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Autologous olfactory mucosal cell transplants in clinical spinal cord injury: a randomized double-blinded trial in a canine translational model

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This study was designed to determine whether an intervention proven effective in the laboratory to ameliorate the effects of experimental spinal cord injury could provide sufficient benefit to be of value to clinical cases. Intraspinal olfactory ensheathing cell transplantation improves locomotor outcome after spinal cord injury in 'proof of principle' experiments in rodents, suggesting the possibility of efficacy in human patients. However, laboratory animal spinal cord injury cannot accurately model the inherent heterogeneity of clinical patient cohorts, nor are all aspects of their spinal cord function readily amenable to objective evaluation. Here, we measured the effects of intraspinal transplantation of cells derived from olfactory mucosal cultures (containing a mean of ~50% olfactory ensheathing cells) in a population of spinal cord–injured companion dogs that accurately model many of the potential obstacles involved in transition from laboratory to clinic. Dogs with severe chronic thoracolumbar spinal cord injuries (equivalent to ASIA grade 'A' human patients at ~12 months after injury) were entered into a randomized double-blinded clinical trial in which they were allocated to receive either intraspinal autologous cells derived from olfactory mucosal cultures or injection of cell transport medium alone. Recipients of olfactory mucosal cell transplants gained significantly better fore–hind coordination than those dogs receiving cell transport medium alone. There were no significant differences in outcome between treatment groups in measures of long tract functionality. We conclude that intraspinal olfactory mucosal cell transplantation improves communication across the damaged region of the injured spinal cord, even in chronically injured individuals. However, we find no evidence for concomitant improvement in long tract function.

Keywords: spinal cord injury; spinal cord injury repair; spinal cord plasticity; models
Abbreviation: ASIA = American Spinal Injury Association

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Introduction

Spinal cord injury has a devastating impact on the emotional and physical well-being of affected individuals and imposes a substantial financial burden on providers of long-term health care (National Spinal Cord Injury Center, https://www.nscisc.uab.edu/). Many interventions have demonstrated beneficial effects on outcome after spinal cord injury in 'proof of principle' experiments in laboratory animals, including pharmacological and biological agents (Kwon *et al.,* 2011*a*, *b*; Tetzlaff *et al.,* 2011), training (Edgerton *et al.,* 2008) and rehabilitation (Wang *et al.,* 2011) strategies. Of these, intraspinal transplantation of olfactory ensheathing cell is one of the most promising (Ibrahim *et al.,* 2006; Ramon-Cueto and Muñoz-Quiles, 2011).

However, although experimental models of spinal cord injury are designed to develop new therapies, success in laboratory animals will not necessarily translate into benefit for patients because of the many differences between models and clinical disease. Firstly, most human patients will be candidates for transplantation therapy after a variable, and often considerable, delay from initial injury. Secondly, there is far greater heterogeneity within even a selected patient cohort than within a typical experimental group of purpose-bred rodents of similar age, mass, injury severity and genetic background, which will tend to obscure detection of the effect of interest. Thirdly, the magnitude of the treatment effect, even if statistically detectable, may not be of sufficient magnitude to be clinically meaningful to patients. The potential of these factors to obstruct effective translation has recently received increasing attention in other fields of medicine (Lowenstein and Castro, 2009; Begley and Ellis, 2012) but can be difficult to discern using conventional laboratory models.

Spinal cord injury is a common condition in companion dogs because of the high prevalence of intervertebral disc degeneration and acute nuclear herniation (Priester, 1976; Fluehmann *et al.,* 2006), resulting in a large population in which the effects of putative therapeutic interventions can be tested. These dogs are evaluated and treated by specialist veterinarians using similar methods to those currently available in human patients with spinal cord injury. To conduct a clinical trial of a putative therapy for spinal cord injury in dogs, we have developed a series of kinematic outcome measures to objectively quantify the effect of an intervention on locomotor function (Hamilton *et al.,* 2007, 2008). Objective quantification of gait coordination in dogs is facilitated by their quadrupedal gait because it allows analysis of the temporal relationships of motion between thoracic and pelvic limbs.

This study constitutes the first double-blinded randomized trial of the efficacy of intraspinal transplants of cells derived from olfactory mucosa cell cultures (containing ~50% p75+ olfactory ensheathing cells) in clinical spinal cord injury and was conceived as a means to address potential obstacles involved in translating a putative therapy from laboratory to clinic. The subjects were a sample of dogs in the chronic phase of the injury recruited from the large population of client-owned (i.e. non-experimental) dogs that had previously sustained severe acute spinal cord injury. This phase was chosen because their established poor prognosis (Olby *et al.,* 2003) greatly increases the efficiency of detection of benefits (Fawcett *et al.,* 2007). The effects of intraspinal autologous olfactory mucosal cell transplantation were compared with injection of cell transport medium alone using a primary outcome measure of forelimb–hindlimb coordination obtained from detailed objective analysis of locomotor coordination; a series of secondary outcome measures were also analysed, with the aim of elucidating the mechanisms by which olfactory mucosal cell transplants might exert an effect.

Materials and methods

Study design and canine subjects

Candidate companion dogs with severe spinal cord injury were recruited for this study between October 2008 and March 2011 through professional contacts, presentations at veterinary conferences and notices on websites. For inclusion, we specified that dogs should weigh <20 kg, have had an acute spinal cord injury located between T10 and L4 spinal cord (the canine spinal cord has seven lumbar segments and terminates at ~L5 vertebra) and had recovered neither conscious pain perception (i.e. no behavioural response to intensely noxious stimuli applied to the hindquarters) nor the ability to ambulate using the hindlimbs, i.e. equivalent to thoracic ASIA (American Spinal Injury Association) 'A' human patients—by at least 3 months after the initial injury. Available literature (Olby *et al.,* 2003; Fawcett *et al.,* 2007) suggests that this time delay is equivalent to ~12 months after injury in human patients. We use the term 'ability to ambulate' here because some dogs will develop reflex hindlimb stepping during treadmill or supported overground walking after complete thoracolumbar spinal cord injury (Handa *et al.,* 1986). Trial exclusion criteria were intercurrent disease requiring constant medication; generalized ill health with a prognosis for survival of <12 months; orthopaedic disease that would preclude accurate locomotor assessment; or unsuitable temperament.

