Plasticity Induced Recovery of Breathing Occurs at Chronic Stages after Cervical Contusion

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Abstract

Severe midcervical contusion injury causes profound deficits throughout the respiratory motor system that last from acute to chronic time points post-injury. We use chondroitinase ABC (ChABC) to digest chondroitin sulphate proteoglycans 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. Through electromyography (EMG) recordings of multiple inspiratory muscles, however, we show that application of the enzyme at chronic time points post-injury initiates the recovery of normal breathing in previously paralyzed 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 fibers 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 of the respiratory motor system and reveal mechanistic evidence of the pathways that govern this effect.

Keywords: cervical contusion injury; chondroitinase ABC; plasticity; respiratory motor system; respiratory recovery

Introduction

PATIENTS WITH SEVERE midcervical spinal cord injuries (SCIs) typically have deficits in respiratory motor function that last from acute to chronic stages post-trauma.¹ This is particularly significant because this is the most prevalent form of SCI,² with deficits in respiratory function being the primary cause of morbidity and death in human patients.^{1,3,4} Modeling these injuries experimentally requires impairment to the respiratory motoneurons and bulbospinal pathways that mediate activity in the diaphragm (primarily responsible for inspiration in mammals)⁵ through innervation of the phrenic motor pool (PMP). Depending on the size and extent of trauma, however, 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 midcervical 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 midcervical contusion injuries show recovery in respiratory parameters four-to-eight weeks after the initial trauma because of the endogenous plasticity of spared tissue and respiratory motor pathways.^{15–19} We hypothesized that through exogenous enhancement of plasticity, we could facilitate recovery of respiratory motor activity at both acute and chronic time points after severe midcervical contusion injury. Of course, functional recovery at chronic time points after injury is typically considered more difficult to accomplish because the injuries tend to be more extensive. This is because of 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 after SCI is through the application of chondroitinase ABC (ChABC). This bacterial enzyme acts to catabolize the glycosaminoglycan (GAG) chains from the inhibitory chondroitin sulphate proteoglycans (CSPGs) that are

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upregulated within the extracellular matrix (ECM) after SCI, facilitating growth and regeneration of damaged tissue.²¹ The application of ChABC, however, also has 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 on functional recovery after contusion injury.^{27–30} Indeed, application of the enzyme most commonly shows significant effects on functional activity after contusion injury when acting in combination with other treatments.^{29,31–33} These data were collected, however, when assessing motor systems that demonstrate little endogenous plasticity after trauma. Contrary to this, the respiratory motor system is capable of plastic alteration and reorganization after trauma.^{34,35} Indeed, modest functional restoration of respiratory function post-ChABC application after acute C2 hemisection injury has been demonstrated previously.³⁶

The application of a plasticity inducing enzyme has never been used to manage respiratory motor dysfunction after the more clinically relevant severe midcervical 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 occurred primarily through the sprouting (or activation) of spared serotonergic fibers and, in part, through recovery of respiratory pathways through the site of injury.

Methods

All animal procedures were performed in accordance with the Animal Care Committee regulations of Case Western Reserve University. Adult male (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 were monitored on a daily basis by the study investigators and veterinary staff at Case Western Reserve University. There were 53 animals included in the

study, divided into six groups: either control or ChABC injected animals (1) injected one week after injury with terminal recordings at week three, (2) injected one week after injury with terminal recordings at week six, and (3) injected four weeks after 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 anesthetized with a mixture of ketamine ($70 \text{ mg} \cdot \text{kg}^{-1}$) and xylazine cocktail ($7 \text{ mg} \cdot \text{kg}^{-1}$; intraperitoneally [IP]) for all surgical procedures. Carprofen (5 mg/kg ; subcutaneously [SC]) and 0.002% bupivacaine hydrochloride were applied before the start of the surgical procedure. Body temperature during all surgical procedures was maintained at $37 \pm 1^\circ\text{C}$. After the completion of recovery operations, yohimbine (1.2 mg/kg ; SC; Tocris) was administered to reverse the respiratory dampening effect of the xylazine. Muscle layers were sutured using (3-0 polyglactin) and the skin closed with wound clips. Animals were given buprenorphine ($30 \mu\text{g/kg}$; maintained for 5 d) and saline SC 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 3 cm 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–C3.¹³ 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.¹³ The completeness of the injury was confirmed through cresyl violet staining.

Spinal injections. Animals received an injection of either ChABC ($20 \text{ U} \cdot \text{mL}^{-1}$; Seikagaku) 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 2 cm midline,

FIG. 1. Schematic of the respiratory motor system after C3 mid-cervical contusion injury and the experimental paradigms used in this study. (A) Schematic of the intact respiratory motor system highlighting the pathways innervating the diaphragm, external intercostals, and genioglossus. (B) The impact of C3 contusion on the respiratory motor system, minimizing ipsilateral diaphragm and external intercostal function and causing the upregulation of chondroitin sulphate proteoglycans (CSPGs) in the surrounding area. Diagram demonstrates the position of control or chondroitinase ABC (ChABC) injection sites. (C) Schematic representation of the groups used in this study for both ChABC or control (saline) injected animals. NaCl, sodium chloride.

