Promoting plasticity in the spinal cord with chondroitinase improves functional recovery after peripheral nerve repair

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Functional recovery after peripheral nerve repair in humans is often disappointing. A major reason for this is the inaccuracy of re-innervation of muscles and sensory structures. We hypothesized that promoting plasticity in the spinal cord, through digestion of chondroitin sulphate proteoglycans (CSPGs) with chondroitinase ABC (ChABC), might allow the CNS to compensate for inaccurate peripheral re-innervation and improve functional recovery. The median and ulnar nerves were injured and repaired to produce three grades of inaccuracy of peripheral re-innervation by (i) crush of both nerves; (ii) correct repair of median to median and ulnar to ulnar; and (iii) crossover of the median and ulnar nerves. Mapping of the motor neuron pool of the flexor carpi radialis muscle showed precise re-innervation after nerve crush, inaccurate regeneration after correct repair, more inaccurate after crossover repair. Recovery of forelimb function, assessed by skilled paw reaching, grip strength and sensory testing varied with accuracy of re-innervation. This was not due to differences in the number of regenerated axons. Single injections of ChABC into the spinal cord led to long-term changes in the extracellular matrix, with hyaluronan and neurocan being removed and not fully replaced after 8 weeks. ChABC treatment produced increased sprouting visualized by MAP1BP staining and improved functional recovery in skilled paw reaching after correct repair and in grip strength after crossover repair. There was no hyperalgesia. Enhanced plasticity in the spinal cord, therefore, allows the CNS to compensate for inaccurate motor and sensory re-innervation of the periphery, and may be a useful adjunct therapy to peripheral nerve repair.

Keywords: spinal cord; nerve repair; plasticity; extracellular matrix; proteoglycans

Abbreviations: ChABC = chondroitinase ABC; CSPG = chondroitin sulphate proteoglycan; ECM = extracellular matrix; PNN = perineuronal net; MAP1BP = phosphorylated microtubule-associated protein 1B

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Introduction

After peripheral nerve repair in human patients, axon regeneration and re-innervation of muscles and skin is usually successful, but recovery of motor and sensory function is often disappointing (Jaquet et al., 2001). The main reason is misdirected re-innervation, i.e. motor and sensory axons reconnecting to inappropriate targets (Valero-Cabre et al., 2004). This is a significant clinical problem, with in excess of 50,000 peripheral nerve repair procedures performed annually in the USA.

Peripheral nerve injury can cause re-organization of projections in the CNS at both the subcortical and cortical level. (Lewin and McMahon, 1993; Chen et al., 2002; Wall et al., 2002). However, in adult mammals this plasticity is insufficient to allow complete functional recovery following peripheral repair (Lundborg, 2003). In children the functional outcome after nerve repair is considerably better than in adults (Hudson et al., 1997; Lundborg and Rosen, 2001) despite the fact that the peripheral...
reinnervation is equally inaccurate (Almquist et al., 1983; Florence et al., 1996). This difference is ascribed to greater CNS plasticity in children. This raises the possibility that if plasticity in the adult CNS could be re-activated, the same degree of functional recovery as is seen in children might be possible for adults (Hensch, 2003). The advent of chondroitinase ABC (ChABC) as a treatment that promotes plasticity in the adult CNS makes it possible to test this hypothesis.

Recent work has indicated that the extracellular matrix (ECM) plays a part in the reduction of plasticity at the end of critical periods (the period of enhanced plasticity seen in young animals during which the final refinement of connections occurs). In the visual cortex critical period closure is associated with the laying down of perineuronal nets (PNNs) containing chondroitin sulphate proteoglycans (CSPGs), and digestion with ChABC reactivates plasticity (Pizzorusso et al., 2002). ChABC treatment also allows sprouting in the cerebellum (Corvetti and Rossi, 2005), promotes sprouting within the superior colliculus following a lesion (Tropea et al., 2003) and in the partially denervated cuneate nucleus following spinal cord injury (Massey et al., 2006). ChABC digestion in the spinal cord promotes functional recovery after spinal cord injury, probably largely through promoting plasticity (Bradbury et al., 2002; Massey et al., 2006), and the enzyme can affect both long-term potentiation and long-term depression in the CA1 region of the hippocampus (Bukalo et al., 2001; Saghatelyan et al., 2001).

We hypothesized that treatment of the spinal cord with ChABC would enhance plasticity and therefore improve functional recovery after peripheral nerve repair. To test this idea, we repaired the median and ulnar nerves in four ways that produced varying degrees of misguidance of regenerating axons. In order to demonstrate the effects of axon misguidance on recovery, we have developed a set of tests for skilled paw function and other parameters, providing a model of the clumsy hand function that is a major problem for human patients after nerve repair. We then treated the spinal cord with ChABC and found improvements in skilled paw function and grip strength.

Material and methods

Subjects

Male Lister-Hooded rats (n = 75; 225–250 g, Harlan) were housed in an enriched environment with seed mix, spaghetti and toys to promote use of their forepaws (Whishaw and Coles, 1996). Experiments were carried out in accordance with the UK Animals (Scientific Procedure) Act, 1986. Rats were anaesthetized in 1–2% halothane in a mixture of 50% nitrous oxide and 50% oxygen.

