Title: Plasticity induced recovery of breathing occurs at chronic stages after cervical contusion

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Abstract:

Severe mid-cervical contusion injury causes profound deficits throughout the respiratory motor system which last from acute to chronic time points post injury. Here we use chondroitinase ABC (ChABC) to digest chondroitin sulphate proteoglycans (CSPGs) within the extracellular matrix (ECM) surrounding the respiratory system at both acute and chronic time points post injury to explore whether augmentation of plasticity can recover normal motor function. We demonstrate that, regardless of time post injury or treatment application, the lesion cavity remains consistent showing little regeneration or neuroprotection within our model. However, through electromyography (EMG) recordings of multiple inspiratory muscles, we show that application of the enzyme at chronic time points post injury initiates the recovery of normal breathing in previously paralysed respiratory muscles. This reduced the need for compensatory activity throughout the motor system. Application of ChABC at acute time points recovered only modest amounts of respiratory function. To further understand this effect, we assessed the anatomical mechanism of this recovery. Increased EMG activity in previously paralyzed muscles was brought about by activation of spared bulbospinal pathways through the site of injury and/or sprouting of spared serotonergic fibres from the contralateral side of the cord. Accordingly, we demonstrate that alterations to the ECM and augmentation of plasticity at chronic time points post cervical contusion can cause functional recovery the respiratory motor system and reveal mechanistic evidence of the pathways which govern this effect.

Key words:

Plasticity, respiratory motor system, cervical contusion injury, chondroitinase ABC, respiratory recovery
Introduction

Patients with severe mid-cervical spinal cord injuries (SCIs) typically suffer deficits in respiratory motor function which last from acute to chronic stages post trauma.¹ This is particularly significant as this is the most prevalent form of SCI² with deficits in respiratory function being the primary cause of morbidity and mortality in human patients.¹³,⁴ Modelling these injuries experimentally requires impairment to the respiratory motoneurons and bulbospinal pathways which mediate activity in the diaphragm (primarily responsible for inspiration in mammals)⁵ through innervation of the phrenic motor pool (PMP). However, depending on the size and extent of trauma, the injury can also affect the accessory respiratory muscles such as the external intercostals (eICs) and the pharyngeal dilator muscles (e.g. the genioglossus).

Current animal models of mid-cervical contusion injury have demonstrated decreases in activity throughout the respiratory motor system by assessment of diaphragm electromyography (EMG), phrenic nerve activity, waveform variance, and ventilatory capacity.⁶⁻¹⁴ A recent model of this disorder shows a robust deficit within respiratory parameters from acute to chronic time points post trauma.¹⁴ Nonetheless, there is evidence of compensatory plasticity within the injured system post contusion. Indeed, increasingly moderate mid-cervical contusion injuries show recovery in respiratory parameters four-to-eight weeks after the initial trauma due to the endogenous plasticity of spared tissue and respiratory motor pathways.¹⁵⁻¹⁹ We hypothesised that through exogenous enhancement of plasticity we could facilitate recovery of respiratory motor activity at both acute and chronic time points following severe mid-cervical contusion injury. Of course, functional recovery at chronic time points following injury is typically considered more difficult to accomplish as the injuries tend to be more extensive. This is due to processes such as increased neuronal degeneration, and axonal entrapment within the glial scar (reviewed in²⁰).

One of the most prevalent experimental methods used to increase sprouting and synaptic plasticity following SCI is through the application of chondroitinase ABC (ChABC). This bacterial enzyme acts to catabolise the glycosaminoglycan (GAG) chains from the inhibitory chondroitin sulphate proteoglycans (CSPGs) which are upregulated within the extracellular matrix (ECM) following SCI, facilitating growth and regeneration of damaged tissue.²¹ However, the application of ChABC has also been shown to promote plasticity in spared tissue after trauma, aiding the sprouting of pathways and formation of functional synapses within numerous models of SCI.²²⁻²⁶
Typically a single application of ChABC has limited effect upon functional recovery after contusion injury.\textsuperscript{27-30} Indeed, application of the enzyme most commonly shows significant effects upon functional activity after contusion injury when acting in combination with other treatments.\textsuperscript{29,31-33} However, these data were collected when assessing motor systems that demonstrate little endogenous plasticity after trauma. Contrary to this, the respiratory motor system is capable of plastic alteration and reorganisation following trauma.\textsuperscript{34,35} Indeed, it has been previously demonstrated modest functional restoration of respiratory function following ChABC application after acute C2 hemisection injury.\textsuperscript{36}

The application of a plasticity inducing enzyme has never been used to treat respiratory motor dysfunction following the more clinically relevant severe mid-cervical contusion injury. Through a single application of ChABC (over four injection sites) we show that alterations to the ECM and enhancement of plasticity can mediate recovery of respiratory function at chronic time points after injury. This primarily occurred through the sprouting (or activation) of spared serotonergic fibers and, in part, through recovery of respiratory pathways through the site of injury.
**Materials and Methods**

All animal procedures were performed in accordance with the Case Western Reserve University’s animal care committee's regulations. Adult male Sprague Dawley rats (350.2 ± 1.54 g; Harlan Laboratories Inc.) were housed in groups of three, exposed to a normal dark-light cycle with access to food, water, and environmental enrichment *ad libitum*. The health and welfare of the animals was monitored on a daily basis by the study investigators and veterinary staff at Case Western Reserve University. 53 animals were included in the study, divided into six groups: either control or ChABC injected animals 1) injected one week following injury with terminal recordings at week three, 2) injected one week following injury with terminal recordings at week six, and 3) injected four weeks following injury with terminal recordings at week six. Figure. 1C summarizes the distribution of animals between groups for analysis.

