Delayed treatment with chondroitinase ABC promotes sensorimotor recovery and plasticity after stroke in aged rats

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Stroke is the dominant cause of sensorimotor disability that primarily affects the elderly. We now show that neuroplasticity and functional recovery after stroke is constrained by inhibitory chondroitin sulphates. In two blinded, randomized preclinical trials, degradation of chondroitin sulphate using chondroitinase ABC reactivated neuroplasticity and promoted sensorimotor recovery after stroke in elderly rats. Three days after stroke, chondroitinase ABC was microinjected into the cervical spinal cord to induce localized plasticity of forelimb sensorimotor spinal circuitry. Chondroitinase ABC effectively removed chondroitin sulphate from the extracellular matrix and perineuronal nets. Three different tests of sensorimotor function showed that chondroitinase ABC promoted recovery of forelimb function. Anterograde and retrograde tracing showed that chondroitinase ABC also induced sprouting of the contralesional corticospinal tract in the aged treated hemicord. Chondroitinase ABC did not neuroprotect the peri-infarct region. We show for the first time delayed chondroitinase ABC treatment promotes neuroanatomical and functional recovery after focal ischaemic stroke in an elderly nervous system.

Keywords: stroke; chondroitin sulphate proteoglycans; plasticity; ageing; perineuronal nets
Abbreviations: BDA = biotinylated dextran amine

Introduction

Stroke is the largest cause of long-term sensorimotor disability (Di Carlo, 2009), with nearly 90% of incidents occurring in individuals aged > 65 years (Truelsen et al., 2006). The elderly population are more likely to become disabled by stroke and they have a reduced capacity to recover from these disabilities compared to younger stroke survivors (Kelly-Hayes et al., 2003). However, the majority of experimental research is still conducted on young animals, despite recommendations by the Stroke Therapy Academic Industry Round Table and other stroke committees urging for the use of aged animals in preclinical studies when modelling the clinical disorder (Macleod et al., 2009; Saver et al., 2009).

Many human stroke survivors have small infarcts (Brott et al., 1989) and the location of these focal strokes predicts the type of functional deficit, thus it is important to model both these aspects in an elderly system. The most common functional deficit following stroke are motor impairments of the contralateral upper limb...
with >80% of subjects experiencing this acutely (Cramer et al., 1997) and 60% chronically (Dobkin, 2004). Additionally, reports also indicate up to 94% of human stroke survivors experience sensory deficits and disturbed performance of motor tasks requiring somatosensory information (Carey et al., 1993). Structural imaging of human stroke subjects demonstrates highly significant correlations between the extent of these sensorimotor impairments and focal ischaemic damage to the corticospinal tract (Lindenberg et al., 2010; Zhu et al., 2010). This indicates that corticospinal tract integrity plays a critical role in functional outcome and that localized ischaemic damage to this pathway is an important goal in animal models of focal stroke. Focal ischaemic lesions can be produced using endothelin-1, which occludes arteries and restricts regional blood flow to produce localized and dose dependent ischaemic injury in various brain regions in young animals (Adkins et al., 2004; Windle et al., 2006). Importantly, we have previously shown that focal ischaemic damage to the corticospinal tract using endothelin-1 produces sustained sensorimotor deficits similar to those observed in human stroke survivors, great reproducibility and low mortality rates in aged animals (Soleman et al., 2010). We now show that this model provides a powerful method for evaluating therapies that promote plasticity, which is vital as there is currently no drug treatment available beyond the acute stage of stroke.

Following injury to the adult CNS, undamaged neurons can undergo plastic responses and neuroanatomical reorganization to replace damaged synaptic connections (Carmichael, 2003; Dancause et al., 2005; Hsu and Jones, 2006). However, the extent of reorganization remains constrained by many intrinsic and extrinsic factors that limit neuronal growth. Age-related changes are also known to alter neuroplasticity and the physiological responses after ischaemic injury (Badan et al., 2003; Ward, 2005; Li et al., 2010) which can influence the effects of some drugs (Fisher and Ratan, 2003). Thus, this reveals age as an important factor when assessing potential therapeutics for neural repair. Various drug interventions that enhance events of structural plasticity have been shown to cause functional recovery after CNS injury (Chen et al., 2002; Wiessner et al., 2003; Lee et al., 2004; Clarkson et al., 2010). Of particular interest is chondroitinase ABC, which degrades inhibitory chondroitin sulphate proteoglycans present in the extracellular matrix. We and others have shown that degradation of chondroitin sulphates with chondroitinase ABC reduces the neurite-inhibitory environment, reactivates plasticity and promotes functional recovery after different central and peripheral nervous system injuries (Moon et al., 2001; Bradbury et al., 2002; Barratt et al., 2006; Massey et al., 2006; Pizzorusso et al., 2006; Galtrey et al., 2007; Cafferty et al., 2008; Garcia-Alias et al., 2009; Allain et al., 2011). However, to date the effects of chondroitinase ABC following stroke have yet to be explored.

Unlike most conventional stroke therapies that typically target the brain or the entire neuraxis, we administered chondroitinase ABC into the denervated cervical spinal cord to induce localized plasticity of spinal circuitry. Here, we tested the efficacy of this intraspinal chondroitinase ABC delivered 3 days after focal ischaemic stroke on neuroplasticity and functional recovery from sensorimotor deficits in aged animals. Our results demonstrate that delayed intraspinal chondroitinase ABC is able to alter the composition of diffuse chondroitin sulphate proteoglycans and condensed chondroitin sulphate proteoglycan-rich perineuronal nets present in the aged spinal cord to promote axonal reorganization and behavioural recovery after stroke. These findings constitute evidence that chondroitinase ABC promotes plasticity of the uninjured CNS after injury and propose chondroitinase ABC as a novel therapeutic candidate in ischaemic stroke for aged individuals.