Peripheral nerve lesions

In the rat, the median nerve supplies all the finger flexors and is most important for hand function while the ulnar nerve plays a secondary role (Bertelli et al., 1995; Galtrey and Fawcett, 2006) hence in this study both nerves were injured and repaired. The median and ulnar nerves were exposed ventrally 5 mm proximal to the elbow. Nerve crush was performed three times for 20 s with a fine needle holder. Nerve transection was performed with iris scissors and repair was performed using one epineurial stitch with 10-0 sutures (Ethicon, Ethilon™). Four types of lesion and repair were performed (Fig. 1). (i) Both nerves were crushed, (ii) both nerves were cut then repaired median to median and ulnar to ulnar (correct repair), (iii) both nerves were cut and repaired median to ulnar and ulnar to median (crossover repair), (iv) both nerves were cut and not repaired. When the nerves were not repaired, a 1 cm section of nerve was excised and the proximal muscle sutured into the pectoralis muscle.

Spinal cord injections

A partial laminectomy was performed at C5/C6 and C7/T1 and then two injections of 1 µl at each site of either ChABC (100 U/ml, Seikagaku) or control bacterial enzyme (penicillinase, 2 mg/ml matched for protein content, Sigma). The injection co-ordinates were 1 mm from the midline and then first lowering the capillary
2 mm and then raised 50 μm and infused for 5 min and then raised a further 50 μm for the remaining 5 min, aiming to target the ventral horn. Injections were performed with a 10 μm pulled glass capillary connected to a 26-gauge 10 μl syringe (SGE, Milton Keynes, UK) driven by a microdrive pump (Harvard Apparatus, Kent, UK) at 6 μl/h. After the injection, the capillary was left in situ for a further 5 min for diffusion. No behavioural deficits were seen after spinal cord injections.

Retrograde tracing
Fifteen weeks after nerve injury and repair, the left flexor carpi radialis (FCR) muscle received an injection of 3 μl of 1% cholera toxin subunit B Alexa Fluor® conjugate (Invitrogen). Three days later, the rats were perfusion-fixed. The spinal cord (C4 to T2) was removed and fiducial marks were made on the white matter with a microcautery at each spinal level to ensure the same section of spinal cord was examined in every case. After postfixing, the tissue was embedded in 5% agarose and 50 μm horizontal sections cut on the vibratome and mounted on gelatin-coated slides. All retrogradely labelled motoneurons containing a nucleus were identified using a Leica DMRD microscope at 10× magnification. Using a grid, motoneuron position was plotted on squared paper and the distance to the nearest neuron measured.

Behavioural testing
Rats were given 2 weeks of daily training on the staircase test and then habituation and training on all the other tests was begun on rats that met the inclusion criteria for the staircase test. All testing was carried out by an experimenter blinded to the injury or treatment group.

Skilled forepaw function was assessed using the staircase test (Montoya et al., 1991) (Model 80300, well depth 9 mm, Rat Motility Staircase Test, Campden Instruments, UK). The apparatus comprises a test box from which extends a narrow corridor with a central plinth and a series of seven steps on either side. Sucrose pellets (Research Diets Inc.) were placed in a well on each step and may be retrieved by the rat reaching down either side of the plinth. The narrowness of the corridor is such that the rat cannot turn around and can only reach the pellets from the left staircase with its left paw and from the right staircase with its right paw. On each test, the staircases were baited with two sucrose pellets on each of the seven steps on each side and the rats placed in the box for 15 min. The number of pellets retrieved (maximum of 14 pellets per side) or the maximum distance reached (maximum of seven steps per side) was recorded. Scoring the number of pellets retrieved (removed from the wells and eaten) gives an indication of the success of grasp and retrieval while scoring the maximum distance reached (the lowest step from which the rat managed to displace a pellet) gives an assessment of how far the rat can reach regardless of its success at grasping (Dobrossy and Dunnett, 2004).

Grip strength was assessed using a lateralized grip strength meter (Dunnett et al., 1998) (Dunnett modification GSM1054 grip strength meter, Linton Instruments, UK). Rats will naturally reach and grasp an object when they are suspended in the air and the object is brought in front of their forepaws. The two grip rods were arranged coaxially and connected to two separate strain gauges and were used at a 45° angle. On each trial, the rat was held around the abdomen and lowered into the apparatus so that it grasps the two grip rods, one with each paw, with its back legs standing on the smooth floor of the apparatus. The experimenter then held the rat by the base of the tail and pulled it gently in a rearward direction. A rat naturally clings to the grip rods to resist the pull until it can no longer hold on and releases. The applied force at which the rat releases the two rods with each paw was measured separately by the strain gauges. Each testing session assessed each forepaw simultaneously three times.