*Surgical procedures and treatment application*

Animals were anaesthetized with a mixture of ketamine (70mg.kg⁻¹) and xylazine cocktail (7mg.kg⁻¹; i.p.) for all surgical procedures. Carprofen (5 mg/kg; s.c.) and 0.002% bupivacaine hydrochloride were applied prior to the start of surgery. Body temperature during all surgical procedures was maintained at 37±1°C. Following the completion of recovery surgeries, yohimbine (1.2 mg/Kg; s.c.; Tocris) was administered to reverse the respiratory dampening effect of the xylazine. Muscle layers were sutured using (3-0 vicryl) and the skin closed with wound clips. Animals were given buprenorphine (30 μg/kg; maintained for 5 days) and saline subcutaneously and recovered in a heated environment before transfer to their home cage.

*Left lateral C3 contusion:* Using sterile techniques and surgical antisepsis, the cervical spinal column was exposed through a 3cm midline, dorsal incision between C1 and C4 and subsequent retraction of the skin and paravertebral muscles. While preserving the facet joints and dura, a laminectomy was performed over C2-C3. The vertebral column was clamped around the C3-C3 lamina using the Infinite Horizon contusion impactor (Precision Systems and Instrumentation). Animals received a single 150 kD left lateral contusion with zero dwell time and a 1.3 mm diameter impact tip. The completeness of the injury was confirmed through cresyl violet staining.
**Spinal injections:** Animals received an injection of either chondroitinase ABC (Seikagaku; ChABC; 20 UmL$^{-1}$) or a saline vehicle control with investigators blind to the treatment condition applied. Similar to the injury, using sterile techniques and surgical antisepsis, the cervical spinal column over C2-C5 was exposed through a 2cm midline, dorsal incision and retraction of the skin and paravertebral muscles. The spinal cord was cleared of scar tissue over C2-C3 and a laminectomy was performed over C4-C5 then the dura cut in line with each of the dorsal roots. A pulled pipette attached to a Nanoject II (Drummond Scientific Company) was sequentially placed at the position of the dorsal roots and stereotaxically lowered to the level of the phrenic motor pool (Figure 1B; 1.1 mm left of midline and 1.6 mm ventral from the spinal cord dorsal surface). After a 5-minute rest period, 250nL (C2/3) or 350nL (C4/5) of drug/vehicle was injected into the spinal cord. Following a 5-minute rest period, the pipette was removed.

**EMG recordings and right lateral C2 hemisection**

**Respiratory muscle EMG recordings:** At pre-determined end points, animals were anesthetised with urethane (1.6 mg/kg; i.p.). Using sterile techniques and surgical antisepsis, a number of incisions were made to enable implantation of EMG electrodes: 1) a 5-cm laparotomy exposed the abdominal surface of the diaphragm; 2) lateral 2 cm incisions were made over the left and right rib cage, with the latissimus dorsi blunt dissected to expose the external intercostals at T1 and T2; and 3) a 1 cm midline incision at the throat exposing the genioglossus following retraction of the digastric muscle. Bipolar electrodes (platinum; Grass Technology, Middleton, WI, USA) were implanted into: 1) the crural region (dorsal to the anterolateral branch of the inferior phrenic artery) of each hemidiaphragm; 2) the left and right external intercostals at T1; and 3) the left genioglossus muscle. The EMG signal was amplified (gain 5000x; Quad-P5II Amplifier; Grass Technology), band pass filtered (30-3,000 Hz; Grass Technology), digitized, and recorded using the CED 1401 (Spike2; Cambridge Electronic Design). The integrated signal was rectified and smoothed at a time constant of 0.08 secs. All EMG recordings were measured during eupneic breathing over a 60-second period, with the animals performing a number of spontaneous deep breaths (sighs) over this time frame. While absolute amplitude of the recordings has been reported for completeness, data were subsequently normalised to the average amplitude of the sigh for each animal.$^{37,38}$ Amplitude was assessed in this way to ensure results were not biased by slight alterations in electrode placement within the muscle. This is a standard method for assessment of such recordings as sigh activity maximally recruits the motor fibres required for eupnoic breathing (type I and IIa) and is a reliable measure for activity and sigh amplitude has been shown to
be unchanged following injury. Representative traces for all respiratory muscle EMG recordings can be seen in Figure 2A.

**Contralateral C2 hemisection:** During end-point EMG recordings, a C2 hemisection contralateral to the contusion was performed. This acted to remove any descending input to the ipsilateral PMP from the contralateral side of the cord and brain stem and isolate breathing activity solely to contused pathways. Using sterile techniques and surgical antisepsis, the cervical spinal column was exposed through a 3cm midline, dorsal incision between C1 and C3 and subsequent retraction of the skin and paravertebral muscles. The C2 spinal cord was re-exposed using micro scissors and a durotomy performed. With a 21G needle, a right hemisection was performed at the level of the C2 dorsal roots. This process was repeated five times and extended from midline to the most lateral point of the spinal cord. The hemisection was always performed while EMG recordings were being conducted, as such the functional completeness of the injury was confirmed through absence of activity on the right diaphragm EMG. Representative traces for all respiratory muscle EMG recordings during the C2 hemisection can be seen in Figure 2B. The time the animal continued to breath following completion of the C2 hemisection was calculated from the last peak of the right hemidiaphragm to the final peak of any other inspiratory muscle using the integrated response.