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 placed sequentially at the position of the dorsal roots and stereotactically lowered to the level of the phrenic motor pool (Fig. 1B; 1.1 mm left of midline and 1.6 mm ventral from the spinal cord dorsal surface). After a 5-min rest period, 250 nL (C2/3) or 350 nL (C4/5) of drug/vehicle was injected into the spinal cord. After a 5-min rest period, the pipette was removed.

Electromyography (EMG) recordings and right lateral C2 hemisection

Respiratory muscle EMG recordings. At pre-determined end-points, animals were anesthetized with urethane (1.6 mg/kg; IP). 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 after retraction of the digastric muscle. Bipolar electrodes (platinum; Grass Technology, Middleton, WI) 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 5000 $\times$; 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 sec. All EMG recordings were measured during eupneic breathing over a 60-sec 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 normalized subsequently 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 because sigh activity maximally recruits the motor fibers required for eupneic breathing (type I and IIa) and is a reliable measure for activity; sigh amplitude has been shown to be unchanged after injury.^{19,39,40} 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.^{13,14} 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 3 cm midline, dorsal incision between C1 and C3, and subsequent retraction of the skin and paravertebral muscles. The C2 spinal cord was re-exposed using microscissors 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 after 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. After terminal recordings, animals were transcardially perfused with 0.1M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA; pH 7.4).⁴¹ The C1-C5 spinal segments were post-fixed overnight at 4°C, cryoprotected in 30% sucrose for 72 h at 4°C, and embedded in optimal cutting temperature (OCT) 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 (20 μ m) were mounted on slides.

Lesion volumetrics. Spinal cord lesion volume was analyzed through iron eriochrome cyanine and 1% cresyl violet staining (Sigma). After imaging (Leica SCN400 Slide Scanner), the lesioned, white and gray matter areas in each section were determined automatically (Photoshop CC7, Adobe). The graft volume was assessed using the equation: $V = \Sigma$ (transplant area $\times$ section thickness $\times$ 18 [the number of sections in each sampling interval]).⁴²

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 recognized as a tight cluster of large motoneurons.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% bovine serum albumin in tris-buffered saline), then incubated overnight in primary antibody at 4°C. The next day, sections were washed and incubated in the appropriate secondary antibody for 2 h at room temperature. After washing, sections were coverslipped using Fluorogold mounting medium (Invitrogen) and viewed using a fluorescence microscope (Leica). The 2B6 was purchased from Seikagaku, serotonin (5HT) from Immunostar, and neuronal nuclei (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 before all experiments to ensure n numbers were sufficient to yield reliable data. Data were subjected to the Shapiro-Wilk test for normalcy before analysis to ensure a normal distribution. No animal was excluded from data analysis based on functional output or lesion size. The parameters were compared between control and the test groups through one-way or two-way analysis of variance with *post-hoc* Bonferroni test (SPSS or GraphPad Prism). Significance values represented as $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$. Data are presented as mean $\pm$ standard error of the mean (SEM).

Results

Unilateral spinal cord contusion

Animals received on average a 192.6 ± 9.49 kDyne injury with 1619 ± 39.4 μ m displacement. Five animals stopped breathing immediately after the contusion injury and were not able to be resuscitated. These data illustrate the severity of the injury produced through the midcervical contusion. During the initial days after the surgical procedure, no animal showed signs of blood in the urine or stool. All animals showed an $\sim 15\%$ drop in weight after the initial injury, which was recovered by 14 days post-operation (Fig. 3).

FIG. 2. Illustration of respiratory muscle electromyography (EMG) recordings after midcervical contusion injury and contralateral C2 hemisection. The EMG data are shown for the contralateral and ipsilateral hemidiaphragms, external intercostals (eICs), and ipsilateral genioglossus (relative to the site of the contusion injury) from a control animal four weeks after the initial trauma. **(A)** Some recovery in ipsilateral hemidiaphragm activity after contusion is evident and activity of the eIC not diminished by the spinal cord injury. **(B)** This activity, however, in the ipsilateral motor system after subchronic midcervical contusion is not sufficient to maintain respiratory motor function when the ipsilateral pathways are isolated through completion of a contralateral C2 hemisection (dashed line). Without the support of the contralateral respiratory motor system and pathways, activity in all respiratory muscles immediately ceases. All panels are recorded in the same animal and all measures relative to the contusion injury.

Interestingly, no significant decrease in weight was noted after the injection of drug/control indicative of the minimally invasive nature of this procedure. Animals in different treatment groups did not show any significant differences in weights over time ($F[1,252] = 49.82$, $p < 0.0001$; Fig. 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 no animal was excluded from further analysis. The injury site was extensive extending through layers I through X of the unilateral gray matter in all animals (Fig. 4A). The gray commissure and central canal, however, were largely left intact.

