Phrenic motor neuron survival below cervical spinal cord hemisection

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Abstract

Cervical spinal cord injury (cSCI) severs bulbospinal projections to respiratory motor neurons, paralyzing respiratory muscles below the injury. C2 spinal hemisection (C2Hx) is a model of cSCI often used to study spontaneous and induced plasticity and breathing recovery post-injury. One key assumption is that C2Hx dennervates motor neurons below the injury, but does not affect their survival. However, a recent study reported substantial bilateral motor neuron death caudal to C2Hx. Since phrenic motor neuron (PMN) death following C2Hx would have profound implications for therapeutic strategies designed to target spared neural circuits, we tested the hypothesis that C2Hx minimally impacts PMN survival. Using improved retrograde tracing methods, we observed no loss of PMNs at 2- or 8-weeks post-C2Hx. We also observed no injury-related differences in ChAT or NeuN immunolabeling within labelled PMNs. Although we found no evidence of PMN loss following C2Hx, we cannot rule out neuronal loss in other motor pools. These findings address an essential prerequisite for studies that utilize C2Hx as a model to explore strategies for inducing plasticity and/or regeneration within the phrenic motor system, as they provide important insights into the viability of phrenic motor neurons as therapeutic targets after high cervical injury.

Keywords

phrenic motor neurons; cervical spinal injury; C2Hx; hemisection; spinal injury; Cholera toxin B; CtB; motor neurons; ChAT; NeuN

1. Introduction

Cervical spinal cord injury (cSCI) disrupts descending bulbospinal pathways to spinal respiratory motor neurons, causing respiratory muscle paralysis, diminished breathing capacity and ventilatory failure (Baussart et al., 2006; Brown et al., 2006; Goshgarian et al., 1986). Restoration of breathing capacity is an important goal since respiratory failure is a leading cause of morbidity and mortality in individuals with cSCI (NSCISC, 2018; Winslow and Rozovsky, 2003).

Considerable research has been devoted to identifying potential strategies to improve breathing ability in people with cSCI. Such efforts have generally focused on either regeneration of severed neural pathways (Alilain et al., 2011; Silver and Miller, 2004) or strengthening of spared neural pathways (Courtine et al., 2009; Fuller et al., 2003; Gonzalez-Rothi et al., 2015a; Goshgarian, 2003; Ling et al., 1994; Lovett-Barr et al., 2012). Cervical spinal hemisection at C2 (C2Hx) is an experimental model frequently used to study breathing recovery and plasticity within respiratory motor circuits after cSCI (Alilain and Goshgarian, 2007; Dougherty et al., 2017; Fuller et al., 2008; Gonzalez-Rothi et al., 2017; Goshgarian, 2009; Navarrete-Opazo et al., 2015). In these studies, one fundamental assumption is that C2Hx dennervates spinal respiratory motor neurons caudal to injury without causing significant neuronal pathology or cell death, thus sparing the necessary substrates for functional recovery of breathing ability. However, a recent study reported substantial presumptive motor neuron death caudal to C2Hx (Satkunendrarajah et al., 2016), using stereological analysis of choline acetyltransferase (ChAT) positive cells to assess motor neuron survival. Since there is considerable evidence for neurochemical plasticity in motor neurons post-injury (Fuller et al., 2005; Ganzer et al., 2018; Grossman et al., 2000; Mantilla et al., 2012; Murray et al., 2010), it is possible that C2Hx may alter motor neuron ChAT expression without actual loss/death of motor neurons themselves. Given the fundamental importance of distinguishing between motor neuron loss *versus* injury-related changes in the neurochemical profile of surviving neurons, it is essential to accurately assess the actual number of cells within the motor pool of interest. Indeed, the report of Satkunendrarajah and colleagues (2016) did not probe the fate of motor neurons in specific motor pools (e.g., phrenic vs. neck vs. forelimb), but performed regional quantification of ChAT expressing cells (e.g. all motor neurons in the cervical spinal cord). This is an important consideration, as there is some historical evidence of caudal motor neuron sparing after C2 spinal cord hemisection(Gonzalez-Rothi et al., 2015b; Lane et al., 2008a), although this was not the primary outcome for either of these studies.

To accurately quantify motor neuron survival caudal to injury, precise identification of the motor neurons of interest is essential. One method frequently used to identify specific motor neuron pools is retrograde tracing of a target muscle or nerve. For example, intrapleural injections of Cholera toxin B fragment (CtB) yield robust retrograde labelling of phrenic motor neurons without non-specific labeling in nearby cells (Mantilla et al., 2009). We utilized a refined version of this technique to test the hypothesis that phrenic motor neuron survival is minimally affected by C2Hx at sub-acute (2 wk) and chronic (8 wk) post-injury time points. We also assessed the impact of chronic spinal injury on expression of commonly used markers of neurons (NeuN-Neuronal Nuclei) and motor neurons (ChAT)

in retrogradely labelled phrenic motor neurons to explore whether injury affected expression of these markers in the absence of frank cell death. We report no effect of C2Hx on phrenic motor neuron survival at 2- or 8-weeks post-injury, nor did we observe altered NeuN or ChAT expression in CtB-labelled phrenic motor neurons. These findings have important implications for studies using the C2Hx model to explore spontaneous and/or induced plasticity within the phrenic motor system, since these neurons are an essential substrate for recovery of breathing function post-injury. Thus, confirmation of their overall survival at chronic post-injury time-points would suggest a robust population of neurons available as targets for therapeutic intervention. Conversely, significant loss of phrenic motor neurons would limit the potential for strategies designed to induce plasticity in surviving neuron populations to effect real and meaningful functional recovery without sufficient viable substrate to target.

2. Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committees of the University of Florida and the University of Wisconsin-Madison, and complied with all standards set forth in the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. Rats were double-housed and maintained on a 12-hour light/dark cycle with access to food and water *ad libitum*.

2.1. Experimental groups

Our primary goal was to assess the impact of chronic C2Hx on phrenic motor neuron survival post-injury. Thus, intrapleural injections of the monosynaptic retrograde tracer CtB were performed in adult male Sprague-Dawley rats (~300 grams, Envigo, Indianapolis IN, Colony 208A, University of Florida) to enable the precise identification of phrenic motor neurons in the cervical spinal cord (Figure 1A). Fourteen days after tracer injections, rats were randomized to one of four groups: 1) spinal intact (no surgery/injury) 2-week time controls (n=7); 2) spinal intact 8-week time controls (n=7); 3) 2-weeks post-C2Hx (n=8); and 4) 8-weeks post-C2Hx (n=9).

