded in an endogenous matrix. Two days before transplantation the cells were transfected with a lentiviral vector expressing GFP (Ruitenberg *et al*., 2002).

Subjects

The total of 85 adult female rats of the same strain (body weight 180–200 g) consisted of 19 normal controls, 20 lesion controls with un-repaired transection of the 4 DRs from C6-T1, 42 rats with C6-T1 4 root lesions into which OECs had been transplanted, 7 of which were used for fibre tracing by injecting biotinylated dextran amine (BD) into the DR ganglia and 4 normal rats used for electrophysiology.

Surgery

Transplantation

In 35 rats hemilaminectomies from C4-T2 were carried out under the operating microscope to expose the spinal cord through a posterior surgical approach, as described in (Ibrahim *et al*., 2009). The dura was carefully opened and once the segmental levels had been clearly defined micro-scissors were used to transect the dorsal roots of C6-T1 close to the spinal cord in the plane of the line in Fig. 5A. The matrix containing GFP labelled cultured OECs was scraped from the dish (with a scraper, Costar, Corning, NY), applied between the cut ends of the DRs and their original entry point on the spinal cord, and retained in place with fibrin glue (Tisseel Kit, Baxter, Thetford, UK).

A further series of seven rhizotomized rats with OEC transplants were used for tracing DR fibres by injection of BD into the ganglia. To avoid tracer passing through anastomoses to adjacent intact roots, extended laminectomies were carried out, and the six roots from C5 to T2 were sectioned. The four roots from C6 to T1 were transplanted with OECs. At 6–8 weeks after transplantation about 0.5 µl of 10% BD in saline was injected by micropipette at three points into each of the two middle (C7 and C8) DR ganglia. Fourteen days after BD injection the rats were perfused and fixed with 4% paraformaldehyde for histology.

Behavioural assessment

Weekly, for 3 weeks before and up to 10 weeks after surgery, each rat was placed facing upwards on the lower bars of a 1-m grid at 15° inclination to the vertical and allowed to climb freely to a horizontal platform at the top of the grid. Video recordings were made during two successive climbs and analysed by an assessor unaware of the experimental status of the rats. The ipsilateral fore-paw was assessed for accuracy in locating and grasping the grid bars. A successful grasp was recorded whenever there was purposeful movement resulting in direct contact with either the horizontal or vertical bars of the grid and ending with full flexion of the digits around the bars so as to provide mechanical purchase for the fore-limb to contribute an upward force for climbing (for details see Ibrahim *et al*., 2009). For each climbing movement, errors in localization were graded from 1 to 4 in order of severity. 1, the paw reaches the grid but does not grasp the bar; 2, the paw overshoots the grid to the wrist level, 3, the paw protrudes through to the elbow level; 4, the paw overshoots the grid to the shoulder level. Other faults (multiple abortive reaches, or holding the paw limp by the side) were present but not included in the score.

Electrophysiology

Rats were anaesthetized with urethane (1.4 gm kg^{-1} ip) and tracheal, arterial and venous cannulae inserted. Hartmann's solution was administered iv, typically 1 ml h^{-1}. Mean arterial blood pressure was maintained above 80 mm Hg with infusion of further fluids if required. Temperature was maintained at 37–38°C and the animal was suspended prone by a spinal clamp at T3, a plate screwed to the skull and a ligature through the lumbar dorsal fascia. A laminectomy was made to expose C4-T2 segments and, in some animals, the caudal medulla was exposed by a small occipital craniotomy and partial C1 laminectomy.

The left median nerve was dissected free a few millimetres above the wrist and cut distally. The ventral aspect of the forelimb was made uppermost by supination and rotation, aided by section of anconeus and some pectoral muscle, together with removal of biceps, and a paraffin oil pool was constructed from skin flaps. The nerve was stimulated (0.1 ms pulses, 1 s^{-1}) via platinum wire electrodes and the evoked nerve volley monitored via an additional wire electrode on the median nerve a few millimetres above the elbow (reference wire on nearby muscle). Here we report responses from stimulation at 10 times threshold for the volley. After opening the dura, evoked potentials were monitored via further platinum wire electrodes on the spinal cord dorsum and on the left medullary dorsum above the cuneate nucleus, 1.3 mm caudal and 0.7 mm lateral to obex. Evoked potential signals were conventionally amplified and band-pass filtered (10 Hz–10 kHz). 50–300 responses were averaged for any one condition.