**Immunohistochemistry (IHC) and lesion volumetrics**

**Tissue collection:** Following terminal recordings animals were transcardially perfused with 0.1M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA; pH 7.4). The C1-C5 spinal segments were post-fixed overnight at 4°C, cryoprotected in 30% sucrose for 72 hrs at 4°C, and embedded in OCT (Optimal cutting temperature compound; Leica). Tissue was collected using the dorsal roots as landmarks and collected as a function of distance from the lesion site to ensure accuracy and consistency in analysis. Serial cryostat spinal cord cross-sections (20μm) were mounted on slides.

**Lesion volumetrics:** Spinal cord lesion volume was analysed through iron eriochrome cyanine and 1% cresyl violet staining (Sigma). Following imaging (Leica SCN400 Slide Scanner), the lesioned, white and grey matter areas in each section was determined automatically (Photoshop CC7, Adobe). The graft volume was assessed using the equation: \[ V = \sum \text{[transplant area x section thickness x 18 (the number of sections in each sampling interval)]}. \]
IHC: Five sections from each segmental level were assessed. The phrenic motor nucleus is located in the mediolateral ventral horn from C3-C6 and is easily recognised as a tight cluster of large motoneurons.\textsuperscript{43} It is at around the putative area of the PMP that all analysis was conducted. Sections were washed, blocked (10% normal goat serum, 0.1% BSA in tris-buffered saline), then incubated overnight in primary antibody at 4\textdegree C. The following day, sections were washed and incubated in the appropriate secondary antibody for two hours at room temperature. Following washing, sections were coverslipped using Fluorogold mounting medium (Invitrogen) and viewed using a fluorescence microscope (Lecia). 2B6 was purchased from Seikagaku, serotonin (5HT) from Immunostar, and neuronal nucli (NeuN) from Millipore. All secondary antibodies were purchased from Life Technologies.

Data Analysis

All experiments were assessed under blinded conditions. All animals in each group underwent treatment (drug or control) application, EMG recordings, contralateral hemisection, and histological analysis of lesion volumetrics. Power analysis was conducted prior to all experiments to ensure $n$ numbers were sufficient to yield reliable data. Data were subjected to the Shapiro-Wilk test for normalcy prior to analysis to ensure a normal distribution. No animal was excluded from data analysis based upon functional output or lesion size. The parameters were compared between control and the test groups through one-way or two-way analysis of variance (ANOVA) with post-hoc Bonferroni (SPSS or GraphPad Prism). Significance values represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data are presented as mean±SEM.
Results

Unilateral spinal cord contusion

Animals received on average a 192.6 ± 9.49 kDyne injury with 1619 ± 39.4 μm displacement. Five animals stopped breathing immediately following the contusion injury and were not able to be resuscitated. These data illustrate the severity of the injury produced through the mid-cervical contusion. During the initial days following surgery, no animal showed signs of blood in their urine or stool. All animals showed an ~15% drop in weight following the initial injury, which was recovered by 14 days post-surgery (Figure 3). Interestingly, no significant decrease in weight was noted following the injection of drug/control indicative of the minimally invasive nature of this surgery. Animals in different treatment groups did not show any significant differences in weights over time (F(1,252)=49.82, p<0.0001; Figure 3). This would indicate that alterations in weight are not a causal factor in the effects described.

ECM modification does not change lesion volumetrics

The level of contusion injury was assessed and verified through comparison with a standard spinal cord atlas (Paxinos). All of our animals had injuries located at C3 and thus none were excluded from further analysis. The injury site was extensive extending through layers I through X of the unilateral grey matter in all animals (Figure 4A). However, the grey commissure and central canal were largely left intact. Importantly, the tissue at C3-4 in layers VII through X was damaged in all animals following injury. These areas encompass the regions of the anterior horn which contain the phrenic motor pool and associated interneurons, thus demonstrate the impairment which has occurred to the pathways which control the respiratory motor system. Damage to the white matter was also broad, covering the posterior, lateral and anterior funiculus, although there was a degree of sparing in the lateral edge of the left dorsal lateral funiculus (Figure 4A). Indeed, at its epicentre, the lesion encompassed ~50% of the total tissue volume (~30% white matter and ~20% grey matter) while the total expanse of the trauma spanned 2-3 cervical levels and approximated 8mm in length (Figure 4B).

The cavity volumes were not significantly different between our control and ChABC treatment groups regardless of the time after injury at which the treatment was applied (Figure 4C). Total volume of tissue approximated 8mm\(^3\) in all groups (F(5,46)=2.17, p=0.076) with a slight trend in animals with longer injuries (groups 2 and 3) to have slightly larger total volumes (Figure 4Ci). This is perhaps reflected in slightly larger amounts of white matter sparing
in these animals (Figure 4Ciii) although it is not significant (F(5,46)=1.20, p=0.326). These data demonstrate that any endogenous processes occurring from acute to chronic time points is not sufficient to alter lesion size or gross anatomical recovery. The lesion volume was constant amongst all treatment and control groups at \( \sim 2.4 \text{ mm}^3 \) (F(5,46)=1.30, p=0.284) as was the degree of grey matter loss (F(5,46)=1.51, p=0.209). As such, at the time points the enzyme was injected, the drug does little to aid aggregated neuroprotection of total tissue. The cavity volumes generated were relatively consistent between and within treatment groups, showing little variance from the mean. This indicates that the injury produced was relatively constant throughout the study showing minimal disparity between animals.