2.2. Intrapleural CtB injections

The basic methods for intrapleural CtB injections were adapted from previously published reports (Allen et al., 2018; Mantilla et al., 2009; Nichols et al., 2015b) as described in Figure 1B. Briefly, anesthesia was induced with 2.5% isoflurane (in 100% O_2) and maintained at 2% via nose cone. Based on findings from an initial study (Fig. 2), intrapleural tracer injections were performed under hypercapnic conditions ($FICO_2$=7.1%, FIO_2=20.8%, balance N_2) to increase breathing, and maximize retrograde labeling of the phrenic motor pool (increased motor neuron activity and/or greater CtB mixing in the pleural space). Upon transfer to the nose cone (Figure 1B), all rats were exposed to hypercapnic normoxia ($FICO_2$=7.1%, FIO_2=20.8%, balance N_2) during and after intrapleural injections to maximize retrograde labelling of phrenic motor neurons with CtB. 12.5 μL of CtB (0.2% w/v CtB; dissolved in sterile H_2O; Calbiochem, Billerica, MA) was loaded into a 25 μL Hamilton syringe attached to a 9.52 mm sterile needle (Hamilton Company, Reno, NV; syringe: 7636-01, needle: 7804-04). Injections were administered bilaterally (2×12.5

μL = 25μL total per animal) at the 5th intercostal space at a depth of approximately 6 mm. Following injections, isoflurane was discontinued, and rats were placed in Plexiglas chambers to recover from anesthesia while under the same hypercapnic normoxic conditions for 15 minutes. Rats were monitored closely for any signs of respiratory distress; however no such signs were observed.

2.3. Surgical preparation

Anesthesia methods and surgical preparation have been previously described (Dougherty et al., 2012; Gonzalez-Rothi et al., 2015b). Briefly, anesthesia was induced with isoflurane (2.5% in 100% O_2) and maintained via nose cone for the duration of the surgical procedure (2–2.5% isoflurane in 100% O_2). Adequate anesthesia was confirmed by the absence of toe pinch and palpebral responses. Body temperature was maintained at 36.5–37.5°C with a heating pad. Artificial tears were applied, nails clipped, and the surgical site shaved and prepped.

2.4. Cervical hemisection

C2Hx methods have been described previously (Dougherty et al., 2012; Gonzalez-Rothi et al., 2015b). Briefly, after anesthetic induction and pre-operative preparation, a dorsal cervical incision was made from the base of the skull to C3, and the skin and dorsal paraspinal muscles were separated to expose the C2 spinous process. The spinal cord was exposed at C2 via dorsal laminectomy. A lateralized hemisection was performed on the left side of the spinal cord just caudal to the dorsal C2 spinal nerve roots using a microscalpel followed by gentle aspiration. After confirming completeness of the C2Hx injury, the dura and overlying muscles were sutured (9–0 and 4–0 sutures, respectively), and the skin closed with stainless steel wound clips. After surgery, rats were administered buprenorphine (0.03 mg/kg, Hospira, IL), meloxicam (2 mg/kg, Portland, ME) and lactated Ringer's (5mL) subcutaneously for pain management and fluid replacement. Intact rats in control groups did not undergo any surgical procedures.

Following surgery, rats were housed in a heated incubator overnight, then returned to their home cages the following day. Subsequent post-operative care and monitoring was provided twice per day (AM/PM) until rats were fully upright, gaining weight, and displaying no overt signs of pain or distress. Pain was managed by administering buprenorphine (0.03 mg/kg, 2x/day, Hospira, IL) and meloxicam (2 mg/kg, Portland, ME) subcutaneously for 72 hours post-injury. Rats received subcutaneous injections of lactated Ringers (5 mL/day) and were manually fed a nutritional supplement (Diet Gel Boost; Clear H_2O; Westbrook, ME) until volitional drinking and eating resumed. Nutritional supplement and chow were provided at the bottom of home cages to ensure food was easily accessible to injured rats.

2.5. Tissue Preparation

Rats were sacrificed at either 2- or 8-weeks post-C2Hx (or the equivalent time point in spinal intact controls). Rats were anesthetized and perfused intracardially with cold 0.1M phosphate buffered saline (PBS), followed by paraformaldehyde (4% w/v in 0.1M PBS, pH 7.4). The spinal cord was removed from the vertebral column and post-fixed in paraformaldehyde overnight. Spinal cords were cryoprotected in 20% sucrose solution

in 0.1M PBS for 3 days, followed by 30% sucrose solution in 0.1M PBS for 3 days. The cervical spinal cord (C3–7) was sectioned longitudinally (40 μm) using a freezing microtome (Leica SM2000R, Buffalo Grove, IL) and individual tissues placed sequentially into cell culture wells. Sections were stored in antifreeze solution at −20°C until processed (30% glycerol, 30% ethylene glycol, 40% 0.1 M PBS, pH 7.4.).

2.6. Immunolabeling

Immunofluorescence was used to identify CTB-positive phrenic motor neurons in the cervical spinal cord of spinal intact and C2Hx rats. Manual counts of CtB-positive phrenic motor neurons were conducted in all tissue sections. All tissue sections were processed for visualization of CtB, with a subset of these tissues co-labeled with antibodies to NeuN (1 out of every 4 sections) and the remaining co-labeled with antibodies to ChAT (3 out of 4 sections). The antibody used against CtB (goat, Millipore Cat# 227040, RRID:AB_211712) recognizes the B subunit of Cholera toxin. Goat anti-CtB does not bind to any protein if CtB has not been introduced to the animal (Seven et al., 2018). The antibody used for ChAT (rabbit, Millipore Cat# AB143, RRID:AB_2079760) has been validated in conditional ChAT knockout mice (Young et al., 2008). The antibody for NeuN clone A60 (rabbit, Millipore Cat# ABN78, RRID:AB_10807945) has been used extensively and targets most neurons (Mullen et al., 1992; Wolf et al., 1996).

Tissue sections were washed 3 × 5 minutes with 0.1M PBS and then incubated in a blocking solution (10% NDS in 0.1M TBS-Triton (0.1%), pH 7.4; SD32–0500, Equitech-Bio, Inc, Kerrville, TX) for 1 hour at room temperature. Tissues were then incubated in primary antibody solution (3% NDS in 0.1M TBS-Triton (0.1%), pH 7.4) with goat anti-CtB (1:2500), and either rabbit anti-ChAT (1:100), or rabbit anti-NeuN (1:500) overnight at 4°C. The following day, tissues were washed 3 × 5 minutes with 0.1M TBS-Triton (0.1%) and then incubated in secondary antibody solution (3% NDS in 0.1M TBS-Triton (0.1%), pH 7.4) with secondary antibodies conjugated to Alexa Fluor® 488 (A-21206, 1:1000; Thermo Fisher Scientific, Waltham, MA) and 594 (A212207, 1:500; Thermo Fisher Scientific, Waltham, MA) for 2 hours at room temperature to label CtB and ChAT or NeuN, respectively. Tissues were then washed 3 × 5 minutes with 0.1M TBS and mounted on positively charged slides using VectaShield Hardset mounting medium (Vector Laboratories, UK) and then coverslipped. Slides were stored at 4°C until staining was assessed. Control experiments included incubating tissues without primary or secondary antibodies. Tissues were processed slightly differently in the initial, pilot study to test the impact of hypercapnia on phrenic motor neuron labeling (see figure legend 2).