Importantly, the tissue at C3-4 in layers VII through X was damaged in all animals after injury. These areas encompass the regions of the anterior horn that contain the phrenic motor pool and associated interneurons,⁴⁴ thus demonstrating the impairment that has occurred to the pathways controlling 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 (Fig. 4A). Indeed, at its epicenter, the lesion encompassed $\sim 50\%$ of the total tissue volume ($\sim 30\%$ white matter and $\sim 20\%$ gray matter) while the total expanse of the trauma spanned two to three cervical levels and approximated 8 mm in length (Fig. 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 (Fig. 4C). Total volume of tissue approximated 8 mm^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 (Fig. 4Ci). This is perhaps reflected in slightly larger amounts of white matter sparing in these animals (Fig. 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 among 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 gray 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

Weight after contusion injury

FIG. 3. Contused animals show $\sim 15\%$ decrease in weight, which is recovered by 14 days post-injury with no effect of treatment on animal recovery. Data show the weight of animals at weekly time points (and three days post any surgery) for control and chondroitinase ABC (ChABC) treated animals. Graphs show means $\pm$ standard error of the mean for control groups: group one = dark blue, group two = blue, group three = light blue; ChABC groups: group one = red, group two = orange, group three = yellow.

A Mid-cervical contusion injury **B** Lesion area **C** Lesion volumetrics

FIG. 4. Chondroitinase ABC (ChABC) treatment at acute and chronic time points does not alter the size of midcervical contusion injury. **(A)** Example sections of the spinal cord stained with iron eriochrome cyanine and cresyl violet to reveal the extent of the cervical contusion lesion. All panels are from the same animal in control group one. **(B)** Average area of (i) the lesion, (ii) white matter, and (iii) gray matter across the spinal cord from the point of initial trauma for control and ChABC injected groups. **(C)** Average volume of (i) total volume, (ii) the lesion, (iii) white matter, (iv) gray matter across the spinal cord for control and ChABC injected groups. Panels show means $\pm$ standard error of the mean for control groups: group one = dark blue, group two = blue, group three = light blue; ChABC groups: group one = red, group two = orange, group three = yellow.

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 after midcervical contusion injury, we evaluated the EMGs of the ipsilateral and contralateral hemidiaphragms (i- or cDia), external intercostals (i- or c.iCs), and the ipsilateral genioglossus (geno)¹⁴ at the terminal end-point of the experiments (Fig. 1C). The hemidiaphragm ipsilateral to the injury demonstrated significant alterations in amplitude in response to management of cervical SCI at chronic time points (Table 1; Fig. 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,³⁷ demonstrating the substantial injury that our contusion formed. Interestingly, there is a trend for the EMG amplitude of the ipsilateral hemidiaphragm to improve spontaneously as the animal transitions from acute to chronic time points without treatment (Fig. 5A+Bi). This is seen through reductions in the activity of the muscle from $82.6 \pm 5.4\%$ of maximal (group 1) to $66.7 \pm 6.4\%$ (group 3).

Treatment of acute injuries with ChABC, however, resulted in similar improvements in functional output (to $\sim 62\%$ of maximal; groups 1,2) occurring at earlier time points (Fig. 5A,Bi). Further, ChABC treatment of the injury at chronic stages (group 3) showed significant reductions in EMG amplitude during eupnea to 42.4% of maximal, demonstrating activity similar to that shown in uninjured animals (Fig. 5A,Bi). Interestingly, decreases in activity of the contralateral hemidiaphragm have similar non-statistically significant trends as the statistically alterations of the ipsilateral hemidiaphragm (Fig. 5A,Bii; $F[5,45]=2.95$, $p=0.023$). Namely, slight improvements from acute to chronic time points in non-treated animals (from $67.2 \pm 5.2\%$ to $50.4 \pm 0.74\%$ of maximal).

Significant improvements, however, are demonstrated in animals where ChABC has been applied in the chronically injured animal (group 3; $45.7 \pm 4.5\%$). These data demonstrate that the contralateral hemidiaphragm was providing compensatory activity after 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 iCs ($F[5,44]=0.77$, $p=0.574$), contralateral iCs ($F[5,45]=0.70$, $p=0.624$) and genioglossus ($F[5,43]=1.39$, $p=0.252$) showed no significant alteration in EMG amplitude either spontaneously over time after injury or as a result of treatment application (all groups $\sim 40\text{--}50\%$ of maximum; Fig. 5A,Biii–v). These data demonstrate the success of having numerous decussating pathways