Materials and methods

Subjects

Fifty aged male and female Long Evans rats (304–595 g; 16–19 months of age) were housed 2–3 to a cage in standard laboratory conditions. Experiments were performed in accordance with guidelines from the Stroke Therapy Academic Industry Round Table and others (Fisher et al., 2005; Macleod et al., 2009; Saver et al., 2009) and our findings were reported in accordance with the Animals in Research: Reporting In Vivo Experiments guidelines (Kilkenny et al., 2010). Surgeries, behavioural testing and analysis were performed with investigators blinded to treatment groups.

Experimental design

We investigated whether delayed intraspinal administration of chondroitinase ABC would promote functional recovery after unilateral stroke in aged (>16 month old) rats. Anterograde tracing was used to assess sprouting of uninjured corticospinal tract collaterals into areas of partial corticospinal tract denervation in the spinal cord.

In our second study, early behavioural improvements observed in the first study following delayed intraspinal administration of chondroitinase ABC were further investigated. Retrograde tracing was used to further assess sprouting of uninjured corticospinal tract collaterals and any neuroprotective effect on surviving corticospinal neurons. Experimental designs and groups are outlined in Supplementary Fig. 1. All surgeries were performed using a randomized block design and the experimenters were blinded to treatment groups during behavioural and histological assessment. Penicillinase served as a control as it is an enzyme shown not to degrade chondroitin sulphate proteoglycans (Moon et al., 2001; Pizzorusso et al., 2002).

Surgery

All procedures were in accordance with guidelines from the UK Home Office and Animals (Scientific Procedures) Act of 1986. Animals were anaesthetized with isoflurane (4% in O₂ for induction) and maintained at 1.5–2% in O₂ delivered via a facemask. Body temperature was monitored via a rectal thermometer and maintained at ~36°C with a heating pad.

Stroke lesions

Prior to surgery, rats were allocated to an experimental group in a counterbalanced fashion to ensure no difference in group mean preoperative performance of the dominant forepaw on the staircase test. Unilateral lesions were performed in the hemisphere contralateral to the dominant forelimb (Supplementary Fig. 3), as determined by the staircase behavioural test. Stroke lesions (n = 32) or sham surgeries were performed as previously described (Soleman et al., 2010) using...
a randomized block design. Briefly, animals were transferred to a stereotaxic frame (David Kopf Instruments) where a midline incision was made, the sensorimotor cortex was then exposed via craniotomy and the dura mater was incised. Since skull thickness varied amongst aged rats, a craniotomy was performed to enable accurate depth placement of endothelin-1 intracortical injections. Four 2 μl injections of endothelin-1 (200 pmol/μl; 0.5 μg/μl dissolved in sterile saline; CalBioChem) were delivered via a glass micropipette connected to a syringe (Hamilton). The first 1 μl was administered at a depth of 1 mm from the brain surface and the subsequent 1 μl applied to the surface of the cortex at four coordinates. Prior to suturing, the animal was left undisturbed for 5 min. Modifying previous work (Soleman et al., 2010), the skull fragment was then replaced and sealed using bone wax (Aesculap, Tuttligen). Sham-operated rats (n = 8) received all procedures up to, but not including, craniotomy. Animals were administered buprenorphine (0.01 mg/kg, subcutaneously) for postoperative pain relief after recovering from anaesthesia. Our method of inducing stroke with endothelin-1 is advantageous for evaluating regenerative stroke therapies for four reasons: (i) our model produces ischaemic lesions that model small focal human strokes rather than larger ‘malignant’ strokes, which tend to be fatal in humans (Carmichael, 2005); (ii) our model targets specific neuronal circuits that are typically affected after stroke including the corticospinal tract pathway that originates in the sensorimotor and agranular cortex; (iii) our stroke model has statistically powerful reproducibility (Soleman et al., 2010) and involves only low mortality rates in aged animals; and (iv) our model causes sustained sensorimotor deficits that are the most common neurological symptoms of human stroke.

### Spinal delivery of chondroitinase ABC

Animals received two unilateral spinal injections of either chondroitinase ABC (10 U/ml; Seikagaku) or the control enzyme penicillinsulfase (same μg/μl of protein; Sigma) delivered into the partially denervated side of the C5 and C8 spinal cord. Chondroitinase ABC was delivered intraspinally as previously described (Wong et al., 2006; Galtrey et al., 2007). Briefly, a partial laminectomy was performed to expose the cervical spinal cord at level C5 and C8. At each site, 1 μl injections of either chondroitinase ABC or penicillinsulfase were delivered using a glass micropipette connected to a 10 μl syringe (Hamilton) at a rate of 0.25 μl/min. The micropipette was positioned 1 mm lateral from the midline and lowered 1.5 mm below the spinal cord surface; this was left in situ for 2 min before being withdrawn. To assess the extent of chondroitin sulphate degradation after chondroitinase ABC treatment, rats were perfused 1 week after injections (n = 2). In our two main experiments, animals received unilateral intraspinal injections 3 days post-stroke injury. The number of rats per treatment group was based on sample size calculations from previous work (Soleman et al., 2010), which showed that a minimum of eight rats per treatment group would be required to identify a treatment which caused a 50% improvement on the staircase test (α = 0.05; power > 0.80).

### Anterograde tracing

In the first experiment, 7 weeks post-stroke, animals were given biotinylated dextran amine (BDA; 10%; 10 000 MW, Invitrogen) unilaterally into the uninjured sensorimotor cortex to visualize uninjured corticospinal tract axons. Animals were placed in a stereotaxic frame and six burr holes were made into the skull at the following coordinates defined as anteroposterior (AP), mediolateral (ML): (i) AP: +1 mm, ML: 1.5 mm; (ii) AP: +0.5 mm, ML: 2.5 mm; (iii) AP: +1.5 mm, ML: 2.5 mm; (iv) AP: +2.0 mm, ML: 3.5 mm; and (v) AP: −0.5 mm, ML: 3.5 mm, relative to bregma. At each site, 0.5 μl injections of BDA were delivered using a glass micropipette attached to a Hamilton syringe inserted 2 mm from the skull surface and delivered at a rate of 0.25 μl/min. Animals were subsequently left for 2 weeks before being perfused.