2.7. Image acquisition

All longitudinal tissue sections were imaged using an epifluorescent microscope with 10x magnification (Keyence BZ-X700, Keyence Corporation of America, Itasca, IL). For tissues dual labelled with CtB and ChAT, a GFP filter was used for CtB (OP-87763; 0.43 seconds) and a Texas Red filter was used for ChAT (OP-87765; 0.10 seconds). For tissues dual labelled with CtB and NeuN, a GFP filter was used for CtB (OP-87763; 0.59 seconds) and a Texas Red filter for NeuN (OP-87765; 0.33 seconds). All image sets (CtB+ChAT and CtB+NeuN) were collected with the same exposure time setting and no binning was

performed. Separate investigators imaged and analyzed data, to ensure injury and time-point remained blinded until analyses completion.

2.8.   Image analysis

CtB-positive phrenic motor neurons were manually counted from single plane 10x images in all longitudinal cervical tissue sections. During tissue blocking, the right side of the cervical spinal cord was marked to ensure accurate determination of "side" during imaging and analyses. Only CtB-labelled neurons with a visible nucleus were counted in order to minimize double counting of labelled neurons. Data were expressed as number of cells.

In addition to manual counts of all CtB labelled neurons, we assessed expression of two proteins commonly used to identify neurons (NeuN) and more specifically motor neurons (ChAT) within CtB-labelled phrenic motor neurons. Optical density of ChAT or NeuN expression was measured within CtB-labelled phrenic motor neurons using semi-automatic quantification software (Keyence Hybrid Cell Count, Keyence Corporation of America, Itasca, IL). This algorithm identified CtB-positive phrenic motor neuron soma and quantified the optical density of ChAT or NeuN immunoreactivity within each soma. Data were expressed as arbitrary units (a.u.).

Group data were averaged and expressed as mean ± standard error of the mean. data were analyzed using three-way Mixed Model ANOVA with repeated measures (independent variables: injury, time-point and side relative to injury as the repeated measure). Statistical analyses were performed using SAS JMP (Cary, NC). Differences were considered significant if $p < 0.05$.

3.   Results

3.1.   Phrenic motor neurons survive caudal to cervical hemisection

Phrenic motor neurons were retrogradely labelled with CtB via intrapleural injections in both intact and C2Hx rats. CtB-positive cells in the C3-C6 ventral cervical spinal cord were identified via immunoreactivity (Figure 2), and were organized in a rostro-caudal column of clustered cells, consistent with prior reports (Goshgarian and Rafols, 1984; Kinkead et al., 1998; Lane et al., 2008b; Mantilla et al., 2009). Labelled cells with a visible nucleus were manually counted from all longitudinal tissue sections. We observed no differences in phrenic motor neuron number regardless of group (intact vs. injured; $p=0.89$), side (left/ipsilateral vs. right/contralateral; $p=0.11$) or time (2 vs. 8 weeks; $p=0.49$). Comparison of the 2- and 8-week intact groups revealed no differences in phrenic motor neuron number (intact, time: $p=0.47$; side: $p=0.09$), so these data were combined into a single intact control group for comparison with injured rat groups. There was no measurable loss of phrenic motor neurons at either 2- or 8-weeks post-C2Hx on either side of the spinal cord *versus* intact rats (intact ipsilateral: 159±15; intact contralateral: 197±19; C2Hx, 2 weeks, ipsilateral: 194±25; C2Hx, 2 weeks, contralateral: 177±12; C2Hx, 8 weeks, ipsilateral: 169±20; C2Hx, 8 weeks, contralateral: 186±28; group: $p=0.93$; side: $p=0.33$; Figure 2).

3.2. Neuronal nuclei immunoreactivity in Phrenic Motor Neurons is not affected by C2Hx

We also explored the impact of C2Hx on expression of NeuN immunoreactivity in large cells in the ventral horn that were co-labelled with CtB (Figure 3), since NeuN is a commonly used neuronal marker in studies assessing cell survival after injury (Gusel'nikova, 2015). NeuN immunoreacitivty was observed in CtB-labelled cells with a distribution consistent with literature reports (Mullen et al., 1992). We observed no injury, side or time-related differences in NeuN immunoreactivity within CtB-labelled phrenic motor neurons (injury: p=0.59; time: p=0.08; side: p=0.79). Similar to our findings with CtB counts, no differences in NeuN immunoreactivity were detected within CtB-labelled phrenic motor neurons among intact groups (intact, time: p=0.25; side: p=0.23), therefore these data were combined into a single control group for comparison with 2- and 8-week injured groups (Figure 3A). We found no effect of C2Hx on NeuN immunoreactivity in CtB-labelled phrenic motor neurons at either time *versus* spinal intact rats, regardless of side (intact, ipsilateral: 236.2±32.4; intact, contralateral: 313.0±51.3; C2Hx, 2 weeks ipsilateral: 295.8±64.7; C2Hx, 2 weeks, contralateral: 225.3±40.0; C2Hx, 8 weeks, ipsilateral: 349.7±47.2; C2Hx, 8 weeks, contralateral: 309.4±31.2; group: p=0.40, side: p=0.93, Figure 3).

3.3. ChAT immunoreactivity in Phrenic Motor Neurons not affected by C2Hx

ChAT immunoreactivity was also assessed within CtB-labelled phrenic motor neurons (Figure 4) since it is a frequently used marker of motor neurons. The distribution of ChAT immunoreactivity in CtB-labelled phrenic motor neurons was consistent with literature reports (Barber et al., 1984). C2Hx had minimal impact on ChAT immunoreactivity on either side of the spinal cord at either 2- or 8-weeks post-injury *versus* intact rats (injury: p=0.18, time: p=0.96, side: p=0.12). Since we did not observe differences in ChAT immunoreactivity in CtB-labelled phrenic motor neurons between intact groups, these data were combined into a single comparison group for 2- and 8-week C2Hx groups (Figure 4A; time: p=0.25, side: p=0.85). When comparing intact *versus* C2Hx at 2- and 8-weeks post-injury, no injury effect was observed (group: p=0.39), however there was an effect of side (p=0.04; intact, ipsilateral: 872.2±67.1; intact, contralateral: 864.9±84.1; C2Hx, 2 weeks, ipsilateral: 1132.1±64.7; C2Hx, 2 weeks, contralateral: 928.5±110.4; C2Hx, 8 weeks, ipsilateral: 1233.2±224.8; C2Hx, 8 weeks, contralateral: 899.8±190.6; Figure 4). Specifically, average ChAT expression is elevated on the side contralateral to injury, although these differences did not reach statistical significance in *post-hoc* analyses.

4. Discussion

The primary finding of this study is that C2Hx had no detectable impact on phrenic motor neuron survival with either subacute (2-weeks) or chronic (8-weeks) spinal injury. Further, no effect of C2Hx on key proteins commonly used to identify neurons (NeuN) or motor neurons (ChAT) within retrogradely labelled phrenic motor neurons were observed. Our results confirm survival of phrenic motor neurons after cervical hemisection, preserving the necessary substrate for recovery of breathing function. These experiments utilize and validate a modified methodology for intrapleural tracer injections that improves retrograde

labelling of phrenic motor neurons and enables more accurate determination of phrenic motor neuron survival post-injury (Figure 2).