The study was conducted under the regulations contained within the Veterinary Surgeons Act (1966); both the interventions were given with therapeutic intent (we had previous data suggesting an immediate effect after intraspinal injections), and the study protocol was reviewed and approved by the Royal College of Veterinary Surgeons and the Ethical Review Committee of the Department of Veterinary Medicine, University of Cambridge, where the study was carried out. The owner of each dog gave written consent to the procedures after being informed of the nature of the study, the comparator groups and the possibility that neither intervention would have a beneficial effect; our previous study had confirmed that transplantation of olfactory bulb-derived cell cultures was safe in dogs (Jeffery *et al.,* 2005).

Randomization and masking

The study was conducted according to CONSORT guidelines (Consolidated Standards of Reporting Trials), (Consort statement, http://www.consort-statement.org/consort-statement/), including a detailed definition of primary and secondary outcome measures before trial onset; subject flow through the study is illustrated in Fig. 1. Consecutive patients who met all of the inclusion criteria and none of the exclusion criteria were admitted to the study. After preparation of the autologous cell culture for transplantation, each dog was anaesthetized, spinal needles were placed into the lesion site (see later in the text), and was then randomized to receive intraspinal injection of either an olfactory mucosal cell suspension or cell transport medium alone. Randomization was achieved through opening a sealed opaque envelope, each containing the word 'cells' or 'medium'. Three blocks of 18

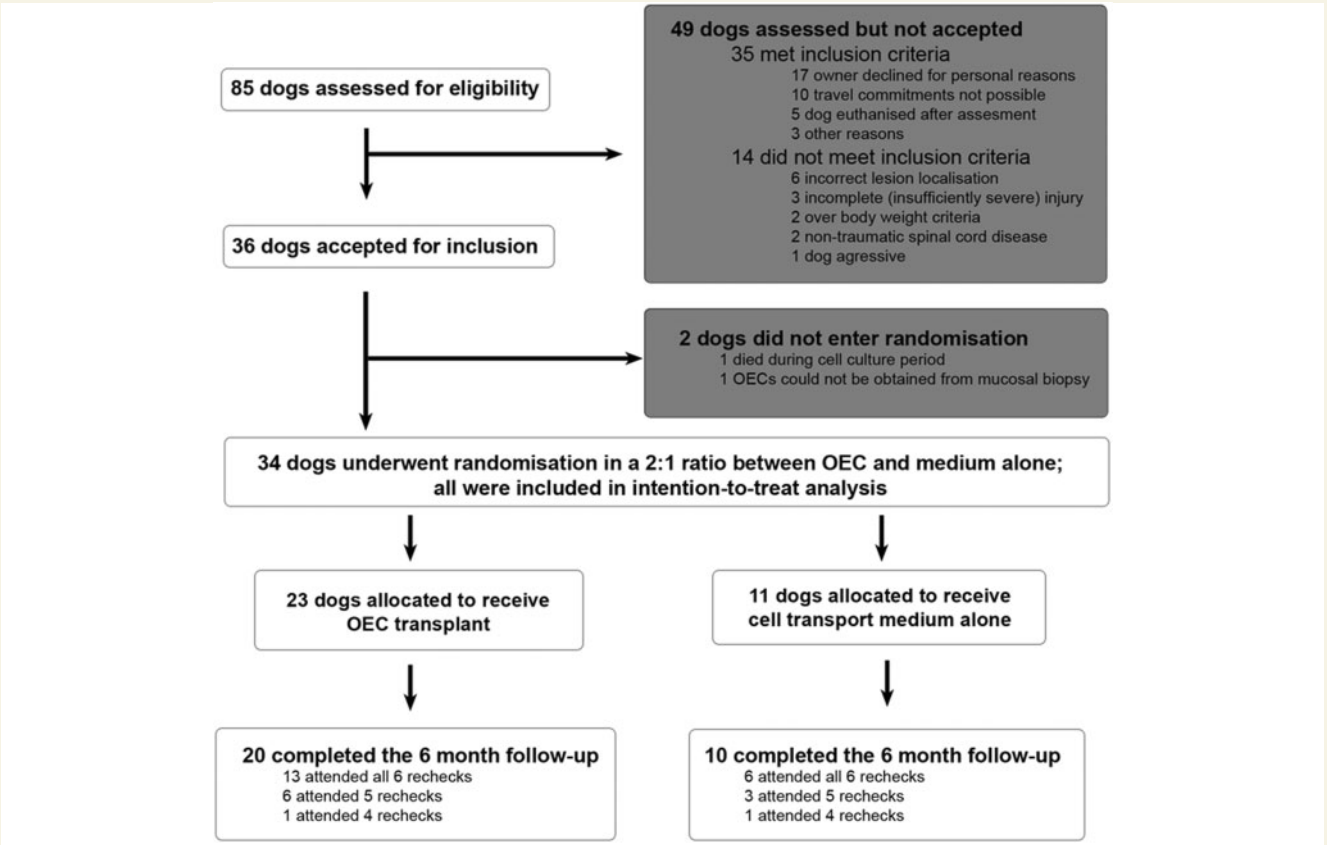

Figure 1 Flow chart to summarize patient recruitment, assessment and follow-up. OMC = olfactory mucosal cell.

envelopes were prepared (of which only two were used, owing to time and financial constraints), each of which contained 12 envelopes containing 'cells' and six containing 'medium'. After sealing, the envelopes were shuffled by hand and kept in a locked drawer with access only available to the unblinded investigator (N.D.J.) who administered the different treatments. We chose a 2:1 ratio because we anticipated that the cell transplant might have effect on only a proportion of transplanted animals. Owners were blinded to treatment allocation until the end of the study period (6 months), and the clinician and technician responsible for recording the outcome measures were blinded to intervention allocation until completion of data analysis.

Procedures

On entry to the study, each dog was examined by a specialist veterinary neurologist (N.D.J. or N.G.) and then underwent standard T_1- and T_2-weighted magnetic resonance imaging scanning (0.2 Tesla Vet MR, Esaote) of the affected region, treadmill gait recording (see later in the text), recording of somatosensory- and transcranial magnetic motor-evoked potentials and urodynamics assessment before receipt of the randomly allocated treatment (given at time = 0).