### Retrograde tracing

In the second experiment, 5 weeks post-stroke, animals were given unilateral intraspinal injections of the retrograde fluorescent tracer Fast Blue (2% in phosphate-buffered saline; Sigma) into the denervated side of the spinal cord and at areas of chondroitinase ABC-mediated digestion. The spinal cord (C6–C8) was exposed as described above. Each animal had five injections of Fast Blue (200 nl/site) spaced at ~1 mm apart along the spinal cord. A glass micropipette was positioned 1 mm lateral and 1.8 mm below the spinal cord surface. Animals were left for 10 days before being perfused. Location of the injections was verified post-mortem. Animals that failed to have consistent unilateral tracing mostly confined to the spinal grey matter were excluded from analysis (chondroitinase ABC group, n = 3; penicillinsulfase group, n = 3).

### Behavioural testing

Tests previously found to be effective in assessing sensory and motor deficits were included (Soleman et al., 2010). Rats were given 4 weeks of daily training on the staircase test to identify forepaw preference. All behavioural testing was carried out by an experimenter blinded to surgery and treatment groups. Baseline values were recorded 3 days before surgery on all behavioural tasks.

### Assessment of fine motor function

The staircase test was used to assess reaching performance; this provides a sensitive measure of skilled forepaw motor function (Montoya et al., 1991). The staircase apparatus (Campden Instruments Ltd.) consists of a chamber with a central platform for the rat to climb onto and a set of seven steps located on either side. Three sucrose pellets (45 mg, Research Diets Inc.) were placed in the well on each step and could be retrieved by the rat reaching down either side of the platform. The number of pellets retrieved (maximum of 21 pellets per side) and the maximum step reached using each forepaw was recorded during each 15 min trial. Scoring the number of pellets retrieved indicates successful grasp and retrieval (fine motor function), while the maximum step reached assesses the lowest step a pellet is displaced regardless of successful grasping (gross limb control). Each weekly session consisted of two trials; mean scores per rat per week were calculated. The minimum criterion to be included in the study was the retrieval of 11 pellets using their dominant paw during pre-operative testing.

### Assessment of forelimb asymmetry

The cylinder test was used to assess asymmetries in forelimb use for postural support during rearing (Schallert et al., 2000) within a transparent 20 cm diameter and 30 cm high cylinder. An angled mirror was placed behind the cylinder to allow movements to be recorded when the animal turned away from the camera. During exploration, rats rear against the vertical surface of the cylinder. The first forelimb to touch the wall was scored as an independent placement for that forelimb. Subsequent placement of the other forelimb against the wall to maintain balance was scored as ‘both’. If both forelimbs were simultaneously placed against the wall during rearing this was scored as ‘both’.
Lateral movements along the wall using both forelimbs alternately were also scored as ‘both’. Scores were obtained from a total number of 10 full rears to control for differences in rearing between animals. Once scores had been acquired, forelimb asymmetry was calculated using the formula: 100 \times (\text{ipsilateral forelimb use} + 1/2 \text{ bilateral forelimb use})/\text{total forelimb use observations} (Hsu and Jones, 2005).

Assessment of somatosensory function

The magnitude of somatosensory asymmetry and sensorimotor impairments in forepaw function after stroke was assessed using the bilateral tactile stimulation test (Schallert et al., 1982, 2000). For each trial, round adhesive patches (13 mm diameter, Ryman) were applied to the plantar surface of both forepaws and the animal was returned to its home cage. Two times were recorded for both forepaws: (i) contact; and (ii) remove; where ‘contact’ represents the time taken for the animal to notice the adhesive patch on its forepaw and bring it to its mouth, and ‘remove’ represents the time taken for the animal to remove the adhesive patch from its forepaw. To determine whether the rats showed bias for their affected or less-affected forelimbs, the order and side of label removal was recorded. This was repeated four times per session until a >75% preference had been found; if this was not the case a fifth trial was conducted. The magnitude of asymmetry was established using the seven levels of stimulus pairs on both forepaws as previously described (Fig. 2D, E) (Schallert and Whishaw, 1984; Schallert et al., 2000). During this phase, to determine the extent of ipsilateral response bias, the size of the stimulus was progressively increased on the affected forepaw and decreased on the less-affected forepaw by an equal amount (14.1 mm²), until the rat removed the stimulus on the affected forepaw first (reversal of original bias). This reversal represents the magnitude of asymmetry, where the higher the score indicates the greater the degree of somatosensory impairment.

Histology

Animals were terminally anaesthetized with sodium pentobarbital (80 mg/kg; intraperitoneally) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains and spinal cords were postfixed for 2 h at 4 °C then transferred to 30% sucrose in phosphate-buffered saline for 2 days. The brain and spinal cord were separately embedded in 10% gelatin in distilled water and blocks were postfixed in 4% paraformaldehyde for 24 h. Free-floating serial sections were then cut using a freezing stage microtome (Kryomat). Ten series of rostral to caudal tissue sections were collected in 24-well plates containing phosphate-buffered saline (with 0.1% sodium azide) and stored at 4 °C.