All sensory testing was performed in a wooden box with Perspex lid on a wire mesh floor (20 cm × 10 cm × 10 cm). In order to ensure that the rats’ forepaws remained flat on the mesh, the rats were given diluted golden syrup to drink via a water bottle inserted in the lid. Testing began after a 10 min acclimatization period. The testing probe was only applied while the rat was drinking. Fine touch and mechanical hyperalgesia were measured using an electronic von Frey Anaesthesiometer (Model 1601C, Life Science Instruments). The probe was applied to the glabrous skin of the plantar surface of the forepaw and the pressure gradually increased until the rat withdrew its paw. The force transducer recorded the maximum force applied in grams. If the rat did not withdraw the paw after 80 g had been applied the test was terminated. This procedure was repeated for nine trials on each paw. The trials were performed on the left and right paw alternately. Temperature sensation and thermal hyperalgesia were measured using ice probes by freezing water in a 1.5 ml microcentrifuge tube (Lindsey et al., 2000). The ice probe was applied to the glabrous skin of the forepaw. A stopwatch was used to measure the withdrawal latency. A test session consisted of nine trials for each forepaw with at least 30 s between trials to allow the skin to return to body temperature. If the rat did not withdraw the paw after 10 s the probe was removed.

Immunofluorescence histology
Rats were terminally anasthetized and the spinal cord was removed and immediately frozen in dry ice. Thick sagittal sections of 20 μm were cut serially on a cryostat, mounted and fixed in cold acetone. Sections were stained with mouse monoclonal unsulphated (1B5), 4-sulphated (2B6) stub (both IgG, 1:200, Seikagaku) or neurocan-N-specific (1B-P, IgG 1:100, Serotec) and MAP1B-P-specific [1B-P, IgM 1:7500 (Nothias et al., 1996)] antibodies and biotinylated goat anti-mouse IgG (1:200, Invitrogen) and biotinylated hyaluronan-binding protein (10 μg/ml, Seikagaku) followed by the streptavidin, Alexa Fluor® 488 conjugate (1:200, Invitrogen).

Immunoperoxidase histology
Rats were deeply anesthetized, transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer and spinal cords (C2–T2) of all the rats were embedded in 5% agarose, sectioned on the vibratome and stained with unsulphated stub, neurocan-N, CD11b (OX42, IgG 1:100, Serotec) and MAP1B-P-specific [1B-P, IgM 1:7500 (Nothias et al., 1996)] antibodies and biotinylated goat anti-mouse IgG (H+L) or IgM secondary antibody (1:200, Vector).

Immunoblotting
Rats were sacrificed, a 10 mm section of cervical spinal cord (C4–C7) was excised, and the meninges removed. The tissue was
homogenized in 1.0 ml of phosphate-buffered saline (PBS) (pH 7.0), and centrifuged at 18 000 g for 10 min at 2°C. Half of the penicillinase homogenate was treated with 20 TRU/ml hyaluronidase (from Streptomyces hyalurolyticus; Calbiochem) to release hyaluronan-bound molecules as a positive control. The saline-soluble proteins were run in 5% or 10% polyacrylamide gels under reducing conditions, and transferred to nitrocellulose. The blots were incubated with mouse monoclonal unsulphated stub (1B5, 0.5 μg/ml; Seikagaku), neurocan-N (1F6, 1:20; DSHB), neurocan-C (1G2, 1:40; gift of A. Oohira) and link protein (8A4, 1:40; DSHB)-specific antibodies and then with HRP-conjugated anti-mouse IgG (1:10 000; Vector, UK). Membranes were washed and proteins visualized using a chemiluminescent substrate (Amersham).

### Toluidine blue histology

The most distal 1 cm of the median nerves was fixed, dehydrated and embedded in resin. One micrometre thick transverse sections were cut from hardened resin blocks with a microtome and stained using toluidine blue and examined using light microscopy to count the total number of myelinated axons and then the total number of myelinated axons per nerve was calculated.

### Image analysis

Digital images were produced using Lucia imaging software (Nikon, UK) with a Nikon DXM 1200 digital camera. Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA) was used to montage and process the images. Semiquantitative optical densitometry on MAP-1BP was performed using images that were saved as TIFF files without compression and exported to NIH Image J. Images were taken at 20× magnification in three areas for each animal. Lighting conditions and exposures were equal within capture sessions and results are expressed as optical density.

### Statistical analysis

All statistical tests were carried out with the Sigma Stat 3.1 (SPSS, Inc.) statistical package. Statistical significance was evaluated for the behavioural testing by using a two-way repeated measures analysis of variance (ANOVA). Post hoc comparisons were made using the Bonferroni test. Statistical significance was evaluated for the histology using a t-test.

### Results

In order to produce graded inaccuracy of axon regeneration, we performed four different repairs of the median and ulnar nerve where they run in parallel just proximal to the elbow: (i) both nerves were crushed; (ii) both nerves were cut and repaired median to median and ulnar to ulnar (correct repair); (iii) both nerves were cut and repaired median to ulnar and ulnar to median (crossover repair); and (iv) both nerves were cut and not repaired (Fig. 1). The accuracy of axon re-innervation after the injury and repair was first assessed anatomically and then the functional effects examined using various behavioural tests.

#### The different types of median and ulnar nerve repair produce graded inaccuracy of re-innervation

In order to provide an assessment of the inaccuracy of re-innervation due to misguidance of axons at the injury site retrograde labelling was used to map the motoneurons re-innervating the FCR. This allowed quantification of the accuracy of median nerve re-innervation of FCR after correct repair and the number of ulnar nerve fibres that re-innervate FCR after crossover repair. The number of retrogradely labelled motoneurons was the same after crush as in uninjured rats, while both correct and crossover repair groups showed a small reduction in the number of labelled motoneurons (Fig. 2B). We were able to map the accuracy of re-innervation because in uninjured rats the motoneurons are arranged in discrete, tightly packed groups. After crush, these tight groups of labelled motoneurons were still present, indicating that axons accurately re-innervate the muscles they previously supplied. However, after correct or crossover repair the labelled motoneurons were no longer tightly clustered, indicating inaccurate re-innervation. This was quantified by measuring the distance between each labelled neuron and its nearest labelled neighbour (Fig. 2C).