Plasticity mediates recovery through the site of injury in the chronically injured animal

To assess the effects of endogenous or ChABC induced plasticity on recovery following mid-cervical contusion injury we evaluated the EMGs of the ipsilateral and contralateral hemidiaphragms (i- or cDia), external intercostals (i.- or c.eICs), and the ipsilateral genioglossus (geno)\(^\text{14}\) at the experiments terminal end point (Figure 1C). The hemidiaphragm ipsilateral to the injury demonstrated significant alterations in amplitude in response to treatment of cervical SCI at chronic time points (Table 1; Figure 5A+Bi; F(5,44)=8.05, \( p<0.0001 \)). Our acutely injured and untreated animals showed ipsilateral hemidiaphragm activity operating at \( \sim 80\% \) of maximal (based on spontaneous sighs) during eupnea. Typically, uninjured animals have diaphragm muscle function operating at \( \sim 40\% \) of maximal,\(^\text{37}\) demonstrating the substantial injury that our contusion formed. Interestingly, there is a trend for the EMG amplitude of the ipsilateral hemidiaphragm to spontaneously improve as the animal transitions from acute to chronic time points without treatment (Figure 5A+Bi). This is seen through reductions in the activity of the muscle from 82.6±5.4\% of maximal (group 1) to 66.7±6.4\% (group 3). However, treatment of acute injuries with ChABC resulted in similar improvements in functional output (to \( \sim 62\% \) of maximal; groups 1+2) occurring at earlier time points (Figure 5A+Bi). Further, ChABC treatment of the injury at chronic stages (group 3) showed significant reductions in EMG amplitude during eupnia to 42.4\% of maximal, demonstrating activity similar to that shown in uninjured animals (Figure 5A+Bi). Interestingly, decreases in activity of the contralateral hemidiaphragm have similar non-statistically significant trends as the statistically alterations of the ipsilateral hemidiaphragm (Figure 5A+Bii; F(5,45)=2.95, \( p=0.023 \)). Namely, slight improvements from acute to chronic time points in non-treated animals (from 67.2±5.2\% to 50.4±0.74\% of maximal). However, significant improvements are demonstrated in
animals where ChABC has been applied in the chronically injured animal (group 3; 45.7±4.5%). These data demonstrate that the contralateral hemidiaphragm was providing compensatory activity following the cervical trauma, and induction of plasticity through application of the bacterial enzyme facilitated in the recovery of the motor system so that such activity was no longer required.

Activity in the inspiratory accessory muscles including the ipsilateral eICs (F(5,44)=0.77, p=0.574), contralateral eICs (F(5,45)=0.70, p=0.624) and genioglossus (F(5,43)=1.39, p=0.252) showed no significant alteration in EMG amplitude either spontaneously over time following injury or as a result of treatment application (all groups ~40-50% of maximum; Figure 5A+Biii-v). These data demonstrate the success of having numerous decussating pathways at the level of the intercostal motor pool which facilitate in strong, patterned activity following cervical trauma. However, as the activity in the eIC muscles does not statistically alter following time or treatment, they demonstrate little compensatory activity for the deficit within the ipsilateral hemidiaphragm (Figure 5A+Biii-v). Similarly, the genioglossus data show how little respiratory muscles innervated above the level of trauma are affected by trauma to the spinal cord, with little evidence of compensatory activity.

Neither time after injury nor the application of treatment altered breath length for any of the muscles assessed (Table 1; iDia: F(5,44)=2.09, p=0.087; cDia: F(5,45)=1.26, p=0.301; i.eICs: F(5,44)=2.87, p=0.267; c.eICs: F(5,45)=1.65, p=0.170; geno: F(5,43)=1.58, p=0.191). Equivalent results were shown for cycle time following injury and treatment (Table 1; iDia: F(5,44)=1.93, p=0.112; cDia: F(5,45)=2.40, p=0.0536; i.eICs: F(5,44)=1.27, p=0.296; c.eICs: F(5,45)=1.83, p=0.129; geno: F(5,43)=1.90, p=0.118). However, breathing frequency did change following treatment in all respiratory associated muscles bar the genioglossus (Table 1; iDia: F(5,44)=2.52, p=0.045; cDia: F(5,45)=2.53, p=0.045; i.eICs: F(5,44)=2.54, p=0.044; c.eICs: F(5,45)=2.54, p=0.044; geno: F(5,43)=2.33, p=0.061). ChABC treated animals tended to show a reduction in breath frequency, particularly in animals where the animals were assessed weeks following treatment application (Table 1). As the respiratory motor system typically compensates for reductions in respiratory motor activity through increasing rates of respiration\textsuperscript{16}, these data suggest that increased plasticity at the PMP facilitates a modest recovery in respiratory function over time after treatment which reduces the need for compensatory activity.
Induced recovery is not sufficient to mediate respiratory function only through contused pathways