In normal rats the cord dorsum responses were measured at the DR entry zone before and after section of the DRs. In all cases the rhizotomy included segments C5 to T1. In the instance of some responses surviving this procedure, the lesions were extended to include C4 and/or T2 to check whether any responses might be transmitted via these segments. If any conducted responses were seen in any of the chronically rhizotomized rats, where the C5 DRs had been purposefully left intact (Ibrahim *et al*., 2009), the C5 DR was sectioned acutely and responses reported from these animals were recorded subsequent to this. In operated animals, the dense connective tissue and dura overlying the previous laminectomy was trimmed medially as close to the root entry zone as was judged safe for the integrity of any regenerating or spared DR fibres, and the recording electrode placed immediately adjacent to this trimmed edge, typically about 0.5 mm medial to the DR entry zone. On account of this more medial placement, signals could have been attenuated relative to the normal animals. The possible extent of this was estimated in two of the rhizotomized rats with OEC transplants and showing substantial CDPs, by recording the CDPs at several points across the dorsal columns. As in Al-Izki *et al*. (2008) (their Fig. 3B), the signal was progressively attenuated going contralaterally. This allowed an estimate by extrapolation back ipsilaterally for 0.5 mm, such as to suggest that the responses in the rhizotomized animals were underestimated by around 20%.

At the end of the procedure, unoperated animals were killed by anaesthetic overdose, operated ones were perfused for histology.

Histology

After behavioural and electrophysiological assessment, the rats were terminally anaesthetized and the tissues fixed by transcardiac perfusion of 50 ml 0.1 M phosphate buffer solution followed by 400 ml 4% phosphate buffered paraformaldehyde. The vertebral column was dissected out from the cranio-cervical junction to the upper thoracic and left to harden in fixative for 24–48 h. The spinal cord and the associated roots were then very gently dissected out from the bony skeleton under the dissecting microscope taking care to preserve the continuity across the DRs and transplants to the spinal cord. The dissected tissues were placed into 10–20% sucrose solution until sinking, frozen in dry ice and sections cut on the cryostat.

For double immunostaining 16 μm sections were incubated in 2% milk containing both 1:1000 anti-glial fibrillary acidic protein (GFAP) antibodies (mouse monoclonal Sigma) and 1:500 anti-laminin antibodies (rabbit polyclonal Sigma, UK) overnight at 4°C. Secondary antibodies were 1:400 anti rabbit and anti mouse (Alexaflour red or green) for 1–2 h in the dark at room temperature. For neurofilament staining sections were incubated overnight with 1:500 heavy chain polyclonal rabbit anti-neurofilament H200 antibodies (Serotec AHP245), followed by 1:500 anti-rabbit secondary antibodies (Alexaflour red). For detection of BD labelling, 30 μm sections were incubated in 1:400 of streptavidin conjugate (Alexaflour 546) overnight at 4°C.

Statistics

Means are quoted with standard errors of the mean.

Results

Fore-paw function

The rats were observed during climbing a 1 m grid. Normal rats accurately placed each of the four paws in regular sequence, with practically no faults reaching the bars, following which they closed the digits in a grasp which provided the purchase for climbing. After C6 to T1 rhizotomy the ipsilateral fore-limb rarely managed to reach the bars, and when it did the digits did not close in a grip. Rats with OECs transplanted into the lesions showed a progressive improvement in locating and grasping the bars, although they did not reach full normal performance. These events were scored from weekly videos over an entire 8 week postoperative testing period by an assessor unaware of the transplantation status of the rats.

As in the previous paper, 19 normal rats scored a mean of 7.1 ± 0.2 grasps, and made hardly any faults in locating the bars (mean 1.3 ± 0.2). Out of 20 rats with C6 to T1 rhizotomy without transplants 16 completely lost the ability to grasp the bars. The remaining four showed only occasional grasps, with a mean score of 0.2 ± 0.08. Overall, these 20 rats had a high incidence of faults, with a mean score of 20.8 ± 0.5.

Of 35 rats where OECs were transplanted into the site of transection, the overall mean fault score was 17.6 ± 0.2 with a mean grasp score of 1 ± 0.09. This group of rats, however, fell into two categories. Ten rats (29%; the 'non-responders') failed to perform more than two grasps over the entire 8-week-observation period. The non-responders had a mean fault score of 19.3 ± 0.4 (comparable to the rhizotomized rats without transplants). In the remaining 25 rats (71%; the 'responders') grasping returned, starting from 2 to 3 weeks after transplantation (Fig. 1). The responders had a mean grasp score of 2.9 ± 0.22, and a significantly lower fault score of 13.8 ± 0.6 (Fig. 2).