TABLE 1. CHABC TREATMENT AT CHRONIC TIME POINTS AFTER CONTUSION INJURY CAUSES INCREASES IN RESPIRATORY AMPLITUDE

Muscle	Control			ChABC		
	Gp1	Gp2	Gp3	Gp1	Gp2	Gp3
Amplitude (mV)						
Lf-dia	1.16 ± 0.53***	1.73 ± 0.40***	3.81 ± 0.60**	3.28 ± 1.17**	1.93 ± 1.30***	10.3 ± 2.24
Rt-dia	0.79 ± 0.23	1.37 ± 1.00	1.93 ± 0.27	2.28 ± 0.72	1.38 ± 0.50	2.95 ± 0.92
Lf-eIC	0.34 ± 0.17*	0.19 ± 0.08*	2.86 ± 0.65	0.81 ± 0.63	0.24 ± 0.21*	3.71 ± 1.32
Rt-eIC	0.56 ± 0.28	0.19 ± 0.05	2.16 ± 0.50	1.08 ± 0.38	0.37 ± 0.17	2.00 ± 0.88
Lf-geno	0.41 ± 0.21	0.40 ± 0.18	0.26 ± 0.06	0.32 ± 0.09	1.0 ± 0.38	0.53 ± 0.20
Breath length (sec)						
Lf-dia	0.22 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	0.28 ± 0.03	0.22 ± 0.01	0.24 ± 0.02
Rt-dia	0.29 ± 0.01	0.29 ± 0.0	0.29 ± 0.00	0.30 ± 0.01	0.29 ± 0.01	0.27 ± 0.00
Lf-eIC	0.20 ± 0.01	0.19 ± 0.02	0.23 ± 0.02	0.25 ± 0.02	0.26 ± 0.0	0.21 ± 0.01
Rt-eIC	0.23 ± 0.01	0.26 ± 0.02	0.24 ± 0.02	0.26 ± 0.01	0.26 ± 0.0	0.21 ± 0.01
Lf-geno	0.26 ± 0.02	0.25 ± 0.02	0.25 ± 0.01	0.30 ± 0.03	0.28 ± 0.01	0.23 ± 0.01
Cycle length (sec)						
Lf-dia	0.40 ± 0.03	0.35 ± 0.01	0.37 ± 0.02	0.41 ± 0.07	0.53 ± 0.06	0.38 ± 0.05
Rt-dia	0.34 ± 0.03	0.28 ± 0.02	0.29 ± 0.03	0.38 ± 0.06	0.46 ± 0.06	0.35 ± 0.03
Lf-eIC	0.42 ± 0.03	0.38 ± 0.02	0.35 ± 0.03	0.43 ± 0.07	0.49 ± 0.06	0.41 ± 0.04
Rt-eIC	0.39 ± 0.03	0.30 ± 0.04	0.35 ± 0.03	0.42 ± 0.06	0.49 ± 0.05	0.41 ± 0.04
Lf-geno	0.36 ± 0.03	0.32 ± 0.02	0.34 ± 0.02	0.39 ± 0.06	0.47 ± 0.06	0.39 ± 0.03
Breath frequency (b/min)						
Lf-dia	98.5 ± 5.3	105.7 ± 3.6	103.8 ± 3.6	91.8 ± 7.4	82.9 ± 5.3	99.5 ± 5.5
Rt-dia	99.8 ± 5.3	105.0 ± 3.6	103.0 ± 3.6	92.0 ± 6.5	82.2 ± 5.3	98.7 ± 5.5
Lf-eIC	98.5 ± 5.3	105.8 ± 3.6	103.9 ± 3.6	91.9 ± 7.4	82.9 ± 5.3	99.7 ± 5.5
Rt-eIC	98.7 ± 5.3	105.8 ± 3.6	103.8 ± 3.6	92.8 ± 6.6	82.8 ± 5.3	99.7 ± 5.5
Lf-geno	98.7 ± 6.4	105.6 ± 3.6	103.8 ± 3.6	92.8 ± 7.7	82.9 ± 5.3	99.6 ± 5.5

Data show amplitude (mV), breath length (sec), cycle length (sec), and breath frequency (breath per minute) for both control and ChABC treated animals at acute and chronic time points following C3 contusion injury. Tables show means ± SEM. Comparisons between groups and ChABC Gp3 shown as: * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

at the level of the intercostal motor pool that facilitate in strong, patterned activity after cervical trauma. Because the activity in the eIC muscles does not statistically alter after time or treatment, however, they demonstrate little compensatory activity for the deficit within the ipsilateral hemidiaphragm (Fig. 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 after 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: $F[5,43] = 1.90$, $p = 0.118$). Breathing frequency did change, however, after 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 after treatment application (Table 1). Because the respiratory motor system typically compensates for reductions in respiratory motor activity through increasing rates of respiration,⁴⁵ these data suggest that increased plasticity at the PMP facilitates a modest recovery in respiratory function over time after treatment that 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.^{7,13,14} The cutting of the contralateral bulbospinal tracts was shown through the total cessation of activity within the hemidiaphragm contralateral to the initial contusion (Fig. 6Cii). The average length of time the animals continued to breath was different between treatment groups (Fig. 6A,B; $F[5,45] = 2.84$, $p = 0.028$). Respiratory activity ceased in all animals that had not received treatment within seconds of hemisection completion (Fig. 6A,B).¹⁴ 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 sec, group 3) injured animals (Fig. 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 the site of injury over time after trauma. Animals treated with ChABC, however, typically breathed for longer periods after 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 sec and 34.7 ± 17.6 sec, respectively, indicating that removal of CSPG at acute time points post-injury modestly facilitates a prolonged recovery of respiratory motor function (Fig. 6A+B). Animals treated with the enzyme at chronic time points post-trauma (group 3), however, continued breathing for 97.9 ± 33.5 sec after the injury (Fig. 6A,B), demonstrating that plasticity had induced a more substantial recovery within these animals.

FIG. 5. Chronic chondroitinase ABC (ChABC) treatment restores ipsilateral hemidiaphragm activity to function at normal levels after midcervical contusion. **(A)** Representative recordings of hemidiaphragm electromyography (EMG) ipsilateral and contralateral to the injury for (i) group one, (ii) group two, and (iii) group three animals treated with either control or ChABC. All panels are recorded in the same animal. **(B)** Average amplitude of (i) ipsilateral hemidiaphragm, (ii) contralateral hemidiaphragm, (iii) ipsilateral external intercostals (eIC), (iv) contralateral eIC, and (v) genioglossus EMG for (i) group one, (ii) group two, and (iii) group three animals treated with either control or ChABC. Graphs show means $\pm$ standard error of the mean for control: group one = dark blue, group two = blue, group three = light blue; ChABC groups: group one = red, group two = orange, group three = yellow.