The decrease in clustering in the correct and crossover repair groups is greater than can be explained by the slightly lower number of labelled motoneurons in these groups (supplementary Figure 1).

#### The number of regenerating axons and muscle weight are unaffected by the type of repair

It was important to know whether the same number of axons had regenerated in the different repair models, in order to be certain that different degrees of functional recovery are not simply due to poor axon regeneration. The numbers of myelinated axons in the distal median nerves at 14 weeks were counted in semithin sections, showing no difference between the types of nerve repair (Fig. 2D). In the non-repaired animals, only a very small number of axons were seen in the nerve distal to the injury. Muscles atrophy rapidly if they are not innervated, and muscle weight is therefore a measure of re-innervation. In animals in which the nerves were not repaired, the FCR and other muscles were very atrophic. Following the nerve crush and the two types of repair there was no difference in muscle weight between the groups (Fig. 2E).

#### Degree of functional recovery depends on the type of median and ulnar nerve repair

We assessed functional recovery after peripheral nerve injury and repair using a battery of tasks (Fig. 3A). Grip strength was measured because inaccurate re-innervation of finger flexors produces poor grip. Fine paw control was
tested using the staircase test, because skilled hand function is poor in humans after nerve repair due to mis-innervation of the many small muscles controlling the hand. Sensation was tested using a von Frey hair method and cold stimuli because fine touch and temperature sensation are often abnormal after nerve repair. In all cases the left side was injured and the right side used as a control.

**Skilled forelimb function**

Skilled forelimb function was examined using the staircase test (Montoya et al., 1991), measuring the number of pellets retrieved and the maximum distance reached. The number of pellets retrieved provides an indication of fine motor skill, since rats must use their digits to extract, grasp and eat pellets from a narrow deep well. After all types of repair...
there was initially complete loss of ability to perform this task. The timing and degree of recovery depended on the type of nerve repair. After nerve crush, rats recovered the ability to retrieve pellets within 2 weeks, after which it was not significantly different from the control side. After correct repair, the majority of rats recovered the ability to retrieve pellets within 4 weeks, and numbers retrieved reached 70% of the control side. After crossover repair, rats recovered limited ability to retrieve pellets within 4 weeks, reaching only 20% of the control side. After non-repair, the rats could not retrieve pellets. The ability of rats to retrieve pellets therefore varied according to the type of repair, with statistically significant differences in skilled paw function between the groups (Fig. 3B and supplementary Table 1).

The maximum distance reached provides an assessment of gross motor recovery because rats can flick pellets from the wells without skilled use of their digits, and in this measure functional recovery was not strongly dependent on the type of repair. After crush, there was full return of function within 2 weeks. After correct repair, rats started to displace pellets within 2 weeks, and were not significantly different from the control side within 4 weeks. After crossover repair, rats started to displace pellets within 3 weeks, and within 4 weeks were not significantly different from the control side. Only the rats that received no repair failed to recover (Fig. 3C and Supplementary Table 1).

**Grip strength**
All median and ulnar nerve injuries initially produced complete loss of grip strength. The degree and speed of recovery depended upon the type of repair. After crush, rats recovered grip strength within 2 weeks, and within 3 weeks there was no significant difference from the control side. After correct repair, rats recovered grip strength within 4 weeks, which gradually increased to ~50% of the control side. A similar time course of recovery was seen after crossover repair, but the degree of recovery was less at 15% of the control side. There was, therefore, a clear relation between the type of repair and degree of recovery of grip strength (Fig. 3D and Supplementary Table 1).

**Injection of ChABC correct into the grey matter of the adult rat spinal cord changes the composition of the ECM**
ChABC has been shown to increase CNS plasticity, but the duration of its effects and the details of how it modifies the ECM are not established. We investigated the effects of injections of the enzyme into the spinal cord in two ways. In the first, tissue sections of treated spinal cord were investigated by immunohistochemistry to demonstrate changes in the distribution of ECM molecules. In the second, tissue from the treated region of the cord was dissected out and the soluble matrix molecules extracted by gentle homogenization in PBS in order to characterize the molecules present in the soluble fraction, and released from binding by the ability of ChABC to digest chondroitin sulphate (CS) and hyaluronan. Some cord tissue was digested with hyaluronidase in vitro before extraction in order to demonstrate the effects of complete digestion of the hyaluronan chains. Injection of ChABC into the spinal cord disrupted the ECM by the digestion of both CS and hyaluronan. One day after injection, carbohydrate stubs, which are created on CSPG core proteins following ChABC digestion of CS, were detected by both immunohistochemistry (Fig. 4A) and immunoblotting (Fig. 4B). In the region of ChABC treatment immunolabelling was almost absent for neurocan-N, indicating that neurocan was no longer attached to the extracellular matrix and had dissolved and dissipated either in vivo or during tissue processing (Fig. 4A). In the tissue extracts, ChABC treatment caused neurocan to be extracted in the soluble matrix fraction, and the removal of the CS chains allowed the core protein to enter gel where it was detected by Western blotting (Fig. 4B). ChABC also digested hyaluronan with a decrease in hyaluronan binding protein (HABP) labelling of sections (Fig. 4A) and an increase in the release of link protein into the saline extracts. Penicillinase injections released little link protein, but if this penicillinase-injected spinal cord was subsequently digested with hyaluronidase in vitro, large amounts of link protein were released. After in vivo ChABC injection the amount of link protein present in the saline extract was almost equal to that seen after in vitro hyaluronidase, indicating that ChABC treatment degraded most of the hyaluronan in the cord (Fig. 4B). These results demonstrate that ChABC digests CS and hyaluronan and by doing so releases link protein and neurocan-N from the ECM.