During the EMG recordings a C2 hemisection was performed on the opposite side of the cord to the contusion, effectively removing any descending control of respiratory function from the contralateral brainstem. The cutting of the contralateral bulbospinal tracts was shown through the total cessation of activity within the hemidiaphragm contralateral to the initial contusion (Figure 6Cii). The average length of time the animals continued to breath was different between treatment groups (Figure 6A+B; F(5,45)=2.84, p=0.028). Respiratory activity ceased in all animals which had not received treatment within seconds of hemisection completion (Figure 6A+B). The average length of time the animals continued to breath did not increase from acutely (6.3±3.0 sec, group 1) to chronically (8.1±2.2 seconds, group 3) injured animals (Figure 6A+B). Further, none of these animals could be resuscitated once breathing had stopped. These data demonstrate that little endogenous recovery had occurred to the respiratory motor pathways at site of injury over time following trauma. However, animals treated with ChABC typically breathed for longer periods of time following contralateral C2 hemisection. Animals given the drug at acute stages post injury (groups 1 and 2) sustained respiratory motor function for 58.8±37.0 secs and 34.7±17.6 secs respectively, indicating that removal of CSPG at acute time points post injury modestly facilitates a prolonged recovery of respiratory motor function (Figure 6A+B). However, animals treated with the enzyme at chronic time points post trauma (group 3) continued breathing for 97.9±33.5 secs following the injury (Figure 6A+B), demonstrating that plasticity had induced a more substantial recovery within these animals. Nonetheless, collectively these data show that ChABC alone was not sufficient to recover pathways through the injury to the extent that it will enable prolonged respiratory activity solely through the damaged ipsilateral bulbospinal pathways. This conclusion is reflected in the assessment of EMG amplitude following completion of the contralateral C2 hemisection.

As expected, the amplitude of activity within the hemidiaphragm ipsilateral to the contusion substantially increases following completion of the contralateral hemisection (Table 2; Figure 6A+Ci). However, there are no significant differences between either groups assessed at different time points post trauma or following the application of treatment (F(5,45)=1.94, p=0.109). This result is also consistent for the accessory respiratory muscles including the ipsilateral eICs (Figure 6Ciii; Table 2; F(5,44)=0.803, p=0.554), contralateral eICs (Figure 6Civ; Table 2; F(5,45)=1.95, p=0.108) and the ipsilateral genioglossus (Figure 6Cv; Table 2; F(5,45)=1.37, p=0.256). Collectively,
these data show that, although compromised by a contusion injury, the respiratory motor system is able to undergo immediate and substantial compensatory plasticity following the C2 hemisection to maximise immediate respiratory output. These endogenous effects are not related to time post-trauma or treatment induced alterations to the spinal cord.

Similar to that described above, neither time after injury nor the application of treatment altered breath length (Table 2; iDia: F(5,45)=1.35, p=0.264; i.eICs: F(5,45)=0.71, p=0.622; c.eICs: F(5,45)=2.83, p=0.028; geno: F(5,45)=1.92, p=0.113) and cycle time (Table 2; iDia: F(5,45)=0.992, p=0.435; i.eICs: F(5,45)=0.72, p=0.613; c.eICs: F(5,45)=2.96, p=0.023; geno: F(5,45)=0.57, p=0.719) for any of the muscles assessed except that the contralateral eICs. Interestingly, breathing frequency did change following treatment in all respiratory associated muscles bar the ipsilateral eICs (Table 2; iDia: F(5,45)=2.47, p=0.049; i.eICs: F(5,45)=0.61, p=0.695; c.eICs: F(5,45)=2.85, p=0.027; geno: F(5,45)=2.61, p=0.039). Data from both the non- and ChABC-treated groups show that the more chronic an animal following injury, the greater the capacity to compensate for respiratory challenge by increasing rates of respiration (Table 2). However, this is marginally increased at all time points with the addition of ChABC treatment, potentially facilitating plasticity within the respiratory motor system as a whole.

**ChABC mediated recovery correlates to an increase in 5HT sprouting**

The activity of ChABC was confirmed through 2B6 (stub antigen) staining at the level of the PMP (Figure 7A+Bi,ii). Indeed, chondroitin sulphate GAGs had been removed from the ECM at the PMP both ipsilateral (F(5,45)=42.0, p<0.0001) and contralateral (F(5,45)=14.7, p<0.0001) to the site of the initial contusion. Intensity of 5HT was also shown to increase in ChABC treated animals on both the ipsilateral (F(5,45)=19.4, p<0.0001) and contralateral (F(5,45)=29.8, p<0.0001) side of the cord at the level of the PMP (Figure 7A+Biii,iv). Indeed, the intensity of 5HT at the ipsilateral PMP was positively correlated with both the raw EMG amplitude (Table 3; R²=0.318, p<0.0001) and the amplitude expressed as a % of maximal output (table 3; R²=0.268, p=0.002) of the ipsilateral hemidiaphragm. Further, increases in 5HT were strongly correlated with 2B6 staining at both the ipsilateral (table 3; R²=0.464, p<0.0001) and contralateral (Table 3; R²=0.406, p<0.0001) PMP, demonstrating that the alterations in 5HT may be specifically induced by the treatment applied. Indeed, 2B6 staining at the ipsilateral PMP was also correlated with ipsilateral hemidiaphragm EMG amplitude expressed as a % of maximal output (Table 3; R²=0.286, p<0.0001).
Collectively, these data show that endogenous increases in 5HT, at least in part, facilitate recovery within the ipsilateral hemidiahragm, caused by the plasticity induced through ChABC treatment.
Discussion

The present study shows the long-term functional deficits caused by unilateral mid-cervical contusion injury. We demonstrate that, while endogenous plasticity and compensatory function are evident, these are not sufficient to facilitate a robust return of function. We hypothesised that enhancement of plasticity through an exogenously applied treatment strategy would facilitate recovery of respiratory motor function following contusion injury. Indeed, this study provides the first direct comparison of a plasticity inducing treatment strategy for respiratory motor dysfunction at both acute and chronic time points following cervical contusion injury. Here we show that ChABC treatment applied at chronic stages following cervical contusion injury can mediate recovery of ‘normal’ activity within the previously compromised ipsilateral hemidiaphragm, reducing the need for compensatory activity in the other muscles. Alternatively, acute application of the drug only marginally aids recovery of respiratory motor function. The mechanism of this recovery is, in part, mediated through serotonergic sprouting of neuronal pathways.