Immunohistochemistry

All sections used were transverse C4–C8 spinal cord sections (40 mm). To confirm chondroitin sulphate proteoglycan digestion, sections were incubated in mouse anti-chondroitin-4-sulphate (1:1000; MP Biomedicals) and goat anti-mouse Alexa 488 (1:500; Invitrogen). To assess degradation of chondroitin sulphate proteoglycan-rich perineuronal nets, sections were incubated in biotin-conjugated Wisteria floribunda agglutinin (15 μg/ml; Sigma) and extra-avidin tetramethyl rhodamine isothiocyanate (1:1000; Sigma). To assess fibre sprouting from the uninjured corticospinal tract, sections were incubated in mouse anti-chondroitin-4-sulphate (1:1000; MP Biomedicals), biotinyl tyramide (1:75; PerkinElmer Life Sciences) and extra-avidin fluorescein isothiocyanate (1:1000; Sigma). All sections were coverslipped with Fluorosave™ mounting medium.

Histological analysis

Images were captured using a Carl Zeiss AxioImager Z1 microscope with Z-stack intervals. All images were re-coded during analysis so the investigator was blinded to treatment groups.

Analysis of biotinylated dextran amine labelled axons

Transverse spinal cord sections in the treated area (C6–C8) were selected. To ensure consistent tracing within animals, the number of BDA-labelled axons present in the dorsal column were quantified and averaged from 10 sections per animal. In the denervated grey matter of these sections, sprouting BDA-labelled axons were quantified. To reduce between animal tracing variations, the axon index was calculated as previously described (Zheng et al., 2005; Lee et al., 2010). For this, the number of BDA-labelled axons present in the denervated grey matter was normalized against the total number of labelled axons present in the dorsal column. The distance (μm) of each BDA-labelled axon along the mediolateral axis from the midline was determined from the central canal.

Analysis of Fast Blue-labelled corticospinal neurons

Coronal brain sections from regions +3.5 mm to −1 mm relative to bregma were selected. The total number of retrogradely labelled corticospinal neurons present in both hemispheres was quantified in 12 sections per animal. In the injured hemisphere, cell area (μm²) was assessed in all corticospinal neurons from two cortical 500 μm-wide regions in the ipsilesional hemisphere: (i) the area directly around the lesion cavity (Supplementary Fig. 2A); and (ii) the area neighbouring the injury site (Supplementary Fig. 2A'). For the latter, as no visible cavity was present, we used the following approach: in sections that had lesion cavities, the distance from the lesion centre to the midline interhemispheric fissure was measured and applied to the sections with no visible lesion. A region 500 μm from this calculated lesion midline was applied on either side and cells within this area were also measured. Cell size distributions were then tabulated.

Quantification of infarct volume

Serial coronal sections from +3.5 mm to −2.5 mm relative to bregma were captured using a light microscope with a high-resolution digital camera (MiniVID Digital Eyepiece Camera, LW Scientific). Infarct size was calculated as previously described (Soleman et al., 2010). For each section, the total area of the ipsilesional hemisphere was subtracted from the area of the contralesional hemisphere. Hemisphere area measurements excluded necrotic tissue, cysts and cavities, and ventricles. Volume of injury (mm³) was calculated as the sum of the area from each section, multiplied by the distance between sections (Buchan et al., 1992). Before the end of the study, one animal died due to age-related pituitary tumours and is thus absent from histological analysis (chondroitinase ABC, n = 1).

Statistical analysis

Behavioural data were analysed using repeated measures analysis of covariance (ANCOVA), using preoperative performances as covariates. Pairwise group comparisons were performed using two-tailed Fisher’s protected Least Significant Difference post hoc tests. Infarct volumes were compared using two-tailed t-tests. The number of Fast Blue-labelled cells and BDA axon counts were compared between groups using the Kruskal–Wallis and Mann–Whitney tests. Cell size
distributions between groups were analysed using repeated measures analysis of variance (ANOVA) and the Kolmogorov–Smirnov test. Data are presented as mean ± standard error of the mean (SEM) and asterisks indicate significance as follows: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. SPSS (IBM) version 18 was used for analysis.

Results

Delayed chondroitinase ABC promotes forelimb motor recovery following stroke in aged rats

To assess motor function, dexterity of the affected forepaw was examined using the staircase test (Fig. 1A; Supplementary Video 1). Here, two parameters were measured: the number of pellets retrieved (skilled motor function) and maximum step from which pellets were retrieved or displaced (unskilled limb control). Concerning the number of pellets retrieved, 1 week after stroke all animals showed a marked reduction in their ability to retrieve pellets with their affected forepaw (sham versus either stroke group, P < 0.001), with no difference between treatment groups at this time-point (chondroitinase ABC versus penicillinase, P = 0.19; Fig. 1B). Compared to preoperative levels, penicillinase-treated animals were able to recover to a maximum of 59%; in comparison, chondroitinase ABC-treated animals were more successful at retrieving pellets (P = 0.007) and were able to recover to a maximum of 85%. Concerning the maximum step, stroke groups remained impaired relative to sham animals (chondroitinase ABC, P = 0.01; penicillinase, P < 0.001; Fig. 1C), with no difference between treatment groups (chondroitinase ABC versus penicillinase, P = 0.24). Thus, our data show that chondroitinase ABC treatment is capable of significantly improving forepaw dexterity to aid successful pellet retrieval, while unskilled limb movements were unaffected by treatment demonstrating some spontaneous recovery.

To further assess forelimb motor function, we employed the cylinder test. This measures forepaw use for postural support during rearing (Fig. 1D). Sham animals demonstrated approximately equal use of forelimbs during postural support (asymmetry score ≈ 50). One week after stroke, animals exhibited an increase in asymmetry score indicating preferential use of their less-affected forelimb for support during rearing (sham versus penicillinase, P = 0.002; sham versus chondroitinase ABC, P = 0.08; Fig. 1E). Sustained functional deficits were apparent in the penicillinase-treated animals, with little recovery occurring beyond the first week of stroke (Week 1 versus Week 9, P = 0.87; Supplementary Video 2). In contrast, chondroitinase ABC-treatment significantly reduced the functional deficits compared to penicillinase animals (P = 0.002), indicating use of their affected forelimb for postural support. By Week 9 post-stroke, there was no significant difference between chondroitinase ABC-treated and sham animals (P = 0.17; Supplementary Video 3).