The duration of ChABC effects is long lasting. Eight weeks after ChABC injection, carbohydrate stub immunoreactivity was still present and the immunolabelling of neurocan-N core protein was also decreased in the digested region (Fig. 4C).

There was no difference in the inflammatory reaction seven days after injection of ChABC or penicillinase as measured by the optical density (OD) of the immunolabelling for CD11b on microglia ($t$-test, $t_6 = 2.419$, $P = 0.052$).

**ChABC improves functional recovery after peripheral nerve injury and repair without causing abnormal pain sensation**
We used a set of behavioural tests to determine whether ChABC injection to the spinal cord improves functional recovery after peripheral nerve repair. Rats received either correct or crossover repair and were then left for 4 weeks to allow for axon regeneration and re-innervation and for spontaneous recovery to progress. Then, either ChABC or a control enzyme, penicillinase, was injected into the spinal cord (Fig. 5A). There was also a parallel series of animals that had a peripheral nerve injury without spinal cord injection.
After correct repair, the ChABC-treated rats showed a progressive and significant improvement in the number of pellets retrieved compared with controls, starting 2 weeks after injection (Fig. 5B). After 80 days, the number of pellets retrieved by ChABC-treated animals was 64.6 ± 13.4% of the uninjured side compared with only 23.4 ± 9.35% of the uninjured side after control treatment, a functional improvement of 2.75 times. The animals that only received a peripheral nerve injury without any treatment recovered to only 7.4 ± 5.49% of the uninjured side.

Fig. 3 The degree of functional recovery depends on the severity of the peripheral nerve injury and repair. (A) At 1, 2, 3, 4, 6, 8 and 14 weeks after peripheral nerve injury and repair, rats were assessed with the staircase test (■) and grip strength meter (●). The staircase test assessed skilled forelimb function by measuring the number of pellets retrieved (B) and the maximum distance reached (C) and the grip strength meter assessed unskilled forelimb function (D). Nerve injury and repair were performed on the left forelimb of the rat; crush (n = 6; ■); correct repair (n = 7; ●); crossover repair (n = 6; ■) and no repair (n = 6; ●) and the uninjured right forelimb function was also measured in the rats that received a left-sided crush (■); correct repair (●); crossover repair (■) and no repair (●). Data are presented as mean ± SEM. By two way repeated measures analysis of variance, there was a significant main effect of both injury level (retrieved, a; distance, b; grip, c) and time (retrieved, a; distance, b; grip, c) and an injury level × time interaction (retrieved, a; distance, b; grip, c). Comparison of final function achieved 14 weeks after nerve injury and repair are shown in Supplementary Table 1.
There was also an effect of ChABC treatment on the maximum distance reached in the staircase test (Fig. 5C), but this was less marked. ChABC treatment had no effect on grip strength (Supplementary Table 2).

After crossover repair, ChABC-treated rats showed improved grip strength. Seven days after treatment some rats in the ChABC-treated group recovered grip strength, whereas no rats in the control group recovered this ability, and the ChABC-treated group maintained significantly better grip strength throughout the testing period (Fig. 5D). After 77 days, the mean grip strength was $18.6 \pm 3.92\%$ of the uninjured side after ChABC treatment compared with only $9.94 \pm 4.47\%$ of the uninjured side after control treatment, a functional improvement of 1.87 times. The animals that only received a peripheral nerve injury without any treatment recovered to only $5.06 \pm 0.86\%$ of the uninjured side. The staircase test detected no difference in functional recovery between the treated and control groups. However, this group of rats had learnt that they were not able to retrieve pellets, probably due to frequent testing, and all rats in the control group and all but three in the treatment group had stopped attempting the task. This is shown by the fact that they showed on average no skilled retrieval or unskilled displacement of pellets from the wells in the control animals, little in the treated animals (Supplementary Table 2).

All rats were tested for mechanical and thermal hyperalgesia. Sensory recovery was incomplete in all groups, with a lower threshold for limb withdrawal in response to fine touch, and a longer latency of withdrawal to cold stimuli. Following both correct and crossover repair, there was no difference between the responses of ChABC-treated and control groups to fine touch (Supplementary Table 3). However, the ChABC-treated rats showed improved recovery of temperature sensation after correct repair (Fig. 5E), and a similar (non-significant) trend after crossover repair (Supplementary Table 3).