At chronic time points post contusion, this treatment induced recovery partially occurs through the contused respiratory fibres but is also governed through the activation of latent pathways, or sprouting, modulatory axons from the undamaged contralateral side of the spinal cord. These data show that enhancement of plasticity can facilitate recovery of the respiratory motor system following severe mid-cervical contusion if applied at chronic time points following trauma.

Mid-cervical contusion injury

The unilateral mid-cervical contusion injury induced substantial damage to the spinal cord encompassing 100% of unilateral white and grey matter. The loss of grey matter within the ipsilateral ventral horn and white matter through the posterior, lateral and anterior funiculus, and thus the loss of phrenic motor neurons, propriospinal interneurons and bulbospinal pathways explains the reduction in EMG activity within the ipsilateral hemidiaphragm. Phrenic motoneuron loss is positively associated with the degree of injury to grey matter following mid-cervical contusion. As such, within our model, these motoneurons critical to diaphragm function would be largely absent at the lesion centre. Further, our contusion injury shows minimal compromise to contralateral spinal tissue, which can facilitate compensatory activity aiding respiratory motor function. The injury caused by this 150 kDyne contusion appears similar to previous reports and marginally larger than those of a 395-400 kDyne trauma, typical of severe SCI trauma. There is substantial variance in the field over reporting of injury size and the extent of injury...
produced from a given force/displacement. Nonetheless, due to the stable configuration of our animals and preparation of the impact site before injury we produce a severe and clinically relevant cervical SCI with little anatomical and functional variance between animals. Further difference between published studies may additionally be explained through strain or sex differences between animals. Nonetheless, it is the production of a reliable and extensive mid-cervical contusion which causes the profound reduction in respiratory function typical of our injury model.

The lesion size of our injury showed a similar magnitude of spinal cord damage regardless of both time after injury or treatment with ChABC. This may explain why we show here no significant return of function within the ipsilateral hemidiaphragm following application of the control treatment, and previously have demonstrated robust reductions in both ventilatory parameters (VE and VT) and ventilatory variance which were persistent from acute to chronic time points. Our data suggests that ChABC treatment at sub-acute and chronic time points does not reduce lesion size or facilitate in tissue sparing but effects plasticity to evoke functional recovery. Previous reports typically describe specific fibre type loss at the site of injury or behavioural deficit rather than the specific lesion volume to show consistency within their model or effect of ChABC treatment. However, hemisection studies have similarly shown no effect of ChABC on lesion size. ChABC has been shown in previous 150 kDyne thoracic contusion models to promote substantial neuroprotection through modulation of the immune response. However, this was induced through constant application of the enzyme, initiated three days post injury. It is, perhaps, this continuous infusion of ChABC which may be key to neuroprotective effects, as well as the acute production of disaccharide units resulting from enzyme activity as similar studies utilising a single injection of the enzyme do not demonstrate a similar outcome. We administer four injections of the enzyme once, a minimum of seven days following the contusion, when the immune response and secondary effects causing widespread neurodegeneration have already begun to occur, thus the effect of ChABC to promote neuroprotection is minimal within our model. We also target the denervated PMP more than neurons at the site of injury which may explain why we do not see these effects. Indeed, the reduced effect of our treatment to induce neuroprotection may explain why we show modest recovery within the respiratory motor pathways through the site of injury, and subsequently why activity within the ipsilateral hemidiaphragm is additionally mediated though latent pathways or sprouting from the contralateral spared tissue.
Compromised diaphragm activity is persistent overtime

Here we show that the severe mid-cervical contusion causes a significant deficit to ipsilateral hemidiaphragm activity. This lasts from acute to chronic time points post injury during eupnea in control treated animals. This has been previously demonstrated within the respiratory motor system through EMG or phrenic nerve recordings.\textsuperscript{6-8,12,14} However, often in these cases the effect of the initial impact are transient, being partially overcome two to eight weeks following the initial trauma.\textsuperscript{7,8,12,17,47} This is likely related to the lesion size and the degree of tissue sparing within the ventral horn and anterior funiculi or the amount of supraspinal reorganisation which occurs following trauma.\textsuperscript{7,59} Indeed, the absence of significant functional recovery within the ipsilateral hemidiaphragm over time\textsuperscript{14} demonstrates that this severe injury yields a lack of endogenous restoration and minimal plasticity in our model. Performing our recordings under anaesthesia could minimise the EMG output achieved and, subsequently, underestimate the scale of recovery achieved.\textsuperscript{10,60} However, any respiratory depressant effect was minimal as our recordings were performed under light ketamine-xylazine anaesthesia\textsuperscript{61} and significant ipsilateral hemidiaphragm recovery has been previously reported in contused animals assessed under similar conditions of anaesthesia.\textsuperscript{12,17}