Interestingly, our data showed a rapid effect of chondroitinase ABC as animals had smaller functional deficits by the first week of behavioural testing (4 days after treatment) compared to the penicillinase group (P = 0.06). To confirm the immediate effects of chondroitinase ABC were not due to baseline differences from the lesion, a second experimental study was repeated with a behavioural testing time point at 2 days post-stroke (1 day before drug intervention). Importantly, both groups prior to treatment showed an equal extent of functional impairment (chondroitinase ABC versus penicillinase, P = 0.68; Fig. 1F) indicating both stroke groups possessed equivalent stroke lesions and behavioural deficits. After treatment, penicillinase-treated rats remained persistently impaired (Day 2 versus Week 5; P = 0.36). Interestingly, chondroitinase ABC-treated rats exhibited immediate behavioural recovery in the first week (Day 2 versus Week 1; P = 0.06) and improved until the end of the study (Day 2 versus Week 5; P = 0.005). By Week 5, chondroitinase ABC-treated animals were significantly different to penicillinase-treated animals (P = 0.04) and no different to sham animals (P = 0.23). Together these two studies show chondroitinase ABC is able to promote use of the affected forelimb during postural support and reduces forelimb deficits following cerebral ischaemia in aged rats.

Delayed chondroitinase ABC promotes forelimb sensory recovery following stroke in aged rats

To assess forepaw impairments and asymmetries in somatosensory function, we used the bilateral tactile stimulation test. A small adhesive patch was stuck to the plantar surface of each forepaw and the time taken to contact and remove both stimuli with their mouth was recorded (Fig. 2A). Sham animals rapidly contacted and removed both stimuli. Stroke rats showed impairments in both sensing and removing the adhesive patch from their affected forepaw (Fig. 2B and C). However, there was no effect of drug treatment as animals displayed a similar extent of recovery in contact (chondroitinase ABC versus penicillinase, P = 0.19; Fig. 2B) and removal time (chondroitinase ABC versus penicillinase, P = 0.71; Fig. 2C).

We also assessed recovery from tactile extinction, a phenomenon manifested in many stroke patients who fail to detect a touch stimulus on their affected hand once it has been applied simultaneously on both hands (Rose et al., 1994). We obtained sensory asymmetry scores using pairs of sensory stimuli (Schallert et al., 2000) (Fig. 2D and E). After stroke, all animals had high asymmetry scores, indicating that animals neglected the larger stimulus on their affected forepaw and preferentially removed the smaller stimulus from their less-affected forepaw first. Penicillinase-treated animals remained persistently impaired relative to sham animals (P < 0.001; Fig. 2F). In contrast, chondroitinase ABC induced significant recovery from asymmetrical sensory impairments with animals able to detect smaller stimuli on their affected forepaw (chondroitinase ABC versus penicillinase, P = 0.04). In conclusion, while chondroitinase ABC had no effect on sensory response times, it overcame somatosensory neglect of the affected forepaw following stroke. This is important as tactile extinction was shown to be the single most important predictor of functional outcome (Rose et al., 1994), thus improving this ability
to detect and process sensory data should enable improvements in motor function.

**Chondroitinase ABC injections effectively degrade diffuse chondroitin sulphate proteoglycans present in the aged spinal cord**

Unilateral intraspinal injections of chondroitinase ABC were carried out at C5 and C8 to encourage localized plasticity and midline crossing of the intact corticospinal tract into chondroitinase ABC-treated areas of the aged cervical spinal cord. We examined the pattern of chondroitin sulphate proteoglycan digestion using an antibody that recognizes the chondroitin-4-sulphate stub epitope on chondroitin sulphate proteoglycan core proteins following chondroitin sulphate removal. As expected, no immunoreactivity for chondroitin-4-sulphate was detected in sham controls or penicillinase-treated animals (Fig. 3A and B). In a pilot study, we assessed the extent and spread of chondroitin sulphate proteoglycan degradation. One week following chondroitinase ABC injections, extensive and predominantly unilateral chondroitin-4-sulphate immunoreactivity spanned segments C4 to T1 (Fig. 3C and D). Even 10 weeks after chondroitinase ABC intraspinal injections, intense immunoreactivity was still present in the cervical cord, confirming chondroitinase ABC effectively degraded chondroitin sulphate proteoglycans and that its effects are long lasting (Fig. 3E). Quantification of chondroitin-4-sulphate immunoreactivity revealed intense digestion in both the spinal cord grey and white matter, (Fig. 3F) and spanning dorsal, ventral and midline regions of the spinal cord (Fig. 3G). The greatest degree of chondroitin sulphate proteoglycan digestion was observed in the
ventral horn and funiculus. Thus, these results indicate that intraspinal chondroitinase ABC injections were effective in degrading growth inhibitory chondroitin sulphate proteoglycans.