Gait analysis

Gait analysis was carried out as previously described (Hamilton *et al.*, 2007, 2008). Briefly, dogs walked on a treadmill with retroflective markers placed on prominent bone landmarks on forelimbs and hindlimbs while recordings were made of limb motion using gait analysis equipment (Qualisys). Digital data were then processed through MATLAB software to extract points of interest to determine (i) coordination between hindlimb and forelimb movements in the sagittal plane, here termed 'fore–hind coordination' and used to determine connectivity across the lesion site; and (ii) reproducibility of hindlimb paw placement in the lateral plane, assessed using the coefficient of variation of the distance between hindlimb paws, here termed 'lateral stability' and used to determine coordination between hindlimb paws and body position. Because it is conceivable that a specific treadmill speed might happen to correspond to the reflex stepping rhythm of the hindlimbs (which is almost constant despite different treadmill speeds)—and also correspond to the voluntary stepping rhythm of the forelimbs—we recorded locomotion at two speeds. For analysis of fore–hind coordination, we used only the data from the speed at which there was worse coordination (thereby eliminating the potential for 'entrainment' to produce inaccurate data). The calculated score for comparison summarized the temporal relationship between each hind paw placement and that of the contralateral fore paw during treadmill stepping; each limb pair was analysed separately, the mean score determined and entered into statistical analysis. During recovery from incomplete spinal cord injury in dogs, clinically observable improvement in locomotion is paralleled by gradual recovery of both fore–hind coordination and lateral stability towards normal values (Jeffery *et al.*, 2011).

Somatosensory-evoked potentials

Somatosensory-evoked potentials were recorded from both hindlimbs of each dog under sedation using standard techniques (Poncelet *et al.*, 1993) of stimulation of the tibial nerve at the hock (ankle) and recording over the contralateral sensory cortex, using signal averaging (256 sweeps).

Transcranial magnetic motor-evoked potentials

Transcranial magnetic motor-evoked potentials were recorded from both hindlimbs of each dog under sedation using established techniques (Sylvestre *et al.,* 1993; Da Costa *et al*., 2006); the single coil stimulator (Magstim 200) was positioned over the motor cortex and discharged, while recording from the cranial tibial muscle.

Urodynamics

Without sedation, bladder cystometry with combined rectal pressure measurement recordings were made using conventional urodynamic equipment (Life-Tech, Urolab Primus 6). Compliance was measured as defined by the International Continence Society (Abrams *et al.,* 2003) as the change in volume divided by the change in pressure at full bladder physiological capacity or first leak.

Cell harvest and culture

After initial gait, electrophysiological and urodynamic recordings were completed, each dog underwent general anaesthesia and the left frontal sinus of the skull was opened aseptically to access the olfactory mucosa lying within the ostium of the frontal sinus and the caudal nasal cavity (Skinner *et al.,* 2005). The small fragments of obtained olfactory mucosa were placed in 20 ml of Leibovitz sterile medium (L15) in a Petri dish, pre-cooled at 4°C, kept on ice and transported from the operating room to the laboratory to be processed. The mucosal biopsies were dissected on ice under a stereomicroscope to remove cartilage fragments, blood vessels, connective tissue and non-olfactory mucosa. The remaining olfactory mucosa was then chopped with a scalpel blade for 1 min and dissociated for 15 min at 37°C, in 1 ml of Dulbecco's modified Eagle medium containing 0.25 mg of 112 U collagenase. After incubation, 1 ml of Dulbecco's modified Eagle medium containing 0.5 mg of bovine pancreas DNase was added and the tissue was triturated for 1 min through glass pipettes, filtered (40 -μm filter), rinsed in 10 ml Dulbecco's modified Eagle medium containing 10% foetal bovine serum and centrifuged (1000 rpm, 5 min). The pellet was resuspended in fresh culture medium containing Dulbecco's modified Eagle medium, 10% foetal bovine serum, 2 μM forskolin in dimethyl sulphoxide, 20 ng/ml neuregulin 1 and 10 mg/ml gentamicin and seeded (10 000 to 50 000 viable cells/ml) onto poly-L-lysine–coated 25-cm^2 flasks. Cultures were maintained at 37°C in 5% carbon dioxide. After initial plating, cultures were undisturbed for 5–7 days to allow cell adhesion. Thereafter, one half of the growth medium was renewed every 3 days. For cell passaging, trypsin solution (2.5% in PBS) was used to detach the cells. Cells of interest were selected by observing detachment of elongated cells under a microscope and blocking the trypsin at this time point (usually <2 min) to prevent further detachment of contaminant cells (i.e. differential trypsinization). The obtained cells were plated to new uncoated 25-cm^2 flasks using the medium described earlier. After replating, the uncoated flasks were left for 24 h and then mechanically agitated to detach elongated cells, as described previously (Ito *et al.,* 2008). The supernatant was plated on new poly-L-lysine–coated 25-cm^2 flasks. After the first 7–10 days in culture, the foetal bovine serum concentration was gradually reduced from 10% to 2.5% over 7 days, depending on cellular growth. When a sufficient number of cells was reached, the cells were plated on larger (75 cm^2 and T175 cm^2) flasks. On the day of transplantation, the cell population was analysed to determine the proportion of p75+ cells and the total population number. We aimed to provide a population of at least 5×10^6 cells, containing at least 2.5×10^6 p75+ cells (i.e. those previously identified as olfactory ensheathing cells in nasal mucosal cultures) (Ito *et al.,* 2008) at the time of transplantation.

When the required number of cells was ready for transplantation (between 3 and 5 weeks after harvesting), each dog was re-anaesthetized and positioned for fluoroscopy (using an image intensifier with a resolution of 441 pixels/cm at the centre of the screen) to guide placement of 20–22-gauge 90-mm long spinal needles percutaneously through the interarcuate ligaments between the vertebral laminae into the affected region of the spinal cord identified previously by MRI. For each dog, three needles were placed: one between the vertebrae overlying the epicentre of the lesion and one each at the neighbouring intervertebral spaces. The needles were inserted through the spinal cord parenchyma to reach the ventral aspect of the vertebral canal and this position was ascertained using fluoroscopy and observing cerebrospinal fluid dripping from the needle hub (and, in some cases, injections of radiographic contrast agent were used to ascertain correct positioning within the vertebral canal). After determining their correct location within the vertebral canal, the needles were withdrawn a few millimetres so that the needle bevel was contained within the centre of the vertebral canal (and therefore within the spinal cord parenchyma). At this stage, dogs were randomized (in a 2:1 ratio of olfactory mucosal cell: medium alone) to receive either olfactory mucosal cell in cell transplant medium or the same volume of cell transport medium alone, injected directly into the parenchyma of the spinal cord. The injection volume was 400 μl in total, delivered over a period of ~5 min: 200 μl injected at the epicentre, divided into two boluses: 100 μl with the needle bevel directed cranially and 100 μl with the needle bevel directed caudally, plus a single bolus of 100 μl at each neighbouring space, with the needle bevel directed towards the epicentre. On recovery, each dog received routine analgesia and was observed for possible adverse effects for 24 h before returning to the owner's care.