4.1. Motor neuron survival caudal to injury C2Hx

The fate of neurons after spinal cord injury has been explored in a number of experimental models, including cervical (Nicaise et al., 2013; Nicaise et al., 2012a; Nicaise et al., 2012b), thoracic (Magnuson, 1999; Yu, 2019), thoraco-lumbar (Wen, 2015), and lumbar contusions (Magnuson, 1999), contusion with compression (Orr, 2017), cervical hemisection (Gonzalez-Rothi et al., 2015b; Lane et al., 2008a; Satkunendrarajah et al., 2016), thoracic transection (Bjugn et al., 1997; Yokota, 2019) and excitotoxic lesions (Magnuson, 1999). Wallerian degeneration is well described after SCI, whereby distal axons and dendrites of neurons directly affected by an injury degenerate over time (A.V., 1850; Becerra, 1995). Transneuronal degeneration, characterized by degeneration of neurons normally innervated by these damaged and/or degenerated axons has also been described (Bose et al., 2005; Cook, 1951; Eidelberg, 1989; Grossman et al., 2001), though there are some reports to the contrary (Kaelan, 1988; McBride and Feringa, 1992; Yokota, 2019).

There appear to be considerable differences in neuronal survival caudal to injury based on the type, level, and severity of injury, as well as other factors such as cell type, distance from the lesion epicenter (Grossman et al., 2001; Nicaise et al., 2013; Nicaise et al., 2012a; Nicaise et al., 2012b) and time post-injury (for review, see (Hassannejad et al., 2018)). For example, motor neuron loss is reported caudal to injury following moderate-severe contusion (Bose et al., 2005; Grossman et al., 2001; Nicaise et al., 2013; Nicaise et al., 2012a; Nicaise et al., 2012b). Conversely, with spinal transection, motor neuron survival has been reported (Hirakawa and Kawata, 1992; Yokota, 2019), even 52 weeks post-injury (L4-S3; (McBride and Feringa, 1992). While some of the disparities observed across studies are likely related to differences in injury type and/or severity, the methods used to evaluate motor neuron survival may also contribute. Indeed, methods used to assess neuronal survival post-injury vary considerably across studies, ranging from retrograde tracing methods (fluoro-gold, horseradish peroxidase, CtB) to immunohistochemistry/staining methods such as hematoxaline and eosin, Cresyl violet, TUNEL, ChAT, etc (Bose et al., 2005; Eidelberg, 1989; Grossman et al., 2001; McBride and Feringa, 1992; Nicaise et al., 2013; Nicaise et al., 2012a; Nicaise et al., 2012b; Satkunendrarajah et al., 2016; Yokota, 2019). Although immunohistochemical methods enable a broader view of injury-associated changes post-injury (e.g. regional/segmental assessments), they are limited by a lack of specificity to defined cell populations and by their susceptibility to injury-associated changes that do not necessarily correlate with cell loss. Conversely, although neuroanatomical tracing enables specificity of the cell population of interest, it is limited to those neurons retrogradely transporting the tracer.

The C2Hx model has been used extensively in studies exploring novel therapeutic strategies to restore breathing function, such as acute intermittent hypoxia (Dougherty et al., 2017; Golder and Mitchell, 2005; Gonzalez-Rothi et al., 2021; Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2015; Vinit et al., 2009), electrical stimulation (Gonzalez-Rothi et al., 2017; Mercier et al., 2017; Sunshine et al., 2021), cellular transplantation (Alilain et al., 2011;

Cheng et al., 2021; Dougherty et al., 2016; Goulao et al., 2019; Gransee et al., 2015; Houle and Reier, 1988; Sandhu et al., 2017; White et al., 2010), gene therapy (Charsar et al., 2019; Martinez-Galvez et al., 2016), and pharmacological manipulation (Basura et al., 2002; Hernandez-Torres et al., 2017; Hsu and Lee, 2015; Nantwi et al., 1996; Nantwi and Goshgarian, 1998; Sieck et al., 2021; Wollman et al., 2020; Zhou and Goshgarian, 2000; Zimmer and Goshgarian, 2006). Since the success of these interventions is largely predicated on having some degree of intact neural substrate innervating the diaphragm (e.g. phrenic motor neurons), significant injury-related neuron loss would likely have a profound functional impact. However, there have been few reports concerning the impact of C2Hx (Gonzalez-Rothi et al., 2015b; Lane et al., 2008a; Satkunendrarajah et al., 2016) on survival of neuron populations caudal to injury; and none have systematically assessed phrenic motor neuron survival specifically.

There is some evidence to suggest that phrenic motor neurons are, at least to some extent, spared after injury. For example, while exploring the effects of C2Hx on pre-phrenic interneurons, Lane et al., reported no significant reductions in ipsilateral motor neuron numbers after injury (Lane et al., 2008a), although methodological considerations related to the use of pseudorabies virus (e.g., time-dependent lysis of infected cells) limit interpretation of motor neuron counts. Functionally, restoration of ipsilateral phrenic/diaphragm output after C2Hx, whether spontaneous (Alilain and Goshgarian, 2008; Fuller et al., 2008; Nantwi et al., 1999; O'Hara and Goshgarian, 1991) or induced (Alilain et al., 2011; Gransee et al., 2013; Nantwi and Goshgarian, 1998; Sieck et al., 2021; Urban et al., 2019; Warren, 2018), requires the existance of viable phrenic motor neurons ipsilateral to the side of injury. This is certainly possible, as C2Hx does not damage these neurons directly, nor does it fully deprive them of synaptic inputs. Indeed, there is considerable evidence of crossed spinal pathways (Boulenguez et al., 2007; Dobbins and Feldman, 1994; Feldman et al., 1985; Goshgarian et al., 1991; Lipski et al., 1994; Moreno et al., 1992; Rikard-Bell et al., 1984), sensory afferent neurons (Nair et al., 2017a; Nair et al., 2017b; Road, 1990; Song et al., 1999; Speck and Revelette, 1987; Vinit et al., 2007) and a robust network of propriospinal interneurons (Cregg et al., 2017; Lane et al., 2008a; Lu et al., 2004; Satkunendrarajah et al., 2018; Streeter et al., 2017; Tian and Duffin, 1996) with known connections to phrenic motor neurons. Sparing of and plasticity within at least some of these synaptic connections after injury (Boulenguez et al., 2007; Lane et al., 2008a; Moreno et al., 1992; Streeter et al., 2020; Vinit et al., 2007) may provide sufficient neurotrophic support and synaptic activity to minimize transneuronal degeneration of phrenic motor neurons, which would be consistent with the findings of the present study. Furthermore, sprouting of neural projections may help restore neurochemical and/or synaptic innervation of phrenic motor neurons, further contributing to their survival.