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 after completion of the contralateral C2 hemisection.

As expected, the amplitude of activity within the hemidiaphragm ipsilateral to the contusion substantially increases after completion of the contralateral hemisection (Table 2; Fig. 6A,Ci). There are no significant differences, however, between either groups assessed at different time points post-trauma or after 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 (Fig. 6Ciii; Table 2; $F[5,44] = 0.803$, $p = 0.554$), contralateral eICs

(Fig. 6Civ; Table 2; $F[5,45] = 1.95$, $p = 0.108$) and the ipsilateral genioglossus (Fig. 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 after the C2 hemisection to maximize 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:

FIG. 6. Chondroitinase ABC (ChABC) treatment restores ipsilateral hemidiaphragm activity through lesioned pathways. **(A)** Representative recordings of hemidiaphragm electromyography (EMG) ipsilateral and contralateral to the injury for (i) group one, (ii) group two, and (iii) group three animals treated with either control or ChABC during contralateral C2 hemisection (dashed line). All panels are recorded in the same animal. Panels are from the same animal used in Figure 4. **(B)** Average time animal continued to show respiratory motor activity after completion of the contralateral C2 hemisection. **(C)** Average amplitude of (i) ipsilateral hemidiaphragm, (ii) contralateral hemidiaphragm, (iii) ipsilateral eIC, (iv) contralateral eIC, and (v) genioglossus EMG for (i) group one, (ii) group two, and (iii) group three animals treated with either control or ChABC. Graphs show means $\pm$ standard error of the mean for control groups: group one = dark blue, group two = blue, group three = light blue; ChABC groups: group one = red, group two = orange, group three = yellow.

$F[5,45] = 0.57, p = 0.719$) for any of the muscles assessed except the contralateral eICs. Interestingly, breathing frequency did change after 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 after injury, the greater the capacity to compensate for respiratory challenge by increasing rates of respiration (Table 2). This is marginally increased at all time points, however, 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 (Fig. 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 (Fig. 7A,Biii,iv).

Indeed, the intensity of 5HT at the ipsilateral PMP was positively correlated with both the raw EMG amplitude (Table 3; $R^2 = 0.318, p < 0.0001$) and the amplitude expressed as a percent of maximal output (Table 3; $R^2 = 0.268, p = 0.002$) of the ipsilateral hemidiaphragm. Further, increases in 5HT were strongly correlated with 2B6 staining at both the ipsilateral (Table 3; $R^2 = 0.464, p < 0.0001$) and contralateral (Table 3; $R^2 = 0.406, p < 0.0001$) PMP, demonstrating that the alterations in 5HT may be induced specifically by the treatment applied. Indeed, 2B6 staining at the ipsilateral PMP was also correlated with ipsilateral hemidiaphragm EMG amplitude expressed as a percent of maximal output (Table 3; $R^2 = 0.286, p < 0.0001$). Collectively, these data show that endogenous increases in 5HT, at least in part, facilitate recovery within the ipsilateral hemidiaphragm, caused by the plasticity induced through ChABC treatment.

TABLE 2. CHABC TREATMENT AT CHRONIC TIME POINTS AFTER CONTUSION INJURY FACILITATES MAINTENANCE OF MOTOR FUNCTION USING ONLY DESCENDING IPSILATERAL RESPIRATORY MOTOR PATHWAYS

Muscle	Control			ChABC		
	Gp1	Gp2	Gp3	Gp1	Gp2	Gp3
Amplitude (mV)						
Lf-dia	0.50±0.31*	0.80±0.80	5.43±1.67	3.06±1.79	3.25±1.78	11.2±4.09
Rt-dia	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Lf-eIC	0.10±0.06	0.11±0.11	0.97±0.62	0.12±0.07	1.29±0.94	1.49±0.65
Rt-eIC	0.00±0.00	0.00±0.00	0.32±0.17	0.00±0.00	0.00±0.00	0.04±0.04
Lf-geno	1.14±1.02	1.02±1.02	2.90±0.80	0.64±0.31	2.77±1.22	1.33±0.57
Breath length (sec)						
Lf-dia	0.12±0.06	0.04±0.04	0.18±0.04	0.13±0.07	0.18±0.06	0.23±0.04
Rt-dia	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Lf-eIC	0.29±0.22	0.05±0.05	0.09±0.04	0.14±0.07	0.17±0.06	0.12±0.05
Rt-eIC	0.00±0.00	0.00±0.00	0.09±0.04	0.00±0.00	0.00±0.00	0.03±0.03
Lf-geno	0.09±0.06	0.08±0.08	0.24±0.06	0.13±0.07	0.25±0.09	0.45±0.18
Cycle length (sec)						
Lf-dia	0.74±0.38	0.18±0.18	0.62±0.14	0.47±0.24	0.61±0.22	1.00±0.30
Rt-dia	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Lf-eIC	0.57±0.38	0.17±0.17	0.37±0.15	0.47±0.23	0.59±0.22	1.31±0.95
Rt-eIC	0.00±0.00	0.00±0.00	0.38±0.16	0.00±0.00	0.00±0.00	0.11±0.11
Lf-geno	0.55±0.38	0.14±0.14	0.57±0.13	0.43±0.22	0.51±0.19	0.74±0.32
Breath frequency (b/min)						
Lf-dia	14.6±7.1	8.0±8.0	39.0±8.7	15.0±7.3	27.1±9.8	39.0±8.9
Rt-dia	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Lf-eIC	14.4±7.1	8.0±8.0	21.8±8.9	15.0±7.3	27.4±10.0	24.4±9.9
Rt-eIC	0.0±0.0	0.0±0.0	21.6±8.8	0.0±0.0	0.0±0.0	6.9±6.9
Lf-geno	8.8±5.9	7.9±7.9	38.4±8.5	17.5±9.0	27.4±10.0	39.4±9.1