Electrophysiology

The electrophysiological investigations were performed by investigators unaware of either the transplantation status or climbing

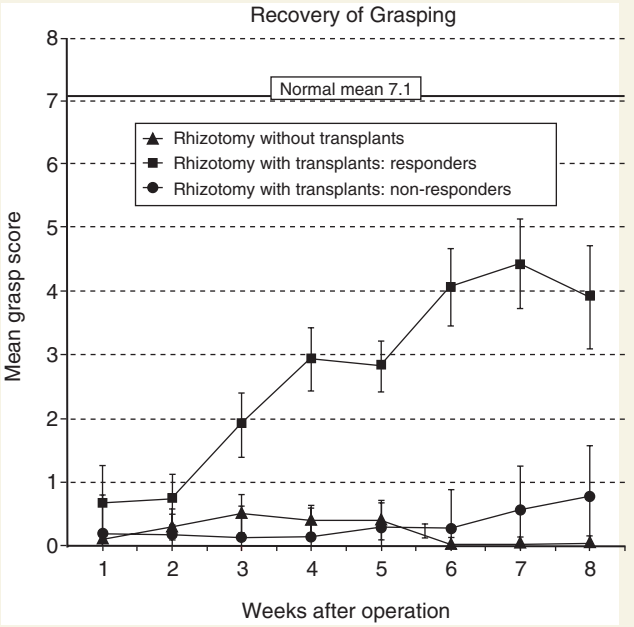

Figure 1 Recovery of grasping. Squares, rhizotomized rats with successful transplants ('responders' $n = 25$) over the observation period. Triangles, non-responders ($n = 10$); circles, rhizotomy without transplants ($n = 20$). Group means (±SEM). Solid line at 7.1 indicates level achieved by unoperated rats.

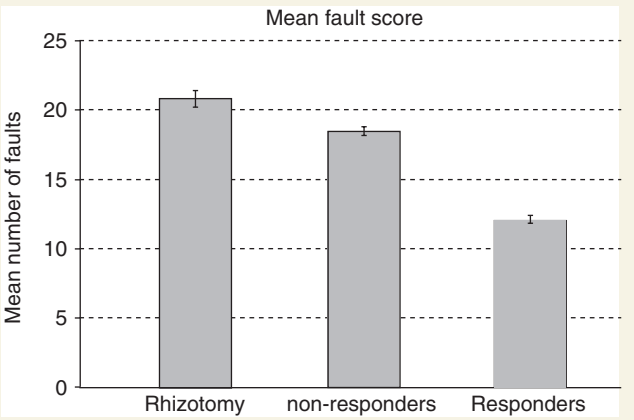

Figure 2 Group mean fault scores (±SEM). Rhizotomized rats without transplants ($n = 20$), Non-responding rhizotomized transplanted rats ($n = 10$) and responding rhizotomized transplanted rats ($n = 25$).