4.2. NeuN and ChAT immunoreactivity following injury

In the present study, NeuN and ChAT immunoreactivity were assessed within CtB-labelled cells since they are frequently used as markers to detect neurons or, more specifically, motor neurons. Since there is considerable evidence for neurochemical plasticity within spinal motor neurons post-injury (Alilain and Goshgarian, 2008; Fuller et al., 2005; Grossman et al., 2000; Kitzman, 2006; Mantilla et al., 2012; Petrov et al., 2007; Yokota, 2019),

we explored whether expression of these proteins was altered in surviving motor neurons post-injury. NeuN antibodies reliably label the nucleus and cytosol of ventral horn neurons, including phrenic motor neurons (Mullen et al., 1992), but NeuN expression is not specific to motor neurons, and may be impacted by neural injury in ways that are independent from cell survival (Gusel'nikova, 2015; Unal-Cevik, 2004). For example, hippocampal NeuN expression is reduced following ischemic injury, despite preservation of neuron numbers (Unal-Cevik, 2004; Xu et al., 2002).

ChAT, an enzyme necessary for acetylcholine synthesis, is normally expressed in motor neurons of the ventromedial, central and lateral columns (Barber et al., 1984), and is often used as a marker of motor neurons. Indeed, phrenic motor neurons robustly express ChAT (Barber et al., 1984; Yuan et al., 2014), although at least some spinal pre-phrenic interneurons are also ChAT positive (Lane et al., 2008b). Thus, ChAT immunoreactivity alone is not a unique identifier of motor neurons. Furthermore, ChAT immunoreactivity is variably altered by spinal injury (Hoang et al., 2003; Nakamura et al., 1996; Yokota, 2019). For example, following mild to moderate cervical compression injury, ChAT immunoreactivity at the site of injury (C6) decreased ~50% 2 days post-injury, an effect that persisted for 28 days post-injury (Nakamura et al., 1996). In this same study, the 50–60% reduction of ChAT immunoreactivity rostral (C4–5) and caudal (C7-C8) to injury had recovered by 7 days post-injury. Conversely, *increased* ChAT expression has been reported caudal to spinal cord transsection (Yokota, 2019), suggesting that in some chronic models, cholinergic activity is selectively maintained in distal motor neurons, despite reduced synaptic inputs and neuronal activity. Since ChAT expression is variably modulated by injury, with its regulation depending on injury type, time post-injury and distance from injury site, ChAT expression is not a reliable indicator of cell number in studies of spinal injury (Lams et al., 1988).

In a recent study, ChAT immunoreactivity was used to determine the extent of spinal motor neuron survival after C2Hx. The authors concluded that over one half of all motor neurons in the cervical spinal cord (C3-C8) die caudal to C2Hx (Satkunendrarajah et al., 2016). This conclusion is problematic since: 1) ChAT immunoreactivity may not be a reliable indicator of motor neuron survival following spinal injury (see above), and 2) ChAT expression is not necessarily limited to motor neurons (Lane et al., 2008b), or uniform across spinal cord regions (Barber et al., 1984). Furthermore, although atrophic changes in spinal regions distal to injury have been described in numerous reports (Bose et al., 2005; David et al., 2019; Gazula et al., 2004; Kitzman, 2005; Yokota, 2019), they are not necessarily the result of neuronal death, nor do they always correlate with reduced ChAT expression. Indeed, a recent report describes sparing of motor neuron size and number after complete spinal cord transection, despite significant reductions in gray matter area; in fact, ChAT expression was actually increased caudal to injury in that study (Yokota, 2019). Thus, factors other than overt cell death may contribute to spinal atrophy caudal to lesions, such as degenerative changes affecting axon tracts that course through spinal gray matter (Yokota, 2019), or reductions in motor neuron somal size or dendritic branching (Gazula et al., 2004; Hirakawa and Kawata, 1992; Kitzman, 2005).

4.3. Retrograde labeling of phrenic motor neurons in anesthetized rats

Accurate identification of phrenic motor neurons is critical to assess cell survival post-injury. Although immunolabeling techniques can be used to identify motor neuron-specific proteins, these techniques are often unreliable after injury, may not be truly reflect cell death, and do not identify specific motor pools. Although endogenous protein markers may exhibit injury-induced neurochemical plasticity not necessarily reflective of motor neuron loss, retrograde labeling techniques prior to injury provide a more definitive approach to determine motor neuron survival post-injury. Indeed, intrapleural injections of retrograde tracers, such as CtB, enable reliable identification of spinal respiratory motor neurons (Allen et al., 2018; Mantilla et al., 2009; Nichols et al., 2015b). Nevertheless, across published studies, there is a range in the reported number of phrenic motor neurons, possibly resulting from methodological differences in tracer administration (Mantilla et al., 2009; Nichols et al., 2015b).

One possible explanation for these disparities could relate to differences in neural activity and or ventilation in anesthetized versus unanesthetized rats. Reduced respiratory motor output in anesthetized rats may decrease phrenic motor neuron CtB labeling versus unanesthetized rats, possibly due to diminished CtB uptake and transport when neural activity is low and/or differences in CtB mixing within the pleural fluid. In an *ex vivo* diaphragm preparation, frequency-dependent CtB uptake was observed, with higher frequencies (80 Hz) increasing CtB uptake at axon terminals (Gonzalez Porras et al., 2019). Accordingly, we hypothesized that intrapleural CtB injections under hypercapnic conditions would increase phrenic motor neuron activity and, thus, CtB uptake due to increased respiratory drive. Alternately, increased breathing during hypercapnia may increase CtB mixing within intrapleural fluid, increasing access to, and uptake by, phrenic axon terminals. Regardless of the mechanism, we hypothesized that greater ventilation in unanesthetized rats leads to a relative increase in phrenic motor neuron counts using these methods (ie. CtB labeling of the phrenic motor pool) versus anesthetized rats, either due to increased neuronal activity and/or pleural mixing. In preliminary experiments exploring the impact of intrapleural injections under hypercapnic conditions (Figure 2), we found increased phrenic motor neuron labeling versus anesthetized rats kept normocapnic, supporting our working hypothesis that phrenic motor neuron CtB labeling is improved by greater breathing activity. A more detailed understanding of mechanisms whereby increased respiratory activity improves phrenic motor neuron CtB labeling is beyond the scope of this study.

Here, we manually counted all retrogradely labeled phrenic motor neurons to obtain accurate assessments of motor neuron numbers (ie. there was no need for unbiased stereology). We also evaluated NeuN and ChAT expression in CtB-labelled phrenic motor neurons to assess injury associated changes in the expression of these commonly used imarkers and observed no changes in NeuN/ChAT expression within CtB-labelled neurons. Thus, C2Hx does not affect phrenic motor neuron survival or neurochemical expression of NeuN or ChAT at the times studied post-injury. Since we focused on phrenic motor neurons, we cannot make conclusions about cell survival or neurochemical phenotype in other spinal motor nuclei.