There is compensatory plasticity demonstrated within our control treated animals shown through the increased activity of the contralateral hemidiaphragm and modulation of respiratory frequency. The drive to increase respiratory frequency likely occurs in order to maintain constant ventilatory performance despite respiratory compromise.\textsuperscript{17,45} Indeed, increases in respiratory rate tend to correlate with the size of the mid-cervical contusion injury.\textsuperscript{9} The increased amplitude of activity in the contralateral hemidiaphragm compared to activity in the uninjured animal\textsuperscript{57} may be mediated through an increase in discharge rate or recruitment of spared phrenic motor units\textsuperscript{38,62} and confirms data reported previously.\textsuperscript{12,18} Similarly, we show increased activity within the ipsilateral eIC muscles to regain function following loss of tissue at the PMP\textsuperscript{12,14,15} likely mediated through propriospinal interneurons.\textsuperscript{44} However, the relative amplitude of activity in these muscles does not change with time, or following treatment, demonstrating that they are not operating to compensate for decreased activity in the diaphragm (as eIC activity would decrease in the chronic animal as diaphragm activity increased, which does not happen). It is likely that the increased activity in the eICs aids to maintain overall respiratory output not compensate for the deficit of one muscle. Confirming previous data, we do not report compensatory activity of the upper airway in response to
severe mid-cervical SCI. However, its cessation of function following completion of the contralateral hemisection in control treated animals shows that our contusion model has induced substantial reorganisation of the supraspinal or sensory feedback networks and ascending pathways leading to chronic changes in respiratory circuitry.\textsuperscript{10,59,63}

Further, compensatory activity within both control and ChABC treated animals allows for an increase in ipsilateral diaphragm, eIC, and genioglossus EMG activity when the respiratory motor system is compromised (e.g. a contralateral C2 hemisection), although this only occurs for a maximum of eight seconds in our control treated animals. This may be mediated through contralateral, decussating pathways.\textsuperscript{64} However, as the muscles under these conditions are typically working at amplitudes far in access of that achieved during eupnoic maximum drive (e.g. spontaneous sigh) it is likely that the pathways activated are not those typically associated with ‘normal’ respiratory motor function.\textsuperscript{39} These data demonstrate that there is little repair through the site of injury in control treated animals. However, animals treated with ChABC are able to breath for significantly longer periods of time, revealing a degree of recovery within these injured pathways. This shows that the recovery we induce within our model occurs through both contralateral spared pathways and regeneration/recovery of fibres through the site of injury.

\textit{Plasticity induced recovery of respiratory motor function after chronic injury}

The functional consequences of ChABC application to respiratory motor function following acute or chronic spinal cord contusion have not previously been assessed. Here we uniquely demonstrate that induction of plasticity through application of the enzyme at chronic time points following unilateral mid-cervical contusion can lead to recovery of normal activity in a previously compromised hemidiaphragm. This was shown through the ‘normal’ activity of the ipsilateral hemidiaphragm and a reduction in compensatory activity of both the contralateral hemidiaphragm and respiration rates, illustrating a reduced need to counteract deficits in respiratory motor activity.

These results are perhaps surprising as ChABC has not typically been shown to mediate significant recovery following severe contusion injury, even when applied over a number of days.\textsuperscript{27-30} However, our treatment paradigm has negated this effect. Unlike previous reports, within this study we are applying the enzyme at the point of desired functional recovery (the ipsilateral PMP) as well as the site of injury to best produce the desired output. It
has been demonstrated within the locomotor system that the plasticity induced by ChABC treatment can facilitate recovery of function only when combined with task-specific rehabilitation. As our animals are constantly breathing (acting as a mild form of task-specific rehabilitation for the ipsilateral hemidiaphragm) the plasticity induced by our enzyme treatment at the point of innervation of the phrenic motor pool may be better able to facilitate functional recovery of the partially paralysed muscle.

Nonetheless, the question remains as to why we get a superior effect of the enzyme when applied at chronic time points post injury rather than acute? Previous studies have determined that ChABC treatment is more effective at restoring locomotor function when applied acutely after thoracic spinal contusion rather than at chronic stages post trauma. Our results demonstrate positive effects of using ChABC on acute injuries through ipsilateral diaphragm EMG activity, but these are relatively modest. However, the secondary processes following contusion injury are still occurring and progressing at acute points post trauma. As such, a single acute application of ChABC to remove inhibitory CSPGs and induce plasticity is likely insufficient to overcome all the aspects of secondary injury which are in the process of occurring to potentially limit functional respiratory motor recovery at these stages. Further, respiratory function following severe mid-cervical contusion is seemingly reliant on the activation or sprouting of spared/latent pathways. But these processes happen only in later stages of spinal cord injury. There is evidence of some endogenous plasticity from two-eight weeks post contusion which may aid respiratory motor function if correctly utilised and augmented. Subsequently, the recovery of respiratory function through induction of plasticity, may be both more successful and prolonged if initiated at chronic time points post trauma. Indeed, the capacity of ChABC application to facilitate in the growth of sprouting axons and formation of functional synapses on inter- and motoneurons following contusion injury has been widely reported. Within the respiratory motor system, we show that this may be more functionally relevant to recovery when applied chronically rather than acutely. This finding potentially has great significant clinical relevance running counter to the established belief that the earlier treatment is applied the more likely it is to induce meaningful recovery. Indeed, we have recently shown that substantial recovery of respiratory motor function is possible up to a year and a half following cervical spinal hemisection and that ChABC induced effects last up to six months following the end of treatment. This recovery was also induced partly through serotonergic mechanisms.
As such, we are currently assessing whether this effect is unique to the respiratory motor system and the exact mechanisms behind functional recovery at chronic time points.