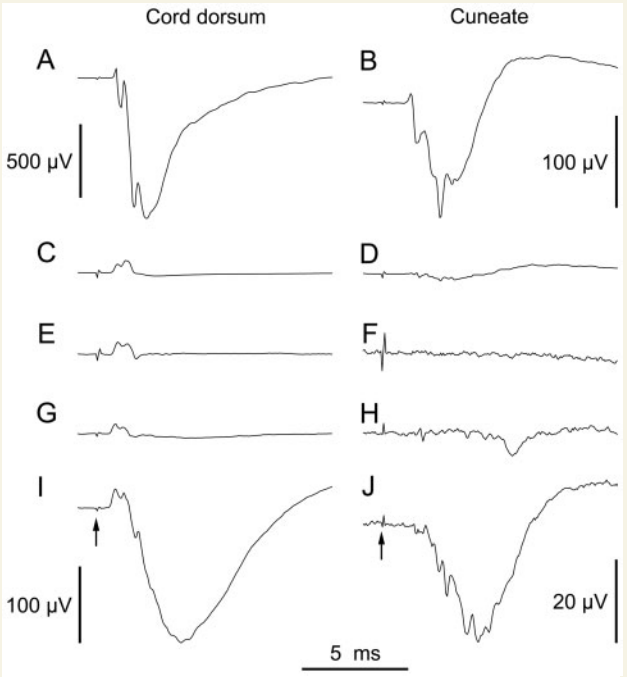

Figure 3 Electrophysiological responses. Stimulation of the median nerve at 10 times nerve threshold (stimulation time indicated by vertical arrows). Simultaneously recorded responses from the cord dorsum of C6 or 7 (left column) and the cuneate nucleus (right column). (**A** and **B)** normal animal; (**C** and **D)** the same animal after acute section of DRs C4-T2; (**E** and **F)** a rat with chronic rhizotomy without transplant; (**G** and **H)** a transplanted rhizotomized rat showing no recovery of grasping ('non-responder'); (**I** and **J)** a transplanted rhizotomized rat showing good restoration of grasping ability. The upper calibrations apply to **A**–**D**, the lower calibrations apply to **E**–**J**. Responses are displayed with negative down. Responses averaged (110–272 per trace).

performance of the rats. In four normal controls before and after acute rhizotomy, in four rats with chronic rhizotomy without transplants and in eight rhizotomized rats with transplanted cells, the median nerve was stimulated under urethane anaesthesia once per second at 10 times threshold, as monitored by a nerve volley recorded in the upper forelimb. Recordings were made at the cord dorsum from C4 to T2 and over the cuneate nucleus.

Normal rats

In the four unoperated rats with intact roots, median nerve stimulation yielded cord dorsum potentials (CDPs, Fig. 3A), comprising a small initial positivity, followed by a negativity representing a conducted volley plus synaptic activity in the dorsal horn (Willis, 1980). CDPs were present from T2 to C4, and were maximal at C6 or C7 (amplitudes 440–1020 μV, mean 761 μV). Cuneate recordings made in two of the normal rats showed evoked potentials in both (Fig. 3B). The complex waveforms (amplitudes 125 and 133 μV) represent a combination of ascending volleys plus synaptic activity (Andersen *et al*., 1964; Sen and Møller, 1991).

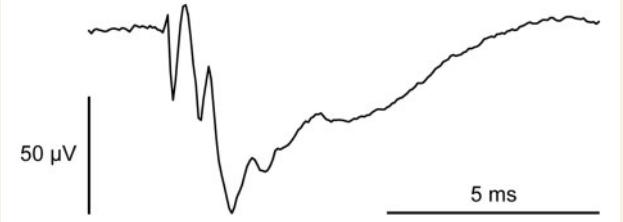

Figure 4 Cord dorsum potential recorded at C6 from median nerve stimulation in a normal rat after sectioning DRs C5-T1, but with about 20% of the rostral end of C7 spared. In fact, a subsequent recording after section of the remaining roots at C7 revealed a small response transmitted via unintentionally spared rootlets in C5/6, so the response above is shown after subtraction of this later recorded response.

Rats with acute or chronic rhizotomy

In the four normal rats, acute transection of all DRs from C5 to T1 completely eliminated the CDP negativity in two, but left small synaptic potentials of 2 μV and 22 μV at C6 in the other two (Fig. 3C). In the first of these rats the cuneate evoked potential was completely abolished, and in the second it was reduced to an amplitude of 7 μV (Fig. 3D).

Electrophysiological data were recorded from four rats which had chronic C6 to T1 rhizotomy without transplants (at survival times of 6–8 weeks). These rats had not recovered grasping and had high fault scores. No negativity was recorded in the CDP (Fig. 3E). Three of these were tested at the cuneate, and no evoked potential could be detected (Fig. 3F). In all eight rhizotomized rats, the initial positive waves remained, representing remote recordings from the cut ends of the DRs (Lorente de Nó, 2008).

Rats with OEC transplants

Recordings were made in eight rhizotomized rats with OEC transplants at survival times of 6–8 weeks. Six of these had restored grasping. The C5 DR was transected at the time of recording. In all animals the CDP showed initial positivities, followed in seven rats by a negative wave, present from C5 to T1, maximal at C6 or C7, but with a notably slower time course than normal, the mean duration at half amplitude being 4.71 ± 0.37 ms as compared to 1.88 ± 0.17 ms for the four normal rats (Fig. 3I and A). Of the six rats that had shown return of grasping five showed a negative CDP wave of amplitude 23–174 μV, mean 114.4 μV. The two rats in which grasping had not been restored, showed amplitudes of six and 37 μV. Recordings were made from the cuneate in four of the eight transplanted rhizotomized rats. All four had synaptic responses, but of longer latency and slower time course than normal. Three rats that had recovered grasping showed responses with amplitudes of 15, 27 (Fig. 3J) and 54 μV. The fourth, which had not recovered grasping, showed a response of lower amplitude, 7 μV (Fig. 3H).