5. Conclusions

Phrenic motor neuron survival after incomplete cSCI has profound implications for spontaneous and/or induced spinal respiratory motor plasticity and functional recovery of breathing ability. Indeed, phrenic motor neurons are often the target of experimental therapeutic strategies, predicated on the assumption that they remain viable post-injury. Although a recent report suggested profound loss of putative motor neurons caudal to C2Hx injury, we demonstrate robust phrenic motor neuron survival with both sub-acute and chronic injury. Our results utilize well-established retrograde labelling methods (prior to injury), refined to improve labelling efficiency, enabling precise identification of phrenic motor neurons. Although we confirm phrenic motor neuron survival following C2Hx, this study does not allow conclusions concerning their viability, or. Survival in other nearby motor neuron pools.

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Highlights

- Phrenic motor neuron survival was assessed 2- and 8-weeks after C2 spinal hemisection

- Improved methods were used to identify phrenic motor neurons via intrapleural CtB injections

- There was no evidence of phrenic motor neuron loss after C2 hemisection

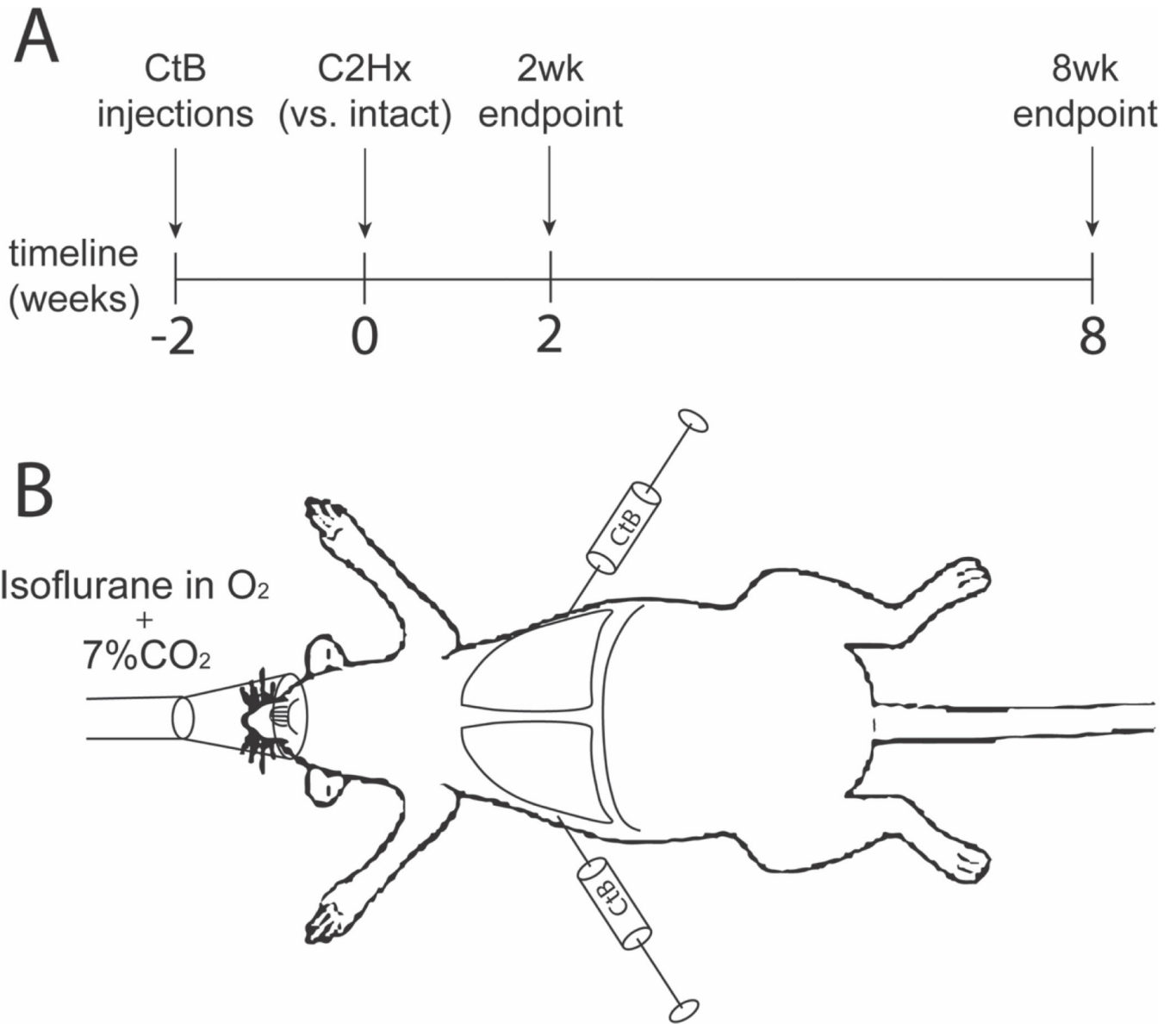- Phrenic NeuN and ChAT immunoreactivity were also unaffected by C2 hemisection