Chondroitinase ABC injections degrade perineuronal nets present in the aged spinal cord

Previous studies have demonstrated that digestion of chondroitin sulphate proteoglycans in perineuronal nets with chondroitinase ABC can reactivate plasticity (Pizzorusso et al., 2002; Massey et al., 2006), leading to the hypothesis that perineuronal nets are involved in the control of plasticity in the CNS (Carulli et al., 2010). We examined W. floribunda agglutinin reactivity, a marker of chondroitin sulphate proteoglycan-rich perineuronal nets, 1 week following chondroitinase ABC delivery. Here, we observed that chondroitinase ABC was able to reduce W. floribunda agglutinin reactivity (Fig. 4A), and this effect was only present in areas of chondroitin sulphate proteoglycan degradation (Fig. 4B and C). Intact perineuronal nets surrounding motor neurons in the ventral horn were observed outside areas of chondroitinase ABC-mediated digestion (Fig. 4D and F) and were completely absent within degraded areas (Fig. 4E and G). Thus, our results confirm that intraspinal chondroitinase ABC delivery not only alters the composition of the diffuse extracellular matrix but also alters the

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**Figure 2** Delayed chondroitinase ABC promotes forelimb sensory recovery following stroke in aged rats. The bilateral tactile stimulation test assesses sensory function using adhesive sticky patches placed on the rats’ forepaws (A). Stroke increased the time animals took to sense (B) and remove (C) these adhesive patches placed on their affected forepaw, and both treatment groups showed a similar extent of recovery [sense: F(2,17) = 1.57, P = 0.24; remove: F(2,17) = 0.63, P = 0.55]. (D, E) The magnitude of sensory asymmetry (tactile extinction) was determined using the seven pairs of sensory stimuli (see text). (D) Stimulus size was progressively increased on the affected forepaw and decreased on the less-affected forepaw by an equal amount, until the rat removed the stimulus on the affected forepaw first. This reversal represents the magnitude of asymmetry. (E) Testing begins at Level 3 (black box) and asymmetry scores indicate the severity of sensory asymmetry (e.g. severe impairment = 7). (F) Stroke increased the magnitude of sensory asymmetry (tactile extinction) in both groups. Penicillinase-treated (P-ase) animals remained impaired whereas animals treated with chondroitinase ABC (ChABC) showed significant improvements in detecting the sensory stimulus on their affected forepaw [group differences F(2,17) = 12.2, P = 0.001]. Results are presented as mean ± SEM and were analysed using repeated measures ANCOVA with post hoc Least Significant Difference tests. Significance is denoted as: *P < 0.05, **P < 0.01, ***P < 0.001 versus sham animals (n = 5) and *P < 0.05, **P < 0.01, ***P < 0.001 versus treatment groups (n = 9 per group).
Figure 3 Intraspinal chondroitinase ABC injections degrade matrix chondroitin sulphate proteoglycans. (A–E) Immunolabelling using the 2B6 antibody that recognizes the chondroitin-4-sulphate stub region of chondroitin sulphate proteoglycans after degradation of chondroitin sulphate by chondroitinase ABC (ChABC). Chondroitin-4-sulphate immunoreactivity in the C6 region of the spinal cord was absent in sham (A) and penicillinase-treated (P-ase) (B) rats. (C) In chondroitinase ABC-treated animals, after 1 week there was an intense immunoreactivity surrounding the injection site (indicated by arrow) revealing extensive chondroitin sulphate proteoglycan digestion from C6–T1 in horizontal sections of the spinal cord. Transverse sections demonstrate unilateral chondroitin sulphate degradation throughout the C5 spinal cord after 1 week (1 W) (D) and 10 weeks (10 W) post-chondroitinase ABC injections (E). (F, G) Quantification revealed intense degradation in both the grey (gm) and white matter (wm) (F), as well as throughout dorsal, ventral and midline areas (G). Scale bars: 500 µm (A, B, D, E), 1 mm (C). Results are presented as mean ± SEM, n = 9.
condensed matrix in these chondroitin sulphate proteoglycan-rich perineuronal nets that are found around neurons present in the spinal cord, particularly around motor neurons.

Delayed chondroitinase ABC promotes collateral sprouting of the uninjured corticospinal tract in the aged spinal cord

In our first experiment, BDA was unilaterally injected into the contralesional hemisphere to anterogradely label intact corticospinal tract axons and collaterals (Fig. 5A). Immunofluorescent visualization of BDA was used to assess axonal sprouting into chondroitinase ABC-treated areas of the spinal cord grey matter. To control for variations in BDA labelling amongst individual animals, axonal counts in the denervated grey matter were normalized against the total number of BDA labelled corticospinal tract axons present in the dorsal column (Zheng et al., 2005). No group differences in BDA labelling were observed ($P = 0.77$; Fig. 5B–D), confirming similar tracing efficiency. Penicillinase-treated animals possessed few BDA-labelled corticospinal tract collaterals in the grey matter of the treated side (Fig. 5E). In contrast, chondroitinase ABC-treatment significantly increased corticospinal tract collateral sprouting on the treated side ($P = 0.02$; Fig. 5F and G). Furthermore, the distance of uninjured corticospinal tract collaterals sprouting from the midline revealed a strong trend for chondroitinase ABC to encourage more axons to sprout at longer distances compared to penicillinase ($F(1,6) = 4.85, P = 0.07$; Fig. 5H). In chondroitinase ABC-treated animals, it was also observed that some BDA-positive axons were sprouting within areas of chondroitin sulphate proteoglycan degradation (Fig. 5I).

In our second experiment, Fast Blue was used to further assess corticospinal tract collateral sprouting by retrogradely labelling corticospinal neurons of the uninjured corticospinal tract (Fig. 6A): Fast Blue was carefully and unilaterally injected into chondroitinase ABC-treated areas of the cervical grey matter (Fig. 6B). We quantified the number of corticospinal neurons present in the intact sensorimotor cortex that projected collaterals into these chondroitinase ABC-treated areas. Labelled corticospinal neurons were primarily located rostral to Bregma, particularly from the primary motor (M1) and somatosensory forelimb cortical regions. Sham and penicillinase-treated animals displayed similar numbers of Fast Blue-labelled corticospinal neurons ($P = 0.13$; Fig. 6C–E). However, chondroitinase ABC-treated animals possessed significantly greater numbers of labelled corticospinal neurons in the sensorimotor cortex (Fig. 6C and F) compared to penicillinase-treated animals ($P = 0.03$). Together these results demonstrate that chondroitinase ABC is able to induce plasticity and cause axonal collateral sprouting from the intact corticospinal tract into chondroitinase ABC-treated areas of the spinal cord in aged rats.