Control for spared fibres

The possible presence of even a small number of spared fibres in the operated animals, as suggested by the small residual responses

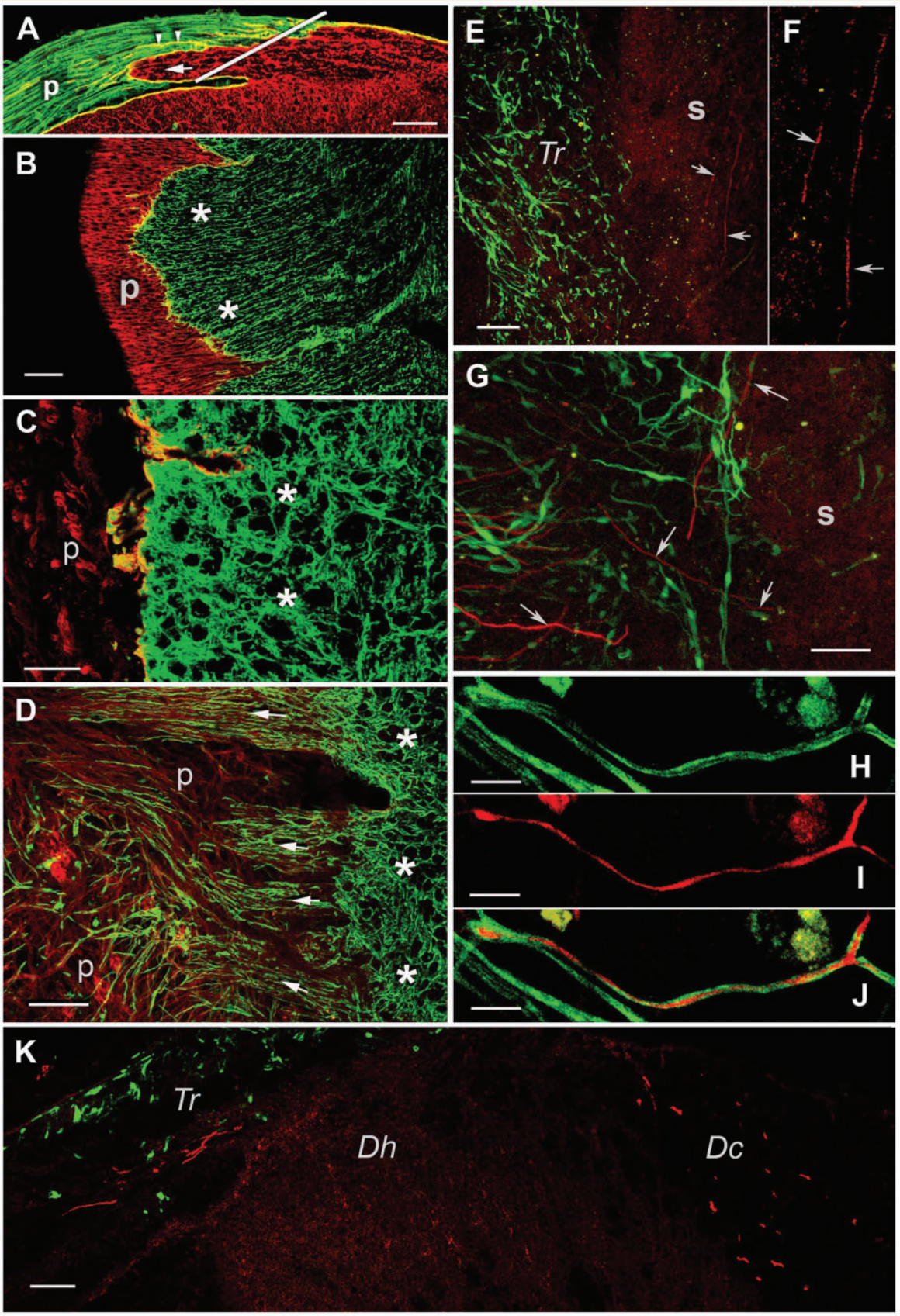

Figure 5 Histology of normal, deafferented and repaired DRs. **A–D**: interaction of the GFAP positive astrocytes (green) of the spinal cord with the laminin positive tissues (red) of the pia and peripheral nerve (colours reversed in A). **E–K**: from transplanted rhizotomized rats show the relationship of the transplanted cells (GFP fluorescence, green) with the DR axons (red; labelled by