ChABC promotes sprouting in the spinal cord after peripheral nerve injury and repair

To investigate whether ChABC treatment promoted sprouting in the spinal cord, we performed immunohistochemistry after behavioural testing. An antibody to phosphorylated microtubule associated protein 1B (MAP1B-P) was used, which has been shown to label newly-grown processes (Soares et al., 2002). There was a significant increase in immunoreactivity in the spinal cord of ChABC-treated rats compared with the control penicillinase-treated rats in the injected region (Fig. 6).

ChABC treatment does not alter the number of regenerated axons

To determine whether any of the recovery observed following ChABC treatment could have been due to changes in the number of myelinated axons that regenerated, we examined axon number and muscle weight. There was no difference in the number of myelinated axons in the

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**Fig. 4** Injection of chondroitinase ABC into the grey matter of the adult rat spinal cord results in disruption of the extracellular matrix. (A) 24 h after injection of chondroitinase ABC (ChABC) there was intense immunolabelling with antibodies that recognize carbohydrate stubs produced after ChABC digestion (IBS and 2B6). Within the same region there was a decrease in hyaluronan demonstrated with hyaluronan binding protein (HABP) and decreased neurocan, shown with an antibody that recognizes the core protein of neurocan (IF6). Scale bar, 1 mm. None of these changes were seen in control enzyme-penicillinses-injected spinal cords (data not shown) (B) Immunoblot analysis of the effect of ChABC digestion after 24 h. ChABC but not penicillinses (Pase) created a number of IBS immunoreactive species with different apparent molecular weights (IBS antibody recognizes degraded chondroitin-0-sulphate). ChABC but not control-Pase caused the appearance of discrete bands of both neurocan-C (IG2) and neurocan-N (IF6) as a result of the removal of the CS chains. ChABC increased the amount of link protein (8A4) present in the saline extract compared with control-Pase injection. The amount of link protein in the saline extract after ChABC digestion is the same as that present after in vitro digestion of the penicillinses-injected spinal cord sample with hyaluronidase (Hase). (C) After the end of behavioural testing, 8 weeks after ChABC injection all animals had their spinal cords sectioned and stained for carbohydrate stub (IBS) and neurocan-N (IF6). There is an intense increase in immunoreactivity of carbohydrate stubs in the area of ChABC digestion and there is a distinct decrease in immunoreactivity for neurocan in the same area on two consecutive sections. Scale bar, 1 mm.
Peripheral nerve injury and repair

Spinal cord injection

Perfusion

**Fig. 5** Chondroitinase ABC improves functional recovery after peripheral nerve injury and repair without causing neuropathic pain. (A) Median and ulnar nerve transection and either correct or crossover repair was performed on day 0; spinal cord injection was performed on day 28 (a) and rats were assessed in a double-blind manner with the staircase test, grip strength and sensory testing at intervals. Functional recovery of both the left paw, that received the median and ulnar nerve injury and repair followed by treatment of the spinal cord with either chondroitinase ABC (ChABC; - - - - ) or penicillinase (Pase - - - ), and the uninjured right paw, after treatment of the spinal cord with either ChABC (- - - - ) or Pase (- - - - ). (B) After correct repair, there was no significant difference in the number of pellets retrieved between the groups before treatment (two-way RM ANOVA, \( t = 0.21; F_{1,51} = 0.0130, P = 0.911 \)). After treatment, there was a significant difference (2.7 times at the end of the experiment) in the number of pellets retrieved between the ChABC \( (n = 10) \) and penicillinase \( (n = 9) \) treated groups (two-way RM ANOVA, \( t = 3.923, P = 0.064 \)) and the difference between the groups altered over time as there was a treatment \( \times \) time interaction (two-way RM ANOVA, \( t = 3.923, P = 0.064 \)) and comparisons at individual time points revealed that the ChABC-treated rats displaced significantly more pellets than the penicillinase-treated rats at 72 and 77 days after the nerve injury (Bonferroni \( t \)-test; \( * P < 0.05 \)). (C) Before treatment there was no significant difference between the groups in the maximum distance reached (two-way RM ANOVA, \( t = 35.77; F_{7,119} = 4.555, P < 0.001 \)). Multiple comparison procedures showed a significant difference between ChABC-treated and control-treated groups from day 63-77 (Bonferroni \( t \)-test; \( * P < 0.05 \)). No treatment - injured No treatment - control (D) After crossover repair, there was no significant difference in grip strength between the groups before treatment (days 0-21; two way RM ANOVA, \( F_{1,90} = 0.000464, P = 0.983 \)). After treatment there was a significant difference (1.87 times at the end of the experiment) in grip strength between the ChABC \( (n = 11) \) and penicillinase \( (n = 6) \) treated groups (two-way RM ANOVA, \( t = 35.77; F_{1,75} = 10.561, P = 0.005 \)). However, the difference between the two groups did not change over time as there was no treatment \( \times \) time interaction (two-way RM ANOVA, \( t = 35.77; F_{7,75} = 3.599, P = 0.875 \)). (E) After correct repair, there was a significant improvement in temperature sensation in the ChABC-treated group compared with controls at day 70 (two way ANOVA; \( F_{1,51} = 8.772, P = 0.009 \); post hoc Bonferroni \( t \)-test \( * P < 0.05 \)). Parallel controls of animals that only received the median and ulnar nerve injury and repair on the left paw without any spinal cord injection (- - - - ) and their uninjured right paw (- - - - ) are shown in B and D and show the same level of recovery as the penicillinase-treated animals \( (n = 6 \) per group).
distal stump of the median nerve after crossover repair followed by either ChABC (1716 \pm 165; n = 11) or penicillase (1726 \pm 141; n = 6) treatment (t-test, t_{15} = -0.0395; P = 0.969, NS). Similarly, there was no difference in the weight of the FCR muscle after crossover repair followed by ChABC (87.7 \pm 3.73; n = 11) or penicillase (88.0 \pm 4.98; n = 6) treatment (t-test, t_{15} = -0.0395; P = 0.969, NS).