Figure 1: Experimental Model.
A. Schema depicting the experimental timeline. All rats received bilateral intrapleural injections of CtB. Two weeks after injection, rats were randomized to one of four groups: 2-wk intact, 8-wk intact, 2-wk C2Hx, or 8-wk C2Hx. Rats were sacrificed at the pre-determined end-point and cervical spinal tissues processed for CtB, NeuN, and ChAT immunohistochemistry. **B.** Schema depicting intrapleural tracer injection methods. Rats were anesthetized with isoflurane (2.5%) while under hypercapnic conditions (FICO$_2$=7.1%, FIO$_2$=20.8%, balance N$_2$). Bilateral intrapleural injections of CtB were administered, isoflurane was withdrawn, and animals were allowed to recover from anesthesia while remaining under hypercapnic normoxia.
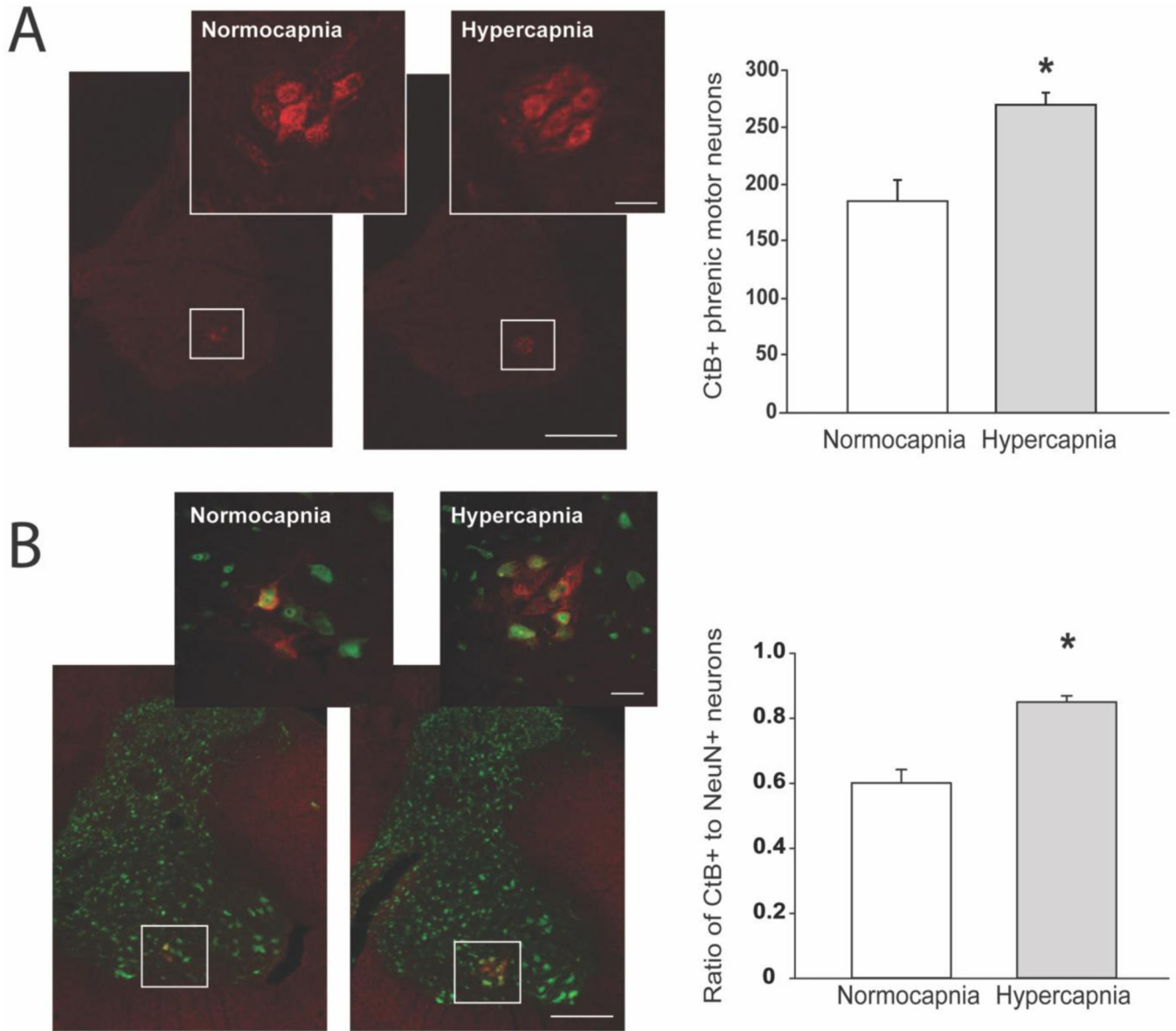
Figure 2. Validation of Modified Intrapleural Injection Methods to Optimize Retrograde Labeling of Phrenic Motor Neurons with Cholera toxin B.

In preliminary experiments, we validated improved intrapleural CtB tracing methods in spinal intact rats by comparing phrenic motor neuron labelling after injections delivered under isoflurane anesthesia with either poikilocapnic (n=7; $FICO_2$=0%, FIO_2=100%; white bars) or hypercapnic conditions (n=7; $FICO_2$=7.1%, FIO_2=20.8%, balanced N_2; gray bars) using previously published methods (Nichols et al., 2013; Nichols et al., 2017; Nichols et al., 2015a). Briefly, transverse sections from the C4 spinal cord were dual labeled for CtB and NeuN. Tissues were incubated in primary antibodies (goat anti-CtB (1:2000); mouse anti-NeuN (1:500)) overnight at 4°C, followed by secondary antibodies (Alexa Fluor® 594 (donkey anti-goat 594; 1:1000); 488 (donkey anti-mouse 488; 1:1000) for 2 hours at room temperature. The total number of phrenic motor neurons within the C4 region was extrapolated from manual phrenic motor neuron cell counts from six C4 sections per animal

(left and right phrenic motor neuron counts were not different from each other, and thus were averaged together for each section for extrapolation); briefly, we divided the total C4 segment length (2000 μm) by the thickness of each section (40 μm), and multiplied by the average number of phrenic motor neurons per animal. **A.** Representative images depict CtB labeling in the cervical spinal ventral horn from isoflurane anesthetized normocapnia and hypercapnia treated rats. (4x with a 20x inset of the phrenic motor pool; Scale bar at 4x = 400μm, 20x = 50μm. We observed increased CtB-labelling with injections performed under hypercapnia vs. normocapnia (*p<0.001). **B.** Tissue sections from these preliminary studies were also dual labeled with NeuN and manually counted by a blinded observer. The number of large, NeuN-labelled neurons was not different between groups (normocapnia: 307±13, hypercapnia: 320±18; data not shown). Thus, the ratio of CtB-labelled to NeuN-labelled cells was increased by hypercapnia (normocapnia: 0.60±0.04; hypercapnia 0.85±0.02; p<0.001), suggesting improved CtB labelling efficiency of phrenic motor neurons. Data were analyzed using Student's *t* test. Differences were considered significant if p< 0.05. Data are displayed as mean±SEM.
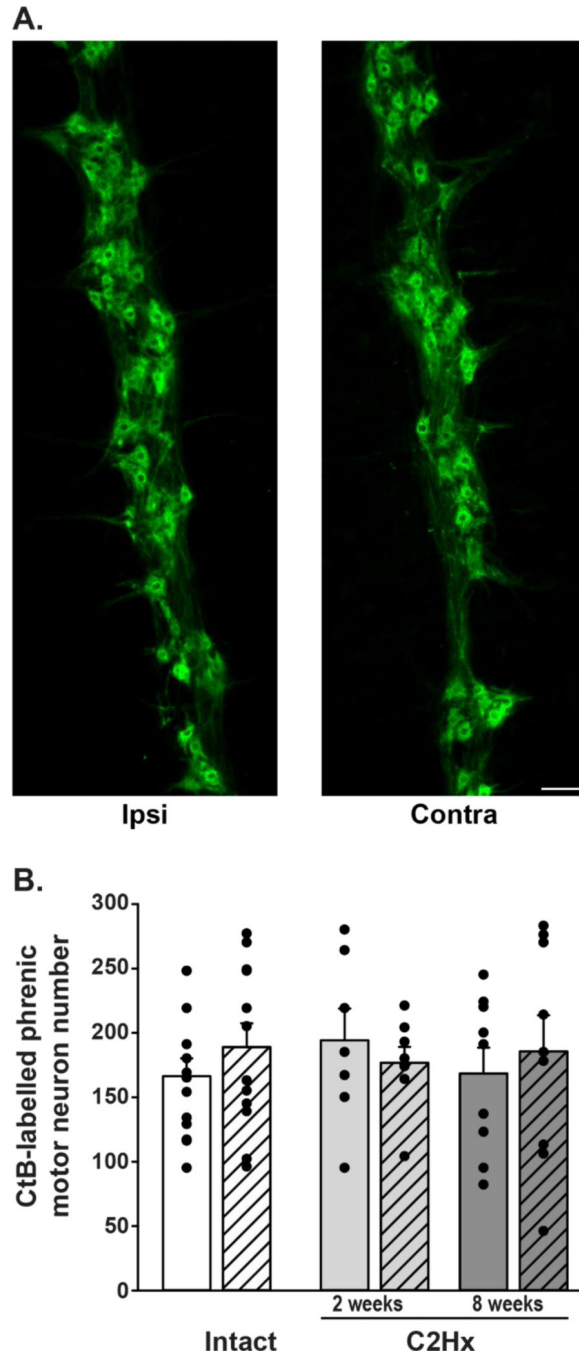
Figure 3: CtB-labelling of phrenic motor neurons in spinal intact and C2Hx rats, 2- and 8-weeks post-injury.