Outcome measure assessment

Each dog was returned at 1-monthly intervals for clinical neurological examination and reassessment of locomotor performance on the treadmill, somatosensory-evoked potential and transcranial magnetic motor-evoked potential recording. Urodynamic assessments were made at 1, 3 and 6 months after spinal cord injection.

Statistical analysis

Pre-study power calculations based on responses in a Phase I trial (Jeffery *et al.,* 2005) suggested the need for 54 dogs in total. To detect 25% improvement to a set value in the olfactory mucosal cell group versus a 0% improvement in the control group at 80% power and $P = 0.05$, produced a projected $n = 48$. Correction for the 2:1 randomization ratio suggested the need for 54 dogs in total. However, here we planned numerical measurements of locomotor coordination and analysis by multivariable linear regression, which increases analytical efficiency, as all of the repeated-measures data can be included and correction for effect modifiers, confounders and covariates (importantly here, the baseline values) is readily incorporated.

Our pre-specified primary outcome measure was a summary of fore–hind coordination, defined as the mean of the score derived for the 'cumulative lag' between each hindlimb/contralateral forelimb pair. Data were logarithmically transformed to normalize distributions. Statistical analysis was preplanned to use group (olfactory mucosal cell transplantation versus medium injection alone) and visit (time) as variables in a random effects model multivariable linear regression analysis for cross-sectional time series data in Stata 11 (command 'xtreg') and adjusting for pre-intervention values by their inclusion as a covariate (i.e. analysis of covariance). A two-sided $P < 0.05$ was

taken as evidence to reject the null hypothesis. Pre-specified secondary outcome measures were lateral stability during treadmill walking (Hamilton *et al.,* 2008), somatosensory-evoked potential, transcranial magnetic motor–evoked potential and bladder compliance.

Use of a kinematic variable as the primary outcome produces objective data that allow rigorous quantitative analysis but do not intuitively translate into a sense of value of the effect in treated cases. This can be addressed through analysis of the relationship between intervention effect and the 'minimal important clinical difference' or similar score-based schemes. Such schemes are best derived from validated quality-of-life instruments because they relate a subjective impression to the objectively measured outcome, but this renders them unsuitable for non-verbal species. Instead, distribution-based methods (Wyrwich and Wolinshy, 2000) can be used, and here we summarized the magnitude of the effect comparing groups (δ) using the formula $\delta = m_2\_m_1/\sigma_1$; where m_2 and m_1 are the mean values of coordination score in the two groups at study termination and σ is the standard deviation (SD) of the whole sample population at baseline.

Finally, in cell transplant recipients, we examined the relationship between functional recovery and the proportion of p75+ cells (assumed to be olfactory ensheathing cells) in the transplanted culture using regression analysis, again controlling for pre-transplant score by inclusion as a covariate.

Results

Eighty-five companion dogs with severe spinal cord injury of at least 3 months' duration were assessed for eligibility for the study, and 34 were entered as randomized subjects (Fig. 1). The characteristics of the dogs and their baseline scores are listed in Tables 1 and 2; 31 of the 34 dogs incurred spinal cord injury as a result of acute intervertebral disc extrusion—the most common cause of acute contusive spinal cord injury in dogs (Fluehmann *et al.,* 2006). The breeds included in this study are typical of those affected by acute intervertebral disc extrusion. All patients when first presented for this study had no volitional motor function or pain sensation to the pelvic limbs (i.e. equivalent to ASIA grade 'A' in humans).

Dogs were randomly allocated to receive intraspinal injections of either autologous olfactory mucosal cells or cell transport medium alone in a 2:1 ratio (Fig. 2). All dogs entered into the study ($n = 34$: olfactory mucosal cell = 23, no cell group = 11) were included in the final results, which therefore constitutes an intention-to-treat analysis. Four dogs died during the study (urinary tract infection: $n = 2$; suspected disseminated intravascular coagulation: $n = 1$; intrathoracic haemorrhage: $n = 1$), but post-mortem examination in each case indicated no evidence to link death to the intervention. Transient (<48 h) mild side-effects (mild spinal pain: $n = 2$; suspected ileus: $n = 2$) occurred in four dogs after intraspinal transplantation, but there was no evidence of long-term detrimental effects in any dog. Adverse effects are summarized in Table 3.

For the pre-specified primary outcome measure, fore–hind coordination, there was a change in score during the course of the study from a mean of 1.35 (SD = 0.71) to 0.95 (SD = 0.74) log units between the baseline and 6-month time point in the olfactory mucosal cell transplant recipients, compared with a change from 1.53 (SD = 0.94) to 1.86 (SD = 0.89) in the no-cell group.

Table 1 Baseline animal data

	Olfactory mucosal cell $n = 23$	Medium alone $n = 11$
Age, years (SD)	5.9 (2.0)	6.1 (3.0)
Number of males (%)	12 (52)	9 (82)
Weight, kg (SD)	9.6 (5.8)	8.0 (2.7)
Time between injury and intervention, months (SD)	13.0 (11.3)	15.5 (18.8)

Table 2 Baseline values for outcome measures

	Olfactory mucosal cell $n = 23$	Medium alone $n = 11$
Mean fore–hind log cumulative lag (SD)	1.59 (0.86)	1.79 (1.0)
Mean lateral stability ratio (SD)	0.41 (0.34)	0.45 (0.37)
Transcranial magnetic motor-evoked potential recordable (%)	10 (33)	3 (27)
Somatosensory-evoked potential recordable (%)	8 (33)	4 (36)
Mean bladder compliance (cm H_2O/ml) (SD)	1.66 (1.53)	2.98 (3.89)

The effect size was also calculated using the distribution-based description of the minimal important clinical difference to be 0.955, which is designated as a 'large effect' (Wyrwich and Wolinshy, 2000), and would therefore be expected to have an appreciable clinical benefit in human patients. The effect size is illustrated in more detail in Fig. 3 and as a video recording in the online Supplementary material, which together illustrate the motion analysis graphs and corresponding sequential video recordings. The transplant effect was investigated in more detail using multivariable regression analysis, controlling for baseline values by inclusion as a covariate, and demonstrated a highly significant effect of olfactory mucosal cell transplantation on hindlimb activity and fore–hind coordination {regression coefficient [β] = −0.455 [95% confidence interval (CI): −0.784 to −0.126]; $P = 0.007$; Fig. 4}. Pre-intervention score (covariate) had a significant influence [$\beta = 0.684$ (95% CI: 0.492 to 0.877); $P < 0.001$] on fore–hind coordination outcome but time after intervention, in the test population as a whole, did not [$\beta = 0.012$ (95% CI: −0.021 to 0.046); $P = 0.474$].