Delayed chondroitinase ABC does not promote neuroprotection after stroke in aged rats

We and others previously reported that surviving corticospinal neurons in the ipsilesional hemisphere following stroke display a degree of cell atrophy (Enright et al., 2007; Soleman et al., 2010). As chondroitinase ABC has been shown to reverse atrophy of corticospinal neurons after spinal cord injury (Carter et al., 2008), we analysed the soma sizes of corticospinal neurons.
retrogradely labelled with Fast Blue at 5 weeks post-injury (Supplementary Fig. 2A). At 7 weeks post-stroke, cell atrophy was evident in penicillinase-treated animals compared to sham controls, particularly for cell sizes between 150 and 300 μm² (Supplementary Fig. 2B, C and E). Following chondroitinase ABC treatment, corticospinal neurons appeared to possess slightly larger-looking somata (Supplementary Fig. 2D), with cell size distribution analysis revealing a trend towards significance between treatment groups for cells between 150–300 μm² (P = 0.07). However, overall drug treatment had no significant effect on cell size distribution [F(2,6) = 0.73, P = 0.52] and cumulative frequency (P = 0.3; Supplementary Fig. 2E and F). There was also no difference in the total number of surviving Fast Blue-labelled corticospinal neurons between treatment groups.

Figure 5 Chondroitinase ABC induces cervical collateral sprouting from the intact corticospinal tract following stroke. (A) Schematic diagram illustrating that BDA injections were administered into the uninjured hemisphere to label projections from the intact corticospinal tract (CST). (B, C) Photomicrographs of BDA-positive fibres present in the dorsal column of the cervical spinal cord in penicillinase-treated (P-ase) animals (B) and chondroitinase ABC-treated (ChABC) animals (C). (D) Quantification of these fibres revealed no significant difference between treatments groups, confirming similar tracing efficiency between animals. (E, F) Photomicrographs of BDA-positive axons projecting to the denervated side of the spinal cord in penicillinase-treated animals (E) and chondroitinase ABC-treated animals (F). Dashed lines indicate the spinal cord midline and arrow-heads highlight axons present on the treated side of the spinal cord. (G) Quantification revealed that animals treated with chondroitinase ABC showed a significant increase in the number of BDA-labelled axons present on the treated side compared to penicillinase treated animals. (H) Mediolateral spatial distribution of BDA-positive fibres throughout the treated side of the spinal cord in chondroitinase ABC-treated animals compared to penicillinase-treated animals. (I) Example of axons (red) in areas of chondroitin sulphate proteoglycan digestion (green). Results are presented as mean ± SEM and were analysed using Kruskal–Wallis and Mann–Whitney tests (D, G) or repeated measures ANOVA (H). Significance is denoted as: *P < 0.05 between treatment groups (n = 4 per group). Scale bars: 100 μm (B, C, E and F).
(P = 0.29; Supplementary Fig. 2G), showing that delayed chondroitinase ABC does not prevent corticospinal neuron cell death in the injured hemisphere.

Additionally, histological analysis of the brain at 10 weeks after stroke revealed the extent of ischaemic damage in lesioned aged rats (Supplementary Fig. 3A and B). Affected areas primarily included the primary motor cortex (M1) with varying amounts of the secondary motor cortex (M2), agranular cortex and primary somatosensory cortex (S1, particularly the forelimb and hindlimb regions). Infarct volume analysis revealed no differences between drug treatment groups (P = 0.1; Supplementary Fig. 3C), confirming both groups had similar extents of ischaemic injury. Thus, our results suggest that intraspinal delivery of chondroitinase ABC does not reverse corticospinal neuron atrophy associated with ischaemic stroke or affect infarct size and therefore we attribute recovery to plasticity of the corticospinal tract and other intraspinal circuits.

**Figure 6** Chondroitinase ABC increases the number of corticospinal neurons projecting to areas of denervation following stroke. (A) Schematic diagram illustrating the five unilateral Fast Blue injection sites which retrogradely labelled corticospinal neurons (CSNs) in the uninjured hemisphere that sprouted uninjured corticospinal tract projections into areas of chondroitinase ABC-mediated digestion. (B) Transverse C5 spinal cord sections confirmed that the Fast Blue injections were unilateral and within the grey matter of the spinal cord. (C) Quantification of the number of labelled corticospinal neurons present in the uninjured sensorimotor cortex revealed that chondroitinase ABC-treatment (ChABC) increased the number of corticospinal neurons projecting to the denervated side of the spinal cord. (D–F) Photomicrographs of the sensorimotor cortex (Bregma + 0.5 mm) showing the number of Fast Blue-labelled corticospinal neurons in sham (D), penicillinase-treated (P-ase) (E) and chondroitinase ABC-treated animals (F). Arrow-heads highlight Fast Blue-labelled corticospinal neurons in sensorimotor cortex. Results were presented as mean ± SEM and were analysed using Kruskal–Wallis and Mann–Whitney tests. Significance is denoted as: *P < 0.05 between treatment groups (n = 4 per group). Scale bar: 1 mm (B), 500 μm (D–F).

**Discussion**

Ischaemic stroke induces sustained sensorimotor impairments and limited spontaneous behavioural recovery. This was supported in our study where control-treated animals showed little improvement in behavioural function after stroke, consistent with our previous work (Soleman et al., 2010). Importantly, we show for the first time that intraspinal delivery of chondroitinase ABC initiated 3 days post-stroke in an aged CNS is significantly able to reduce sensorimotor impairments of the affected forelimb, including fine
motor function, forelimb use during rearing and somatosensory function (reversal of neglect). Our results extend previous work showing chondroitinase ABC promotes behavioural recovery after different neurological injuries (Bradbury et al., 2002; Pizzorusso et al., 2006; Galtrey et al., 2007; Cafferty et al., 2008; Garcia-Alias et al., 2009; Allain et al., 2011). Approximately 16 million people worldwide suffer from stroke every year (Strong et al., 2007) and there is still no restorative drug treatment beyond the acute stage of stroke. Our study proposes chondroitinase ABC as a potential treatment to promote neural repair after stroke that is clinically attractive as it also counteracts existing problems of treatment time windows and age as chondroitinase ABC was administered 3 days post-stroke in an elderly CNS system.