in two of the acutely lesioned animals, is clearly a matter of concern for the interpretation of recovery of function in terms of regeneration. Control measurements were, therefore, made to put such responses in context. In one animal, the C5-T1 DR section was, at first, left incomplete. A small part (~20%) at the rostral end of C7 was left intact, chosen to be approximately equivalent to the spared part of one root in Fig. 4 of Ibrahim *et al*. (2009). The negativity transmitted by this spared rootlet was then seen to be was 78 μV in amplitude, recorded at C6 (Fig. 4). Since the amplitude of the negativity before root section was 950 μV, this survival of 8% in the CDP corresponds well to 20% of one root out of four or five originally transmitting (see Discussion section).

In summary, of the eight rhizotomized rats with transplants, six showed substantial spinal cord and/or cuneate responses to stimulation of the median nerve. In contrast, in the eight rhizotomized rats without transplants, none of the four rats with chronic rhizotomy showed any response, and of the four acutely rhizotomized rats two showed a very small response.

Histology

A full description of the histological findings is beyond the scope of this article, and will be published separately. Continuous serial sections were taken through the entire series of DRs from C6 to T1 and the adjacent dorsal spinal cord, mainly in the longitudinal plane, in some cases in cross section. Neurofilament immunohistochemistry was used to confirm the completeness of the rhizotomies (for illustration see Ibrahim *et al*., 2009).

For the present study, we describe the key histological events in the rhizotomized rats with functionally effective transplants. The data show the interaction of the peripheral and central tissues induced by the transplants (compared with the rhizotomized rats without transplants), the presence and location of the transplanted cells and the passage of the DR axons through the transplants and into the spinal cord.

The normal DR entry zone consists of domes of astrocytic tissue which extend into the peripheral nerve [Fig. 5A (arrow), B (asterisk); Fraher (1992)]. The surgical transection, in the plane shown by the line in Fig. 5A, damages these domes. Hypertrophy and loss of alignment of the astrocytic processes (asterisks in Fig. 5C and D) was seen after chronic rhizotomy, presumably induced by such damage, together with the loss of DR fibres. Transplantation of OECs into the region of re-apposition of the severed DRs stimulated a massive outgrowth of fine, reactive astrocytic processes into the transplants and DR tissue (arrows in Fig. 5D).

Use of the matrix method of transplantation (Li *et al*., 2003) effectively retained a high concentration of OECs between the cut roots and the spinal cord throughout our observation period (Fig. 5E). The transplanted cells were identified by the green fluorescence of the transduced GFP. They were located as a dense mass (Fig. 5E) incorporated into the tissue at the interface where the astrocytic processes extend out into the re-apposed DR. The transplanted cells had small ovoid cell bodies and thin elongated processes.

DR fibres were identified by biotinylated dextran amine (BD) injected into the C7 and eight DR ganglia. In favourable section planes individual axons could be traced across the transplant area and into the spinal cord (Fig. 5E–G). A proportion of the fibres can be seen to be ensheathed in a one-to-one fashion by elongated OEC processes (Fig. 5H–J). However, since in our experience the lentiviral GFP construct (Ruitenberg *et al*., 2002) labels around 70–80% of the transplanted cells, and BD injected into two DR ganglia labels only a small proportion of axons carried by the four transplanted roots we do not have direct evidence that all crossing axons had been ensheathed in this way.

Having crossed the OEC tissue bridge fine diameter DR axons arborized in the grey matter of the dorsal horn (Dh in Fig. 5K). Large diameter, longitudinally arranged axons were dispersed in the white matter of the dorsal columns (Fig. 5E and F and Dc in Fig. 5K), which is the normal location of ascending and descending