**Anatomical and physiological mechanisms of recovery**

The substantial recovery the induction of plasticity caused within our animals at chronic time points following mid-cervical contusion was partially mediated through spared desiccating pathways from the undamaged contralateral side of the cord and the potential interaction with propriospinal interneurons. We demonstrate that this is, at least in part, through serotonergic projections. The sprouting of serotonergic fibres have previously been shown to be important to recovery of respiratory function following mid-cervical contusion injury. Our observations that ChABC brought about the increased serotonergic fibre innervation at the PMP correlate with other models of contusive SCI showing that application of the enzyme increases serotonergic sprouting and functional activity. Of course, it is likely that other mechanisms of recovery are additionally functioning. For example, the recruitment of V2a interneurons has been shown critical in rat and mouse models of cervical SCI to the promotion of respiratory function. Nonetheless, these findings demonstrate that CSPG digestion and the induction of plasticity within the PMP at both sides of the spinal cord facilitates alterations in serotonergic fibre growth (through either, or both, sprouting or sparing of pathways) which facilitate recovery of function within the damaged respiratory motor system.

Through application of ChABC, respiratory motor recovery was partially mediated by pathways through the site of injury. As far as we are aware, this is the first time that treatment induced recovery following mid-cervical contusion has come about through pathways previously so damaged they could not sustain respiratory function alone. The observed recovery in both pathways is likely instigated by a reduction in inhibitory molecules at the PMP allowing for anatomical sprouting, plasticity and regeneration of fibres (reviewed in), alleviation of potential blocks to neuronal conduction, and increased myelination/remyelination of newly growth or spared fibres. These data demonstrate that plasticity induced restoration of respiratory motor activity following severe mid-cervical contusion can occur through a spectrum of pathways and means acting in concert to facilitate functional recovery.

**Physiological and clinical significance**
Our model of unilateral mid-cervical contusion injury accurately reflects the clinical population showing a rapid deficit in respiratory motor function due to reduced activity in the ipsilateral hemidiaphragm.\textsuperscript{70} Some compensatory activity is demonstrated within the accessory muscles to maximise respiratory performance.\textsuperscript{1,71,72} However, the deficit in diaphragm function is pronounced and lasts from acute to chronic stages post injury. As such, this is an ideal model in which to assess the potential treatment methods for respiratory motor recovery following cervical contusion. The use of ChABC to augment endogenous plasticity, facilitating recovery of respiratory motor function in theory has many benefits. The enzyme has been shown to induce functional effects in multiple models of SCI and in numerous motor systems\textsuperscript{36,54,65,73-75} without increasing pain or sensitivity.\textsuperscript{33,76} However, application of the drug requires invasive surgery. Our data show that restoration of normal respiratory motor functioning can be achieved at chronic time points post trauma with a single application of the drug (over multiple injection sites). However, the success of this treatment strategy is partially reliant upon the sprouting of this spared tissue. As such, with more complete bilateral, severe contusion injuries this approach is unlikely to be successful. Induction of plasticity could facilitate further regeneration and restoration of function within pathways through the site of injury. However, the enzyme (or pharmaceutical equivalent) may need to be applied for longer,\textsuperscript{42,51,77,78} stabilised,\textsuperscript{79} applied through a different route,\textsuperscript{80} or in combination with a cavity spanning bridge\textsuperscript{29,36,81,82} to achieve the desired outcome.

Through the enhancement of plasticity, we demonstrate the rapid recovery of the respiratory motor system at chronic time points following severe mid-cervical contusion injury. This restoration of function reduced the need for compensatory plasticity within the system and was primarily mediated through the activation or spared or sprouting contralateral serotonergic fibres. Recovery of pathways through the site of injury was achieved in part. These findings have significant implications for the treatment of severe trauma to the spinal cord and other pathological disease states.
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Figure 2

A) Post contusion

B) Post hemisection

Ipsilateral Genioglossus

Contralateral etC

Ipsilateral etC

Contralateral Hemidiaphragm

Ipsilateral Hemidiaphragm

100μV

1s
Figure 3

Weight after contusion injury

Control
-ChABC

Days after injury

Weight (g)
Figure 4

A) Mid-cervical contusion injury

B) Lesion area

i) Lesion area

ii) White matter area

iii) Grey matter area

C) Lesion volumetrics

i) Total volume

ii) Lesion volume

iii) White matter volume

iv) Grey matter volume
Figure 5

A) Hemidiaphragm EMG activity

Control

ChABC

i) Ipsilateral hemidiaphragm

ii) Contralateral hemidiaphragm

iii) Ipsilateral eICs

iv) Contralateral eICs

v) Ipsilateral genioglossus

B) Respiratory EMG amplitude

i) Ipsilateral hemidiaphragm

% Amplitude of Maximal Output

Weeks after injury


** Control  ChABC

ii) Contralateral hemidiaphragm

% Amplitude of Maximal Output

Weeks after injury


* Control  ChABC

***  **  *
**Figure 7**

**A) Immunohistochemistry**

- **Control**
  - i) NeuN 2B6
  - iii) NeuN 5HT

- **ChABC**
  - ii) NeuN 2B6
  - iv) NeuN 5HT

**B) Intensity recordings**

- **i) Ipsilateral 2B6 intensity**
- **ii) Contralateral 2B6 intensity**
- **iii) Ipsilateral 5HT intensity**
- **iv) Contralateral 5HT intensity**

Graphs show the intensity of 2B6 and 5HT in different conditions: Control and ChABC, with groups Gp1, Gp2, and Gp3.