Data show amplitude (mV), breath length (sec), cycle length (sec), and breath frequency (breath per minute) for both control and ChABC treated animals at acute and chronic time points following C3 contusion injury. Tables show means±SEM. Comparisons between groups and ChABC Gp3 shown as: *= $p < 0.05$.

A Immunohistochemistry

B Intensity recordings

FIG. 7. Chondroitinase ABC (ChABC) treatment increases 2B6 and serotonin (5HT) intensity readings at both the ipsilateral and contralateral phrenic motor pools (PMPs). (A) Representative images of ipsilateral 2B6 (i,ii) and 5HT (iii,iv) staining from group 3 animals at the level of the C5 PMP after either control (i and iii) or ChABC (ii and iv) treatment. Panels from each treatment group were recorded in the same animal. (B) Average intensity readings from immunohistochemistry sections for all groups for (i) ipsilateral and (ii) contralateral 2B6 staining and (iii) ipsilateral and (iv) contralateral 5HT imaging. Graphs show means±standard error of the mean for control groups: group one=dark blue, group two=blue, group three=light blue; ChABC groups: group one=red, group two=orange, group three=yellow.

TABLE 3. HEAT MAP OF CORRELATIONS BETWEEN PHYSIOLOGICAL AND IMMUNOHISTOCHEMICAL OUTPUTS SHOWING 5HT INTENSITY POSITIVELY RELATES TO IPSILATERAL DIAPHRAGM EMG AMPLITUDE

	Actual amplitude (pre)	Actual amplitude (post)	% Max amplitude (pre)	% Max amplitude (post)	Time after C2Hx (sec)	Lesion vol.	2B6 intensity (ipsi)	5HT intensity (ipsi)	2B6 intensity (contra)	5HT intensity (contra)
Actual amplitude (pre)		0.202 $p=0.002$	0.111 $p=0.0002$	0.0000593 $p=0.67$	0.000697 $p=0.86$	0.00222 $p=0.76$	0.143 $p=0.11$	0.318 $p=0.0001$	0.180 $p=0.004$	0.104 $p=0.031$
Actual amplitude (post)	0.202 $p=0.002$		0.0524 $p=0.14$	0.208 $p=0.007$	0.158 $p=0.007$	0.0179 $p=0.38$	0.000455 $p=0.89$	0.00371 $p=0.69$	0.00212 $p=0.77$	0.000287 $p=0.91$
% Max amplitude (pre)	0.111 $p=0.0002$	0.0524 $p=0.14$		0.0312 $p=0.76$	0.0117 $p=0.79$	0.00586 $p=1.00$	0.286 $p<0.0001$	0.268 $p=0.002$	0.184 $p=0.015$	0.0771 $p=0.025$
% Max amplitude (post)	0.0000593 $p=0.67$	0.208 $p=0.007$	0.0312 $p=0.76$		0.0405 $p=0.27$	0.0796 $p=0.05$	0.00505 $p=0.64$	0.00940 $p=0.47$	0.00356 $p=0.77$	0.00190 $p=0.78$
Time after C2Hx (sec)	0.000697 $p=0.86$	0.158 $p=0.007$	0.0117 $p=0.79$	0.0405 $p=0.27$		0.0270 $p=0.27$	0.0142 $p=0.43$	0.0511 $p=0.13$	0.0000386 $p=1.00$	0.117 $p=0.02$
Lesion vol.	0.00222 $p=0.76$	0.0179 $p=0.38$	0.00586 $p=1.00$	0.0796 $p=0.05$	0.0405 $p=0.27$		0.00368 $p=0.69$	0.000579 $p=0.87$	0.00637 $p=0.60$	0.0336 $p=0.22$
2B6 intensity (ipsi)	0.143 $p=0.11$	0.000455 $p=0.89$	0.286 $p<0.0001$	0.00505 $p=0.64$	0.0142 $p=0.43$	0.00368 $p=0.69$		0.464 $p<0.0001$	0.728 $p<0.0001$	0.360 $p<0.0001$
5HT intensity (ipsi)	0.318 $p=0.0001$	0.00371 $p=0.69$	0.268 $p=0.002$	0.00940 $p=0.47$	0.0511 $p=0.13$	0.000579 $p=0.87$	0.464 $p<0.0001$		0.518 $p<0.0001$	0.705 $p<0.0001$
2B6 intensity (contra)	0.180 $p=0.004$	0.00212 $p=0.77$	0.184 $p=0.015$	0.00356 $p=0.77$	0.0000386 $p=1.00$	0.00637 $p=0.60$	0.464 $p<0.0001$	0.518 $p<0.0001$		0.406 $p<0.0001$
5HT intensity (contra)	0.104 $p=0.031$	0.000287 $p=0.91$	0.0771 $p=0.025$	0.00190 $p=0.78$	0.117 $p=0.02$	0.0336 $p=0.22$	0.360 $p<0.0001$	0.705 $p<0.0001$	0.406 $p<0.0001$	
		0.0 to 0.1	0.1 to 0.2	0.2 to 0.3	0.3 to 0.4	0.4 to 0.5	0.5 to 0.6	0.6 to 0.7	0.7 to 0.8	