Dogs in the olfactory mucosal cell transplantation group were injected with a cell preparation containing 6.24 [±standard error (SE) = 0.39] × 10^6 cells in total, enriched in p75+ cells to a mean purity of 49.4% (±SE = 6.8). Therefore, the cell preparation was composed of 3.20 (±SE = 0.49) × 10^6 p75+ cells (assumed to be olfactory ensheathing cells), 2.82 (±SE = 0.49) × 10^6 fibronectin-expressing cells (likely to be fibroblasts derived from the sub-mucosa) plus 0.21 (±SE = 0.06) × 10^6 unidentified cells (Supplementary Fig. 3). In cell transplant recipients, there was no significant relationship between forelimb–hindlimb coordination and the proportion of p75+ cells contained within the transplant

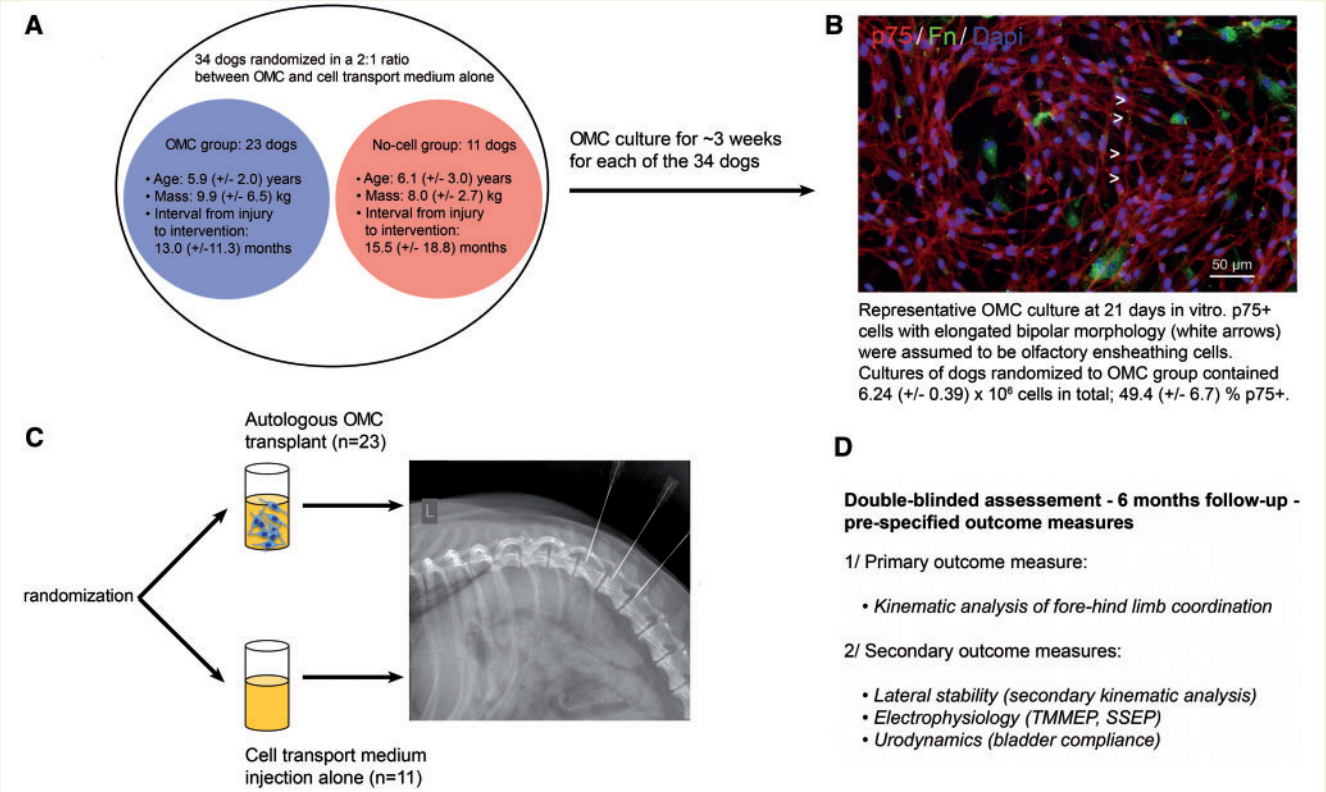

Figure 2 Flow chart to illustrate trial design. (**A**) Trial dogs were recruited from the population of companion dogs that had incurred severe spinal cord injury at least 3 months previously and had not regained behavioural evidence of pain perception from the hindlimbs or voluntary hindlimb locomotor function or urinary continence. They were randomly allocated to receive olfactory mucosal cells (OMCs) or cell transport medium alone in a 2:1 ratio. (**B**) Olfactory mucosa was harvested from each dog and olfactory mucosal cells were multiplied in culture for ~3 weeks, aiming for a total population of $\sim 5 \times 10^6$ cells containing ~50% olfactory ensheathing cells (defined as p75 + by immunocytochemistry) before transplantation—values stated here are mean ± SEM of the cell population among all 34 dogs. (**C**) Each dog received an intraspinal transplant of either autologous olfactory mucosal cells or cell culture medium alone. Transplants were injected percutaneously, using fluoroscopic guidance to ensure correct needle placement. (**D**) A blinded observer assessed outcome at monthly intervals for 6 months after intervention. Each test and statistical analysis method was pre-specified to avoid biased analysis; the primary outcome measure was objective quantification of forelimb–hindlimb temporal coordination using computerized analysis of digitized kinematic data. SSEP = somatosensory-evoked potential; TMMEP = transcranial magnetic motor-evoked potential.