the ganglionic injection of BD). Spinal cord on the right in all figs, DR on the left; section plane horizontal in B-I, coronal in A and K. Confocal images. Survival, 6–8 weeks. **(A** and **B)**. Normal cervical DR (cut in coronal plane of spinal cord in **A** and longitudinal in **B**) showing domes of aligned astrocytic tissue (arrow in **A**; asterisks in **B**) extending into the peripheral nerve tissue (p). Line in A shows plane of surgical transection; downward arrow heads indicate a 'streamer' of astrocytic tissue extending from the dome into the peripheral nerve. Note: in A the colours are reversed (astrocytes red, peripheral tissue green) compared with **B–D**. Overlap in yellow. (**C**) Higher power view of original entry region. The root has been cut along the line shown in A. This damages the astrocytic domes projecting into the DR. Together with the loss of the afferent DR fibres, this lesion induces hypertrophy and loss of alignment of the underlying astrocytes (asterisk). The pial surface becomes sealed over the lesion (yellow, due overlap of green GFAP and red laminin). p, free cut ends of detached DR fascicles. (**D**) Low power view to show massive, transplant-induced outgrowth (arrows) of long fine astrocytic processes from the hypertrophic region (asterisks) and projecting through the region of transplanted OECs into the laminin immunoreactive tissue (red) of the re-apposed DR (p). (**E**) Low power view of mass of transplanted GFP expressing OECs (Tr, green) in the region of the astrocytic outgrowth shown in (**D**). s, spinal cord; arrows, regenerating DR axons running vertically in the dorsal columns, enlarged in (**F**). (**G**) Higher power view to show regenerating BD labelled DR fibres (arrows) crossing the zone where the GFP expressing OECs are apposed to the spinal cord (s). (**H**). Typical elongated process of a transplanted OEC with a central 'hollow' (clear) lumen containing a single BD labelled regenerating DR axon (red) in (**I**); overlap in (**J**). (**K**) Montage of cross sections of the spinal cord. The GFP positive transplanted cells (Tr) are apposed to the surface of the dorsal horn (Dh) which contains very fine labelled axon arborizations (merging with background at this low magnification). A number of large intensely BD labelled regenerating axons, seen in cross section, run vertically in the dorsal white columns (Dc). Such axons would have been responsible for the postsynaptic responses in the cuneate nucleus on stimulation of the median nerve. Scale bars = 100 μm in **A**, **B**, **D**, **E** and **K**; 50 μm in **C** and **G**; 10 μm in **H–J**.

branches of DR afferent fibres. These axons are presumably the mediators of the restored responses seen electrophysiologically on stimulation of the median nerve and the return of grasping function by the fore-paw.

Discussion

There have been a number of recent studies of DR repair. They include different methods of injury [cut (Li *et al*., 2004; Riddell *et al*., 2004; Ramón-Cueto and Nieto-Sampedro, 1994) versus crush (Ramer *et al*., 2000; Wang *et al*., 2008)]. In our present study, we have chosen the option of cutting the roots to minimize the possibility of fibres being spared.

Of the different types of intervention (e.g. transplantation of cells Ramón-Cueto and Nieto-Sampedro, 1994; Li *et al*., 2004; Riddell *et al*., 2004), peripheral nerve injury Neumann and Woolf (1999), electrical stimulation Udina *et al*. (2008) or application of growth factors (Bradbury *et al*., 1999; Ramer *et al*., 2002; Steinmetz *et al*., 2005; Wang *et al*., 2008), we have chosen transplantation of OECs because of previous positive results with these cells in repair of intraspinal tracts and DRs.

The outcome measures of repair include anatomical demonstration of regeneration of DR axons (Li *et al*., 2004; Ramón-Cueto and Nieto-Sampedro, 1994), electrophysiological evidence of transmission (Ramer *et al*., 2000; Steinmetz *et al*., 2005; Wang *et al*., 2008) and/or behavioural recovery (Ramer *et al*., 2000; Wang *et al*., 2008). Functional outcomes have included modality specific observations (Wang *et al*., 2008). In the present study, we show that the rats can integrate the restored sensory inputs so as to perform a complex, goal-directed behaviour.

In the previous paper (Ibrahim *et al*., 2009), we showed that unilateral transection of the four consecutive DRs from C6 to T1 permanently disables the ipsilateral fore-paw both for locating and for grasping the frame bars during a climbing test. In the present study, we show that transplantation of OECs into C6 to T1 rhizotomies leads to a progressive restoration of fore-paw grasping over a 2-month observation period, with a concomitant reduction in faults in locating the bars. This functional recovery is associated with regeneration of DR axons into the spinal cord and resumption of synaptic transmission to the grey matter of the dorsal horn and the cuneate nuclei (Massey *et al*., 2008).