Data show the r^2 value (3dp) for correlations between the actual and % maximum ipsilateral hemidiaphragm amplitude (mV) pre and post C2Hx, time animal maintains breathing after the C2Hx, lesion volume, and the intensity readings for 2B6 and 5HT at ipsilateral and contralateral sides of the cord.

Discussion

The present study shows the long-term functional deficits caused by unilateral midcervical 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 hypothesized that enhancement of plasticity through an exogenously applied treatment strategy would facilitate recovery of respiratory motor function after 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 after cervical contusion injury.

We show that ChABC treatment applied at chronic stages after 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 fibers 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 after severe midcervical contusion if applied at chronic time points after trauma.

Midcervical contusion injury

The unilateral midcervical contusion injury induced substantial damage to the spinal cord encompassing 100% of unilateral white and gray matter. The loss of gray 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^{18,44} explains the reduction in EMG activity within the ipsilateral hemidiaphragm. Phrenic motoneuron loss is associated positively with the degree of injury to gray matter after midcervical contusion.¹⁹ As such, within our model, these motoneurons critical to diaphragm function would be largely absent at the lesion center. 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^{13,46} and marginally larger than those of a 395–400 kDyne trauma,^{17,19,47–49} typical of severe SCI trauma.¹¹ There is substantial variance in the field over reporting of injury size and the extent of injury produced from a given force/displacement.^{6,9} Nonetheless, because of the stable configuration of our animals and preparation of the impact site before injury,^{7,11,13} we produce a severe and clinically relevant cervical SCI with little anatomical and functional variance between animals. Further difference between published studies may be explained additionally through strain or sex differences between animals.⁵⁰ Nonetheless, it is the production of a reliable and extensive midcervical contusion that 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 no significant return of function within the ipsilateral hemidiaphragm after application of the control treatment, and previously have demonstrated robust reductions in both ventilatory parameters (VE and VT) and venti-

latory variance that were persistent from acute to chronic time points.¹⁴ Our data suggest that ChABC treatment at subacute 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 fiber type loss at the site of injury or behavioral deficit rather than the specific lesion volume to show consistency within their model or effect of ChABC treatment.^{30,32,51–53} Hemisection studies, however, similarly have shown no effect of ChABC on lesion size.^{54,55} ChABC has been shown in previous 150 kDyne thoracic contusion models to promote substantial neuroprotection through modulation of the immune response.⁵¹ This was induced, however, through constant application of the enzyme, initiated three days post-injury. It is, perhaps, this continuous infusion of ChABC that may be key to neuroprotective effects,⁵⁶ as well as the acute production of disaccharide units resulting from enzyme activity⁵⁷ because similar studies utilizing a single injection of the enzyme do not demonstrate a similar outcome.^{33,58}

We administer four injections of the enzyme once, a minimum of seven days after the contusion, when the immune response and secondary effects causing widespread neurodegeneration have already begun to occur²⁰—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 mediated additionally through latent pathways or sprouting from the contralateral spared tissue.

Compromised diaphragm activity is persistent over time

We show that the severe midcervical 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 demonstrated previously within the respiratory motor system through EMG or phrenic nerve recordings.^{6–8,12,14} Often in these cases, however, the effect of the initial impact is transient, being partially overcome two to eight weeks after the initial trauma.^{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 reorganization that occurs after trauma.^{7,59}

Indeed, the absence of significant functional recovery within the ipsilateral hemidiaphragm over time¹⁴ demonstrates that this severe injury yields a lack of endogenous restoration and minimal plasticity in our model. Performing our recordings under anesthesia could minimize the EMG output achieved and, subsequently, underestimate the scale of recovery achieved.^{10,60} Any respiratory depressant effect was minimal, however, because our recordings were performed under light ketamine-xylazine anesthesia⁶¹ and significant ipsilateral hemidiaphragm recovery has been reported previously in contused animals assessed under similar conditions of anesthesia.^{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 to maintain constant ventilatory performance despite respiratory

compromise.^{17,45} Indeed, increases in respiratory rate tend to correlate with the size of the midcervical contusion injury.⁹