Table 3 Adverse events

	Olfactory mucosal cell *n* = 23	Medium alone *n* = 11
Death (%)	3 (13)	1 (9)
Post-injection ileus (%)	2 (9)	0 (0)
Post-injection pain (%)	1 (4)	1 (9)

each dog had received when transplanted [β = −0.002 (95% CI: −0.009 to 0.005); $P = 0.52$].

We next examined a series of secondary outcome measures, designed to detect the effect of the intervention on sensory and motor spinal cord long tract function. For hindlimb lateral stability (Hamilton *et al.*, 2008), a measure of hindlimb coordination with centre of gravity, no significant effect (using multivariable regression analysis, as described previously) was found associated with olfactory mucosal cell transplantation [β = 0.049 (95% CI: −0.072 to 0.170); $P = 0.428$], but both time [β = −0.018 (95% CI: −0.034 to −0.002); $P = 0.029$] and pre-intervention score [β = 0.386 (95% CI: 0.209 to 0.563); $P < 0.001$] had a significant effect. Somatosensory-evoked potentials could be recorded over the scalp in 12 of the 34 dogs at trial entry, and the latency of this response altered very little (mean latency reduction = 0.85 ms) during the study period. An additional two dogs regained somatosensory-evoked potentials by the end of the trial, of which one had received olfactory mucosal cells. Transcranial magnetic motor-evoked potentials could be recorded in the tibialis cranialis muscle before intervention in 13 dogs. An additional three dogs regained this response during the 6-month trial period, two of which had received olfactory mucosal cells. There was no difference in incidence of recovery of somatosensory-evoked potentials or transcranial magnetic motor-evoked potentials between groups (Fisher exact test, $P = 1.00$ for each). Urinary bladder compliance, a measure of CNS modulation of bladder activity (Biering-Sørensen *et al.*, 2008), was determined in 31 dogs throughout the trial and

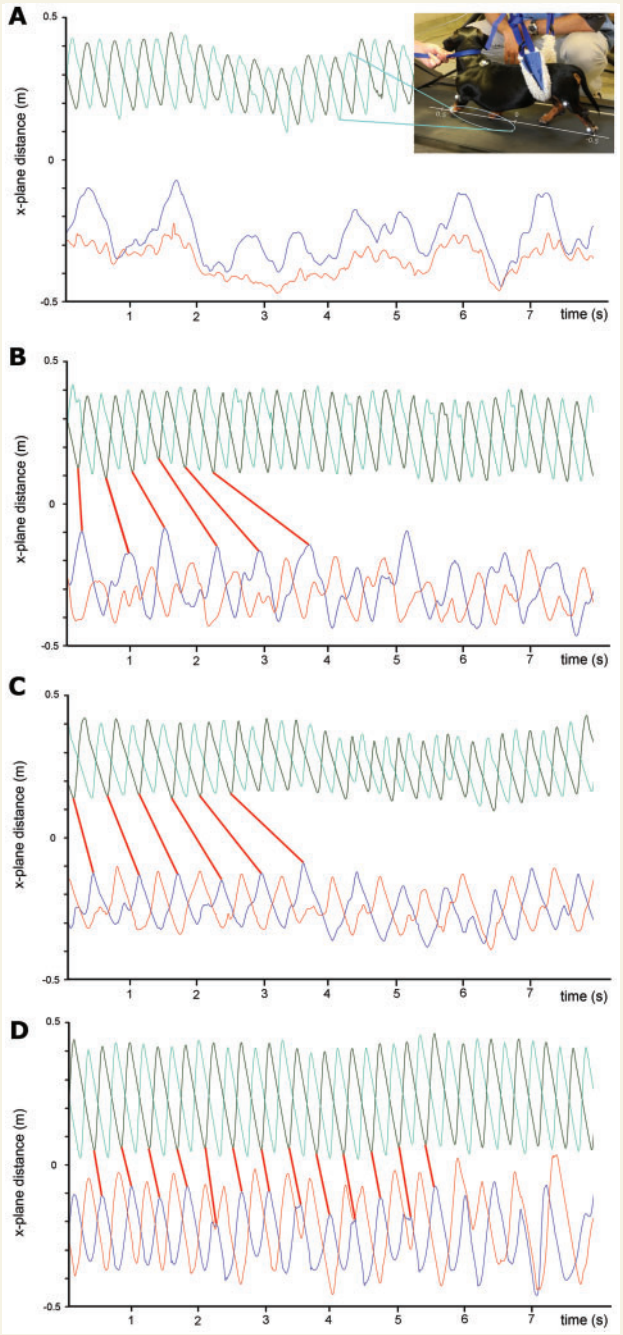

Figure 3 Digital recordings of locomotor activity can be represented by sine wave patterns, here corresponding to forward and backward motion of the paws during walking on a treadmill. In this series of wave patterns, corresponding to video recordings of Dog 8 in Supplementary Fig. 2, the fore paw movement is shown by the dark and light green traces and the hind paw movement by the red and blue traces. The generation of the sine wave pattern is illustrated in **A**, illustrating the correspondence between forward and backward motion of the paw during treadmill walking (the oval shape on the image in **A**) and the wave pattern. Temporal coordination between forelimb and hindlimb motion is then analysed using a MATLAB script to determine the time interval between the peaks of the 'sine wave' patterns (red lines between the curves in **B**, **C** and **D**), corresponding to the furthest extent of each step by each limb.

did not differ between olfactory mucosal cell and no-cell groups [$\beta = -0.077$ (95% CI: -0.891 to 0.736); $P = 0.852$; analysis by multivariable regression as described previously].

Discussion

The results of this study show unequivocally that injection of olfactory mucosa-derived cells in a blinded randomized trial is associated with improvements in locomotor outcome in chronic clinical spinal cord injury. This result is a substantial advance on previously available laboratory data regarding intraspinal olfactory cell transplantation because it establishes that the beneficial effects are sufficiently robust to be detectable even in clinical cases, in which the 'noise' of other uncontrolled variables, such as precise character of the injury, makes an effect more difficult to detect. Furthermore, the effects were notable for occurring in cases with chronic spinal cord injury that had previously reached a plateau of recovery – some of the dogs had been paraplegic for >12 months – and were of sufficient magnitude to change locomotor patterns with a clinically useful effect (Supplementary Fig. 2). It is unusual, even in experimental animals, for interventions in chronic spinal cord injury to have a detectable benefit because of the numerous obstacles to regeneration and functional recovery at this stage (Kadoya *et al.*, 2009). Altogether, these data suggest that improvement in spinal cord connectivity would most likely be detectable in human patients if they were to receive a similar intervention, such as those enrolled in the recently reported Phase I human trials (Lima *et al.*, 2008; 2010; Mackay-Sim *et al.*, 2008; Chhandra *et al.*, 2009). However, it is important to recognize that improvement on our primary outcome measure is a composite between increase in spinal stepping activity (as a non-stepping dog cannot coordinate forelimbs and hindlimbs) and increase in hindlimb step coordination with the forelimbs and does not necessarily imply restoration of brain control over hindlimb motion.