Commenting on the minimal regeneration of DR axons seen after injection of OECs into a re-anastomosed L4 DR entry zone and adjacent dorsal horn Riddell *et al*. (2004) suggested that 'simply injecting cells may not be the most effective means of achieving a high density of cells at the critical CNS-PNS interface'. The more consistent regeneration seen in the present study suggests that the matrix method of transplantation (Li *et al*., 2003) may provide a more effective placement of the cells at the interface.

Our assignment of afferent axon regeneration to be the substrate for the observed restoration of function is based on: (i) the failure to observe any spared fibres histologically; (ii) the complete surgical separation of the roots from the spinal cord becoming bridged by a mass of transplanted OECs through which BD labelled DR axons cross into the spinal cord; (iii) the absence of electrophysiological responses in the chronically rhizotomized rats without transplants; and (iv) further quantitative arguments, below, related to the likely sizes of responses from spared fibres

The CDP shown in Fig. 4 was 78 μV in amplitude, close to the median value for the rhizotomized rats with transplants. A spared bundle similar to the one which produced this would have been easily detected in the histology of the operated animals (as it was in Ibrahim *et al*., 2009), but nothing was seen even approaching this size. The two observed residual CDPs after rhizotomy at the time of recording (2 and 22 μV) represented 0.4 and 2.3%, respectively of the CDPs before root section, and thus were also much smaller than the 8% represented by the response in Fig. 4. The amplitudes of the CDPs in the rhizotomized rats with transplants were up to eight times larger than even the largest of these residual CDPs. Moreover, these measurements of response amplitude in the transplanted rats probably underestimate the strength of synaptic input that they represent, in comparison with normal animals, because (i) the response durations were also on average 2.5 times greater than normal; and (ii), as explained in Methods, the recording electrode in the transplanted animals could not be ideally positioned.

Moreover, although two of the acute lesions had small responses (minute in one case), none of the four chronically rhizotomized rats investigated electrophysiologically showed any responses. Together, these considerations indicate that any fibres that might have been spared in the transplanted animals would have needed to have amplified their effectiveness (e.g. by collateral sprouting) by at least an order of magnitude to have produced the observed responses. We suggest this is an unlikely occurrence.

We cannot give a definitive explanation for the failure of 10 of the transplants to restore function or electrical transmission. There is considerable individual variation in the pattern of the DRs; the operation requires two surgeons cooperating in order to place the cells so as to accurately fill and close the gap and is currently on the limits of microsurgical skill. Failure would occur if the transplanted cells had not survived, or the gap between the roots and the spinal cord had not been physically bridged.

There is a widely held view that a major factor preventing regeneration of connections in the adult CNS is the presence of axon growth inhibitory molecules associated with myelin (Schnell and Schwab, 1993; Fournier and Strittmatter, 2001). The present observations indicate that once the regenerating DR fibres are able to enter the CNS, they follow quite complex and correct routes, including long distance elongation through and dispersed in a myelinated tract (see also Davies *et al*., 1997, 1999), and they form functional contacts in a distant synaptic destination.

Clinically, the current practice of surgical repair of brachial plexus avulsion by re-implantation of avulsed roots via a peripheral nerve graft provides a degree of motor recovery that is sometimes of functional use but can be disturbed by synkinetic activity. Useful hand function is mostly not restored in adult patients. There is no recovery of sensation, although pain is usually alleviated for the re-implanted segments (Carlstedt *et al*., 2000; Kato *et al*., 2006; Carlstedt, 2007). Our present rat study indicates that transplantation of OECs into the re-implantation site in severed DRs is able to restore the sensory input needed to re-establish

the fine control of the distal musculature of the fore-paw required to perform a functionally significant task.

Whereas the clean cuts used in the rat study do not reproduce the combined pattern of neural and vascular trauma to the peripheral nerve and the dorsal horn which are features of clinical avulsion, the present findings suggest that OEC transplants may provide a route for improving hand function in patients with brachial plexus injury.

A number of groups, including our own, are currently studying the properties of human OECs (Féron *et al*., 1998; Barnett *et al*., 2000; Bianco *et al*., 2004; Miedzybrodzki *et al*., 2006; Choi *et al*., 2008*a*, *b*) with a view to translation to a clinical trial, and various different OEC preparations have already been used in human spinal cord injury (Huang *et al*., 2003; Féron *et al*., 2005; Lima *et al*., 2006). Success in a first clinical application of OECs in brachial plexus repair would open the way to a future extension of the benefits of this approach in the wider field of spinal cord injury, subcortical strokes and cranial nerve lesions.

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