Human and animals studies have shown that the adult CNS is capable of some functional reorganization that is associated with the limited spontaneous recovery observed following stroke (Cramer, 2008). Two main mechanisms considered for reorganization and plastic changes after stroke are: (i) formation of new neuronal circuitry; and (ii) unmasking/strengthening of existing pathways. In the present study, chondroitinase ABC was administered into the denervated side of the spinal cord to encourage localized plasticity of spinal circuitry and limit the extent of chondroitinase ABC-mediated digestion throughout the neuraxis, thus minimizing effects on other brain regions. As delivery of chondroitinase ABC was spatially restricted this suggests recovery was possibly a consequence of enhanced plasticity within local spinal circuitry. It is likely that chondroitinase ABC stimulated anatomical changes in a number of spinal pathways that may have contributed to the observed functional improvements. However, here we anatomically traced and primarily focused on the intact corticospinal tract, a major pathway known to modulate both sensory and motor function. Our results demonstrate that chondroitinase ABC is able to enhance plastic changes and axonal sprouting of the intact corticospinal tract from the contralesional hemisphere. This is consistent with other stroke studies that have enhanced anatomical sprouting of uninjured spinal pathways from the contralesional hemisphere and consequently improved recovery of function (Chen et al., 2002; Papadopoulos et al., 2002; Lee et al., 2004; Markus et al., 2005). Other human and animal studies have also shown that the contralesional hemisphere is involved in recovery following stroke, including changes in dendritic arborization (Jones and Schallert, 1992; Biernaskie and Corbett, 2001; Uryu et al., 2001) and enhanced cortical activation (Dijkhuizen et al., 2003; Schaechter and Perdue, 2008). It has been reported that stroke induces reorganization in two regions of the cortex: an area immediately adjacent to the infarct core that has a substantial increase in chondroitin sulphate proteoglycans, and a more distant area from the infarct that has reduced levels of chondroitin sulphate proteoglycan and a loss of perineuronal nets that contributes to anatomical plasticity (Carmichael et al., 2005). Whether further digestion of chondroitin sulphate proteoglycans in these peri-infarct regions enhances local plasticity and functional recovery has yet to be determined, but may reveal the potential for local as well as distant chondroitinase ABC treatment in stroke.

Additionally, the rapid recovery of function may be attributed to the unmasking of physiologically silent synapses (Carmel et al., 2010) which may be recruited after alterations in neural activity following treatment. Recently, it has been shown that intraspinal injections of chondroitin sulphate proteoglycan acutely depresses axonal conduction in a dose-dependent manner (Hunanyan et al., 2010) and intraspinal chondroitinase ABC injections prevent the decline in axonal conduction in intact fibres after spinal cord injury (Hunanyan et al., 2010). This demonstrates an inhibitory action of chondroitin sulphate proteoglycans on axonal conduction and that their removal using chondroitinase ABC can enhance neural activity. Thus, it may also be possible that chondroitinase ABC enhanced conduction and synaptic transmission in the present study.

Another mechanism for both rapid and progressive recovery may be the removal of perineuronal nets. In the adult spinal cord, chondroitin sulphate proteoglycans are distributed diffusely in both the grey and white matter extracellular matrix (Tang et al., 2003) and are also highly condensed into structures known as perineuronal nets that surround specific neuronal cell bodies, such as motor neurons (Takahashi-Iwanaga et al., 1998). The time of the appearance of perineuronal nets is closely correlated with the termination of plasticity and the end of the critical period in development (Pizzorusso et al., 2002). Developmental attenuation of perineuronal nets extends this critical period (Carulli et al., 2010) and digestion of chondroitin sulphate proteoglycan-rich perineuronal nets in the adult visual cortex using chondroitinase ABC is known to reactivate plasticity (Pizzorusso et al., 2002), confirming that perineuronal nets control plasticity in the CNS. A recent study has also shown that removal of perineuronal nets around phrenic motor neurons using chondroitinase ABC promotes rapid functional recovery of diaphragmatic function after spinal cord injury 1 week after treatment (Allain et al., 2011). We have shown that microinjections of chondroitinase ABC directly into the spinal cord produces rapid, localized and long-lasting chondroitin sulphate proteoglycan digestion throughout both the grey and white matter, consistent with previous studies (Galtrey et al., 2007; Cafferty et al., 2008; Garcia-Alias et al., 2009). We also found chondroitinase ABC effectively removed both diffuse chondroitin sulphate proteoglycans in the cervical spinal cord and chondroitin sulphate proteoglycan-rich perineuronal nets present around motor neurons. Our data indicate that removal of perineuronal nets may be a probable mechanism through which chondroitinase ABC rapidly reactivates plasticity and recovery of function.

In summary, our work is the first to demonstrate that chondroitinase ABC can promote recovery of sensorimotor forelimb function after stroke and plasticity in the uninjured CNS. Excitingly, we show that recovery and neuroplasticity occurs in an elderly CNS, even when treatment is delayed by 3 days, and proposes chondroitinase ABC as a novel therapeutic candidate in ischaemic stroke for aged individuals.

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Supplementary material

Supplementary material is available at Brain online.

References


Hsu JE, Jones TA. Contralateral neural plasticity and functional changes in the less-affected forelimb after large and small cortical infarcts in rats. Exp Neurol 2006; 201: 479–94.