Our secondary outcome measures were designed to provide insight into mechanisms by which olfactory mucosal cell

Figure 3 Continued
Pre-transplantation in Dog 8 (**A**), there are only occasional stepping movements in the hindlimb and this is reflected in the very high 'coordination score' (here = 2.71), which is a summation of the 'lag' between corresponding forelimb and hindlimb movements. At 1 month (**B**) and 6 months (**C**), the same dog shows more regular hindlimb stepping, and overall coordination is improved but imperfect; there still remains an increasing 'lag' time between corresponding fore paw and hind paw placement during the recording period (the 'coordination score' is 1.21 in **B** and 0.78 in **C**). In **D**, Dog 30 illustrates a return of near-normal coordination between fore paw and hind paw placement after transplantation of olfactory mucosal cells 6 months previously (the 'coordination score' = 0.26). In this panel, there are prolonged periods of regularity in the time interval between fore paw and hind paw placement, as occurs in normal dogs, interspersed with periods in which coordination between limb girdles is lost.

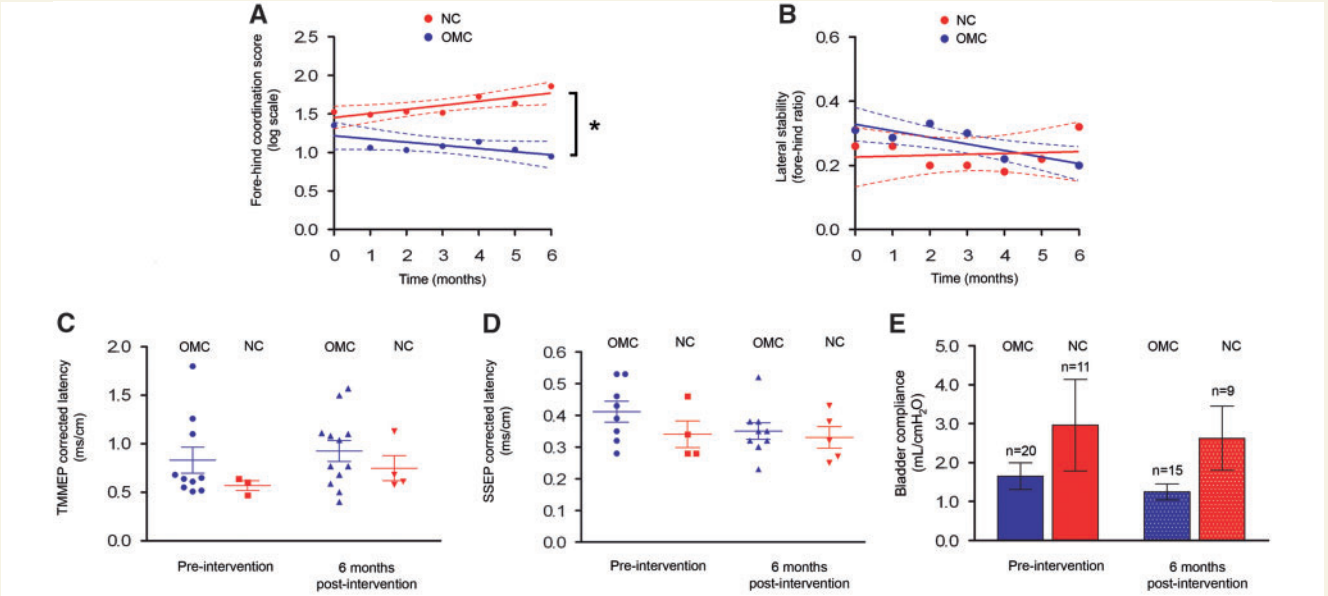

Figure 4 Analysis of effect of intraspinal injection of olfactory mucosal cells (OMC, blue) compared with cell transport medium alone (NC, red) on measures of forelimb–hindlimb coordination (**A**) and spinal cord long tract function (**B–E**). (**A**) Linear regression plot of fore–hind coordination scores during the 6-month trial period (low scores indicate good performance). *The sum effect of olfactory mucosal cell transplantation compared with the no-cell group, when controlling for the effects of time and baseline scores, was highly significant ($\beta = -0.455$; $P = 0.007$). (**B**) Linear regression plot of lateral stability ratio scores during the 6-month trial period (low scores indicate poor performance). The scores did not differ between the olfactory mucosal cell and no-cell groups ($\beta = 0.049$; $P = 0.428$). In **A** and **B**, solid lines indicate linear relationships and dashed lines indicate 95% confidence intervals. (**C**) Latencies of transcranial magnetic motor-evoked potentials recorded before and 6 months after intervention and adjusted for individual dog size (each symbol represents a single individual). There was no difference in recovery incidence (Fisher exact test $P = 1.00$) or final latencies ($P = 0.544$, Mann–Whitney test) between the olfactory mucosal cell and no-cell groups. (**D**) Latencies of recorded somatosensory-evoked potentials before and 6 months after intervention adjusted for individual dog size (each symbol represents a single individual). There was no difference in recovery incidence ($P = 1.00$, Fisher exact test) or final latencies ($P = 0.788$, Mann–Whitney test) between the olfactory mucosal cell and no-cell groups. In **C** and **D**, lines indicate mean and SEM. (**E**) Bladder compliance measures recorded before and 6 months after intervention. There was no difference in compliance between the olfactory mucosal cell and no-cell groups during the trial period ($\beta = -0.077$; $P = 0.852$; multivariable regression analysis). Bars indicate mean, lines indicate SEM.