**Discussion**

In this study, we developed a rat forelimb model of peripheral nerve repair that reproduces the weak and clumsy limb that follows nerve repair in humans, together with a set of functional tests that quantify these defects. We demonstrated that the functional tests could quantify the behavioural consequences of axon misdirection by testing animals in which the median and ulnar nerves were repaired in four different ways, leading to progressively less accurate regeneration of axons back to their original targets. The nerves were (i) crushed; (ii) repaired correctly median to median and ulnar to ulnar; (iii) crossed over with median connected to ulnar and ulnar to median; (iv) cut and not repaired.

After nerve crush the endoneurial tubes surrounding axons remain intact, providing a structure that guides axons accurately back to their correct target (Nguyen et al., 2002). Nerve transection disrupts both the axons and endoneurial tubes. Regenerating axons may then enter wrong and multiple endoneurial tubes, resulting in a complex misdirection of regenerating axons and the almost random re-innervation of the denervated target (Valero-Cabre et al., 2004). Our correct repair should,
therefore, lead to randomization of axonal targeting, but with axons remaining within the correct innervation field of the median and ulnar nerves, respectively, while crossover repair should lead to median nerve axons innervating ulnar targets and vice versa. These expectations were confirmed by our retrograde mapping of motoneurons connected to the FCR muscle, showing a graded inaccuracy of muscle re-innervation after the three types of repair.

We used the staircase skilled paw reaching task to assess motor recovery because rats require fine finger dexterity which depends on accurate control of the intrinsic muscles of the forepaw to retrieve small sugar pellets from deep wells. We also measured grip strength, for which connections between skin and muscle afferents and motor output are important (Maurissen et al., 2003). Sensory function is quantitatively and qualitatively abnormal after nerve repair, so we tested fine touch and temperature sensation.

Recovery in motor tests differed in a graded fashion between the types of repair, and correlated with accuracy of re-innervation. After nerve crush, there was complete functional recovery, as might be expected following topographically correct axon regeneration demonstrated by us and in previous studies (Bodine-Fowler et al., 1997; Valero-Cabre et al., 2004). Recovery following correct repair was better than in crossover repair rats, as shown by a greater ability to retrieve and eat sugar pellets and by greater grip strength. However, recovery of unskilled limb movement, as measured by the ability of rats to knock sugar pellets out of wells without picking them up recovered almost equally and completely after all types of repair. The defects in grip strength recovery were not due to hyperalgesia (Woolf et al., 1992), because we tested for this and did not observe it, and importantly, the differences in recovery in the repair models were not due to different numbers of axons having regenerated to the muscles that control the digits. We noticed that, while within single experiment rats that received the same injury recovered to a similar degree, there was variation in the absolute degree of recovery between experiments. For this reason, we included a parallel set of nerve-injured controls that did not receive a spinal cord injection in the later experiments, and these show a similar level of recovery to the penicillinase-injected controls. The group of rats whose spontaneous recovery is shown in Fig. 3 showed better recovery than the control group in our treatment experiment. It is interesting that these animals were younger than those treated with chondroitinase.

We hypothesized that increasing CNS plasticity would allow for central compensation of the incorrect peripheral connections (Fig. 7). To promote plasticity, we used ChABC, which has been shown to increase plasticity and sprouting in the CNS (Pizzorusso et al., 2002; Corvetti and Rossi, 2005; Massey et al., 2006). ChABC produced long-lasting changes in the CNS ECM. Previous studies have demonstrated that the enzyme treatment exposes the sugar stubs that form the attachment site for chondroitin sulphate chains on the CSPG core proteins (Moon et al., 2001; Bradbury et al., 2002). Here we demonstrate that ChABC has more widespread effects on the ECM including digestion of hyaluronan, a recognized substrate of ChABC (Hamai et al., 1997) as shown previously in the cortex (Bruckner et al., 1998). This digestion led to the release of link protein and neurocan-N, two proteins known to bind to hyaluronan in the ECM. Immunoreactivity to the carbohydrate stubs which remain after ChABC digestion remained for at least 7 weeks, similar to previous reports in the cortex (Bruckner et al., 1998) confirming that turnover of CSPGs in the uninjured spinal cord is very slow (Bruckner et al., 1996, 1998). Moreover, the decreased level of neurocan core protein lasted 7 weeks, indicating that new synthesis of CSPGs in the undamaged spinal cord is also slow.