The increased amplitude of activity in the contralateral hemidiaphragm compared with activity in the uninjured animal³⁷ may be mediated through an increase in discharge rate or recruitment of spared phrenic motor units^{38,62} and confirms data reported previously.^{12,18} Similarly, we show increased activity within the ipsilateral eIC muscles to regain function after loss of tissue at the PMP^{12,14,15} likely mediated through propriospinal interneurons.⁴⁴ The relative amplitude of activity in these muscles, however, does not change with time, or after treatment, demonstrating that they are not operating to compensate for decreased activity in the diaphragm (because 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 midcervical SCI. Its cessation of function following completion of the contralateral hemisection in control treated animals, however, shows that our contusion model has induced substantial reorganization of the supraspinal or sensory feedback networks and ascending pathways leading to chronic changes in respiratory circuitry.^{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.⁶⁴ Because the muscles under these conditions are working typically at amplitudes far in excess of that achieved during eupneic maximum drive (e.g., spontaneous sigh), however, it is likely that the pathways activated are not those typically associated with normal respiratory motor function.³⁹

These data demonstrate that there is little repair through the site of injury in control treated animals. Animals treated with ChABC, however, are able to breath for significantly longer periods, 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 fibers through the site of injury.

Plasticity induced recovery of respiratory motor function after chronic injury

The functional consequences of ChABC application to respiratory motor function after acute or chronic spinal cord contusion have not been assessed previously. We demonstrate uniquely that induction of plasticity through application of the enzyme at chronic time points after unilateral midcervical 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 because ChABC has not been shown typically to mediate significant recovery after severe contusion injury, even when applied over a number of days.^{27–30} Our treatment paradigm has negated this effect, however. 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.²⁵ Because our animals are breathing constantly (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 paralyzed 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.^{51,52} Our results demonstrate positive effects of using ChABC on acute injuries through ipsilateral diaphragm EMG activity, but these are relatively modest. The secondary processes after contusion injury, however, are still occurring and progressing at acute points post-trauma.²⁰ 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 that are in the process of occurring to potentially limit functional respiratory motor recovery at these stages. Further, respiratory function after severe midcervical contusion is seemingly reliant on the activation or sprouting of spared/latent pathways.^{7,8,10,18}

These processes happen, however, only in later stages of spinal cord injury. There is evidence of some endogenous plasticity from two to eight weeks post-contusion^{8,9,12,19,47} that may aid respiratory motor function if correctly utilized 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 after contusion injury has been reported widely.^{31,42,53,65}

Within the respiratory motor system, we show that this may be more relevant functionally 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 shown that substantial recovery of respiratory motor function is possible up to a year and a half after cervical spinal hemisection and that ChABC induced effects last up to six months after the end of treatment.⁵⁵ This recovery was also induced partly through serotonergic mechanisms.⁵⁵ As such, currently we are 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 caused within our animals at chronic time points after midcervical contusion was mediated partially through spared desiccating pathways from the undamaged contralateral side of the cord and the potential interaction with propriospinal interneurons.^{6–8,10,12,18} We demonstrate that this is, at least in part, through serotonergic projections. The sprouting of serotonergic fibers have been shown previously to be important to recovery of respiratory function after midcervical contusion injury.^{8,15}

Our observations that ChABC brought about the increased serotonergic fiber innervation at the PMP correlate with other models of contusive SCI showing that application of the enzyme increases serotonergic sprouting and functional activity.^{30–33,42,51} Of course, it is likely that other mechanisms of recovery are functioning additionally. For example, the recruitment of V2a interneurons has been shown to be critical in rat and mouse models of cervical SCI to the promotion of respiratory function.^{66–68} Nonetheless, these findings demonstrate that CSPG digestion and the induction of plasticity within the PMP at both sides of the spinal cord facilitate alterations in serotonergic fiber 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 mediated partially by pathways through the site of injury. As far as we are aware, this is the first time that treatment induced recovery after midcervical contusion has come about through pathways so damaged previously that they could not sustain respiratory function alone.^{13,14} The observed recovery in both pathways is instigated likely by a reduction in inhibitory molecules at the PMP allowing for anatomical sprouting, plasticity, and regeneration of fibers (reviewed in²⁰), alleviation of potential blocks to neuronal conduction,⁶⁹ and increased myelination/remyelination of newly growth or spared fibers.^{51,58} These data demonstrate that plasticity induced restoration of respiratory motor activity after severe midcervical 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 midcervical contusion injury accurately reflects the clinical population showing a rapid deficit in respiratory motor function because of reduced activity in the ipsilateral hemidiaphragm.⁷⁰ Some compensatory activity is demonstrated within the accessory muscles to maximize respiratory performance.^{1,71,72} The deficit in diaphragm function is pronounced, however, 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 after 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^{36,54,65,73–75} without increasing pain or sensitivity.^{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). The success of this treatment strategy, however, is reliant partially on 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. The enzyme (or pharmaceutical equivalent), however, may need to be applied for longer,^{42,51,77,78} stabilized,⁷⁹ applied through a different route,⁸⁰ or in combination with a cavity spanning bridge^{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 after severe midcervical contusion injury. This restoration of function reduced the need for compensatory plasticity within the system and was mediated primarily through the activation or spared

or sprouting contralateral serotonergic fibers. Recovery of pathways through the site of injury was achieved in part. These findings have significant implications for the management of severe trauma to the spinal cord and other pathological disease states.

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