transplantation may have exerted its effects. In experimental studies on focal lesions in rodents, there is strong evidence that olfactory ensheathing cells can promote regeneration of long tract (e.g. pyramidal tract) axons (Li *et al.*, 1998), and olfactory ensheathing cell-mediated functional improvement in complete transection models has been thought to result from serotonergic fibre regeneration (Ramon-Cueto *et al.*, 2000). The improved forelimb–hindlimb coordination we observed implies enhanced communication across the damaged region of spinal cord and could therefore be attributed to long-range axon regeneration. However, our data strongly suggest that this may not have occurred in these dogs – most notably the lack of improvement in any of our measures of long tract function (i.e. lateral stability, somatosensory-evoked potential, transcranial magnetic motor-evoked potential and urodynamics) despite the concomitant improvement in fore–hind coordination. Instead, plastic changes in propriospinal connections provide an explanation for improvement in fore–hind coordination that is more consistent with the data. Such an effect could result from many processes including, but not limited to, local axon sprouting. Many other previously documented properties of olfactory ensheathing cells may be relevant, including modulation of immune responses (Arnold and Hagg, 2011; Chuah *et al.*, 2011), provision of neurotrophic factors (Chiu *et al.*, 2009), remyelination of demyelinated axons (Franklin *et al.*, 1996; Sasaki *et al.*, 2011) or modulation of glia and neuronal function (Chuah *et al.*, 2011).

It is also possible that the effects of the olfactory mucosal cell transplant are not dependent solely on effects mediated by the olfactory ensheathing cell component of the transplanted population. Our data show a clear and strong overall effect of olfactory mucosal cell transplants, but extent of recovery does not appear to depend on the total proportion of p75+ olfactory ensheathing cells contained within the transplant population. Interestingly, a similar observation has also been made after transplantation of olfactory mucosa-derived cells in rat models of spinal cord injury (Yamamoto *et al.*, 2009). This finding does not exclude the possibility that the effect could be mediated by a threshold number of olfactory ensheathing cells within the population, which might be quite low; alternatively it could suggest that the precise type of cells in the transplant is not critical to the success of mucosal-derived transplants. It has instead been suggested that their effects might be a result of local axon sprouting

(Yamamoto *et al.,* 2009) rather than the long-range axon regeneration associated with formation of 'bridges' across the lesion site that have been postulated for other types of olfactory cell transplants (Raisman and Li, 2007). This explanation corresponds well with the observation that the effect in our study appeared to be to alter the intraspinal connectivity (i.e. acting at a local level) rather than communication between spinal locomotor centres and the brain. To define more closely the precise relationship between transplanted olfactory mucosal cell and functional outcome, it would also be useful to analyse the survival of transplanted cells within host tissue.

A percutaneous cell transplantation technique was used in this study, mainly to facilitate blinded comparison between intraspinal injections that did and did not contain cells. Percutaneous delivery of cells avoids the need for a sham surgical procedure in the control animals (needed for blinding purposes), and similar avoidance of the ethical problems associated with sham neurosurgical procedures (Galpern *et al.,* 2012) would also be of value during translation into human patients. Our previous intraspinal cell transplantation technique (Jeffery *et al.,* 2005) via surgical exposure of the spinal cord ensures accurate needle placement into the spinal cord parenchyma but is not necessarily more reliable in delivering cells to their target site. Obtaining cerebrospinal fluid during spinal puncture provides unequivocal evidence of needle bevel position within an intradural location, and needle stability during injection is excellent because of the surrounding soft tissue, thus minimizing transplant leakage. It is simple and quick to perform, appeared to have no detrimental effects on the recipients and would be straightforward to translate into human medicine. In human patients with spinal cord injury, introduction of a larger number of needles may be required to ensure delivery of cells to the whole length of lesions, such as after severe fracture luxations, which may be of much greater relative size than the lesions treated in the current study.

A major reason for carrying out this study was to determine whether intraspinal transplantation of olfactory mucosal cell would be an appropriate subject for expeditious clinical efficacy trials in human patients with spinal cord injury. Several groups throughout the world have already carried out Phase I trials on olfactory ensheathing cell transplantation for spinal cord injury in humans (Lima *et al.,* 2006, 2010; Mackay-Sim *et al.,* 2008; Chhabra *et al.,* 2009), demonstrating the safety of this approach. Efficacy testing will require much greater resources and therefore needs very careful consideration. In this study, although we demonstrate that olfactory mucosal cell transplantation improves communication across the lesion, allowing recovery of 'automatic' coordination between forelimbs and hindlimbs, there are few data to support improvement of spinal long tract function. Human spinal cord injury patients most value recovery of arm, bladder and sexual function (Anderson, 2004), all of which are dependent on spinal long tract function, through which the brain is able to modulate lower motor neuron activity. Therefore, although it would be expected that transplanting olfactory mucosal cells into human spinal cord injury patients would be associated with clinically detectable effects, it is improbable that patients would experience useful benefit in their everyday lives (and see Barnett and Riddell, 2007). For that reason, the main implication of our study is that it does not provide encouragement to consider that olfactory mucosal cell transplants as a sole treatment would provide significant clinical benefit to human spinal cord injury patients. Nevertheless, the effects we show here may provide some worthwhile benefit if the olfactory mucosal cell transplant were to be part of a multimodal intervention, as is more likely to be developed as a future therapeutic strategy (Thuret *et al.,* 2006). In some previous human studies, olfactory mucosal cell transplantation has been accompanied by rehabilitation therapies (Chhabra *et al.,* 2009; Lima *et al.,* 2010), which in themselves may have a beneficial effect on outcome and may thus confound interpretation of the origin of the observed effects. In our current study, although the extent of physical exercise varied between individuals, there was no systematic difference between groups of transplanted and control dogs, and it does not form an alternative explanation for our findings.

The information gained in this project illustrates the contributions that can be made by study of spinal cord injury in pet dogs, in providing a bridge between the laboratory and human clinic. Thus, promising therapies identified through previous research in rodents can be screened for efficacy in this 'clinical model' before embarking on human trials. This translational aspect is highlighted by the provision of relatively heterogeneous patient groups (similar to human patients), while also permitting assessment using electrophysiological testing and finely calibrated quantitative functional neurological data. The conclusion drawn from this approach is that olfactory mucosa-derived cell transplants can mediate substantial change in function in a clinical spinal cord injury model. However, the effects are likely to be on local intraspinal circuitry rather than on long tract function, which leads us to conclude that this intervention alone is unlikely to have appreciable benefits in the treatment of human spinal cord injury but, nonetheless, may form a useful component of a multi-faceted approach.

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Supplementary material

Supplementary material is available at *Brain* online.

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