After nerve repair, we allowed 4 weeks for spontaneous behavioural recovery to begin and axonal regeneration to reach their distal targets. ChABC was then injected into the spinal cord, leading to a further improvement in functional recovery. Our observations indicate that the spinal cord must have been the site of action of the treatment rather than the peripheral nerves. First, the axons had 4 weeks to regenerate and innervate their distal targets before the ChABC was administered. Second, the number of regenerated axons and their effect on muscle atrophy was the same in both the ChABC-treated and control groups. Third, ChABC digestion in our experiment was restricted to a circumscribed region of the spinal cord. We were able to demonstrate an increase in axonal plasticity in the spinal cord directly by showing an increase in the density of processes stained for the axonal plasticity intrinsic marker phosphorylated MAP1B (Black et al., 1994). MAP1B is a developmentally regulated phosphoprotein that is localized in the distal portion of growing and regenerating axons. In axons of the adult CNS, MAP1B-P is maintained in regions exhibiting continuous plasticity (Nothias et al., 1996) and is upregulated in response to physiological changes or to injury-induced axonal remodelling (Soares et al., 2002).

ChABC treatment produced its main effects on skilled-reaching after correct repair and on grip strength after crossover repair. The lack of improvement in the paw-reaching task in the crossover repair rats was because the rats in this group learned that they could not do the task and therefore stopped trying. The difference between these rats and our first experiment (where some animals continued to reach) may have been due to much more frequent testing in the treatment experiment leading to extinction of the behaviour. However, rats in the crossover repair group showed a 1.87-fold increase in grip strength compared with controls despite the fact that the treatment did not affect the number of axons regenerating to muscles. The inaccuracy of muscle re-innervation was probably so severe that it affected even the unskilled grip-strength task, and enhanced spinal cord plasticity allowed partial functional correction. There was no evidence of hyperalgesia after ChABC treatment. This is encouraging because...
neuropathic pain can be a consequence of aberrant neuronal plasticity (Woolf and Salter, 2000) and ChABC treatment has been shown to promote sprouting of sensory axons in the injured cord (Bradbury et al., 2002).

The mechanism by which ChABC is able to promote plasticity is not proven, but recent studies have shown that ChABC induces axonal sprouting of Purkinje cells both in slice cultures (Tanaka et al., 2003) and in vivo (Corvetti and Rossi, 2005), and sprouting of optic axons (Tropea et al., 2003) and of the ascending sensory projection in the cuneate nucleus (Massey et al., 2006). In the present study, the level of MAP1B-P increased in rats that had received peripheral nerve injury and ChABC digestion, suggesting that axonal sprouting may be part of the plasticity leading to functional recovery. This plasticity may involve input from descending motor pathways, such as the rubrospinal (Murakami et al., 1984; Fujito and Aoki, 2002) and corticospinal (Chen and Wolpaw, 2002) pathways, and plasticity of the local spinal circuitry connecting sensory inputs to appropriate motoneurons (Holmberg et al., 1997; Rossignol et al., 2004).

It is probable that CSPGs in the PNNs are particularly involved in the control of plasticity (Pizzorusso et al., 2002; Rhodes and Fawcett, 2004; Apostolova et al., 2006; Dityatev and Schachner, 2006). The motoneurons in the ventral horn of the spinal cord are surrounded by PNNs (Takahashi-Iwanaga et al., 1998) that develop postnatally in an activity-dependent manner and then are stable in the adult (Kalb and Hockfield, 1988, 1990). It has recently been shown that tenascin-R deficient mice that have abnormal PNNs (Haunso et al., 2000) show better functional recovery after facial nerve injury and repair (Guntinas-Lichius et al., 2005). Removal of the PNNs with ChABC digestion is therefore probably the event that allows plasticity after peripheral nerve injury. Exactly how PNNs might have their effects on plasticity is unknown. The PNNs contain CSPGs and tenasin both of which inhibit axon growth and might therefore directly inhibit contact between sprouting axons and the surface of target neurons. CSPGs bind and present a variety of molecules including midkine, pleiotrophin, members of the fibroblast growth factor family (Milev et al., 1998; Deepa et al., 2002; Kawashima et al., 2002) and growth inhibitory factors, such as semaphorins 3a and 5 (Kantor et al., 2004; De Wit et al., 2005). They also block the access of axonal integrins to growth-promoting molecules such as laminin (Condic et al., 1999).

This study shows that ChABC is able to promote functional recovery after peripheral nerve repair adding further evidence to the emerging picture that ChABC can promote plasticity in the uninjured CNS. The treatment has potential for enhancing recovery from peripheral nerve repair by increasing spinal cord plasticity. It is also possible that enhanced spinal cord plasticity could be helpful in conditions that damage other parts of the CNS.

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Fig. 7 Hypothesis of the mechanism by which injections of ChABC into the spinal cord improved functional recovery after peripheral nerve injury and repair. In the normal spinal cord, sensory afferents enter the spinal cord into the dorsal horn, connecting with motoneurons and other neurons via interneurons in the intermediate grey of the spinal cord. This reflex pathway is modified by descending motor pathways including the corticospinal tract (CST) and the rubrospinal tract (RuST). Motoneurons and many interneurons are surrounded by perineuronal nets (PNNs). After peripheral nerve injury and repair the inaccuracy of reinnervation results in misdirection of the regenerating axons. However, the ECM and PNNs in the grey matter prevent central reorganization, so functional recovery is poor. Digestion of the grey matter of the spinal cord with the enzyme ChABC allows sprouting and reorganization of connections leading to improved functional recovery.
References


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