A: Representative images from spinal intact rats illustrating CtB-labelled phrenic motor neurons in longitudinal sections from the cervical spinal cord (40μm). B: Phrenic motor neuron counts in spinal intact (white bars) and C2Hx rats, 2- and 8-weeks post-injury (light grey and dark grey bars, respectively). Left (ipsilateral) and right (contralateral) sides of the cervical spinal cord are indicated by solid and hashed bars, respectively. Data were initially analyzed with repeated measures ANOVA with injury (intact vs. C2Hx) and time-point (2 weeks vs. 8 weeks) as between subject variables, and side (left/ipsi vs. right/contra) as the

within subject repeated measure. Since we observed no differences in motor neuron number between 2 and 8 week intact groups (time: p=0.47, side: p=0.09), these data were combined to form a single control group for comparison with the 2 and 8 week C2Hx groups (panel B). Similar to our findings with separate control groups, we found no effect of injury, nor did we observe differences in motor neuron counts between sides (group: p=0.93, side: p=0.33). Scale bar: 100μm. Differences were considered significant if p<0.05. Data are displayed as mean ± SEM.
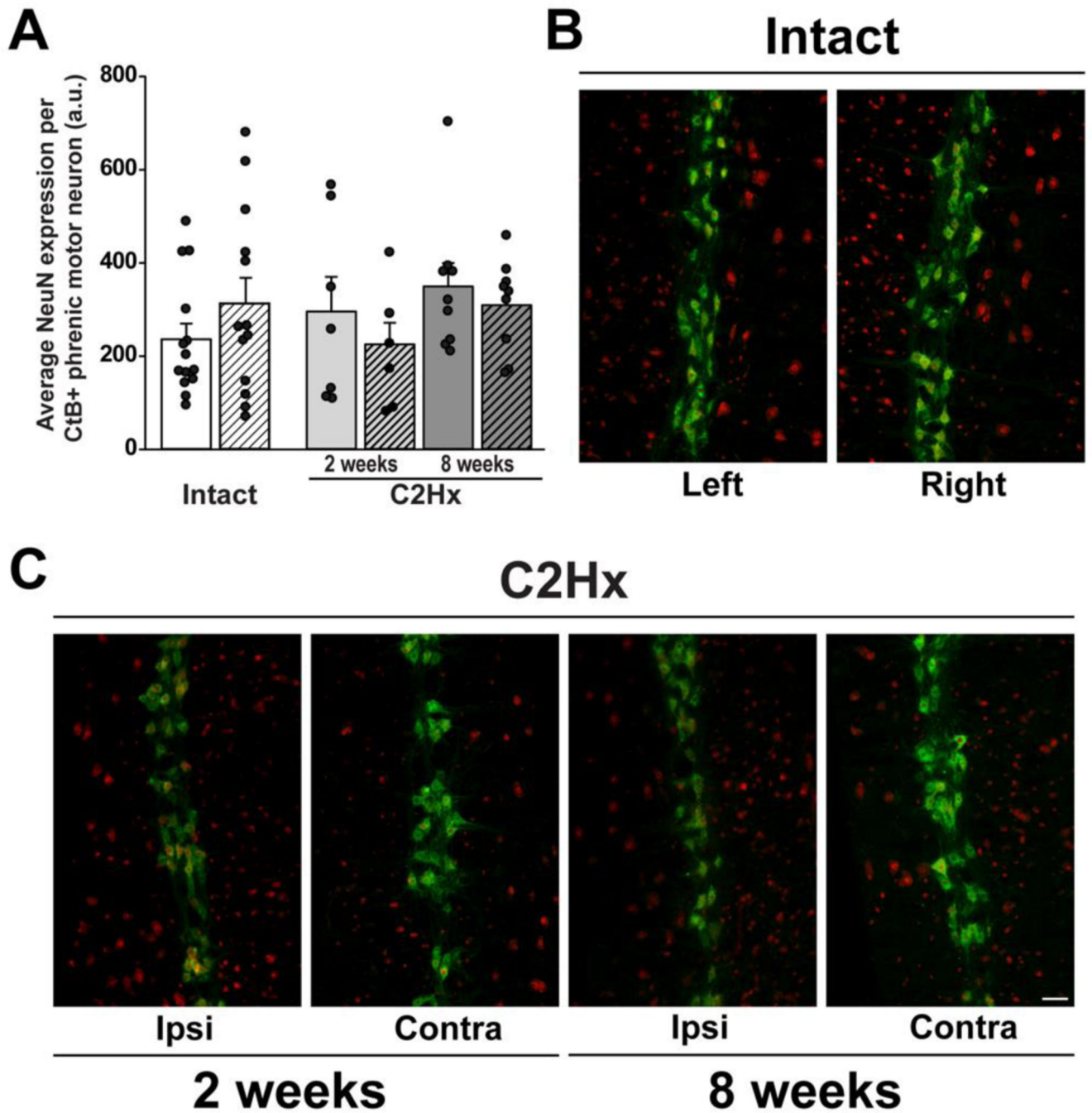
Figure 4: NeuN expression in CtB-labelled phrenic motor neurons in spinal intact and C2Hx rats, 2- and 8-weeks post-injury.

A. Quantification of NeuN expression in CtB-labelled phrenic motor neurons in spinal intact (2- and 8- week groups combined; white bars) and C2Hx rats at 2- and 8-weeks post-injury (light and dark grey bars, respectively). Left/ipsilateral and right/contralateral sides of the cervical spinal cord are indicated by solid and hashed bars, respectively.Data were analyzed with repeated measures ANOVA with "group" (intact vs. 2wk C2Hx vs. 8wk C2Hx) as the between subjects variable and "side" (left/ipsilateral vs. right/contralateral) as the within subject repeated measure. For this analysis, intact groups (2 vs. 8 weeks) were

combined to form a single control group, as there were no differences between the two time points in intact rats (time: p=0.25, side: p=0.23). NeuN expression in CtB-labelled phrenic motor neurons was not affected by C2Hx compared to spinal intact rats at 2- or 8-weeks post-injury (group: p=0.40) on either the left/ipsilateral or right/contralateral sides of the spinal cord (side: p=0.93). B-D: Representative images illustrating NeuN expression (red) in CtB-labelled phrenic motor neurons (green) in spinal intact (B) and C2Hx rats at 2- (C) and 8-week (D) time-points (10x). Scale bar: 100μm. A.U.: arbitrary units. Differences were considered significant if p<0.05. Data are displayed as mean ± SEM.
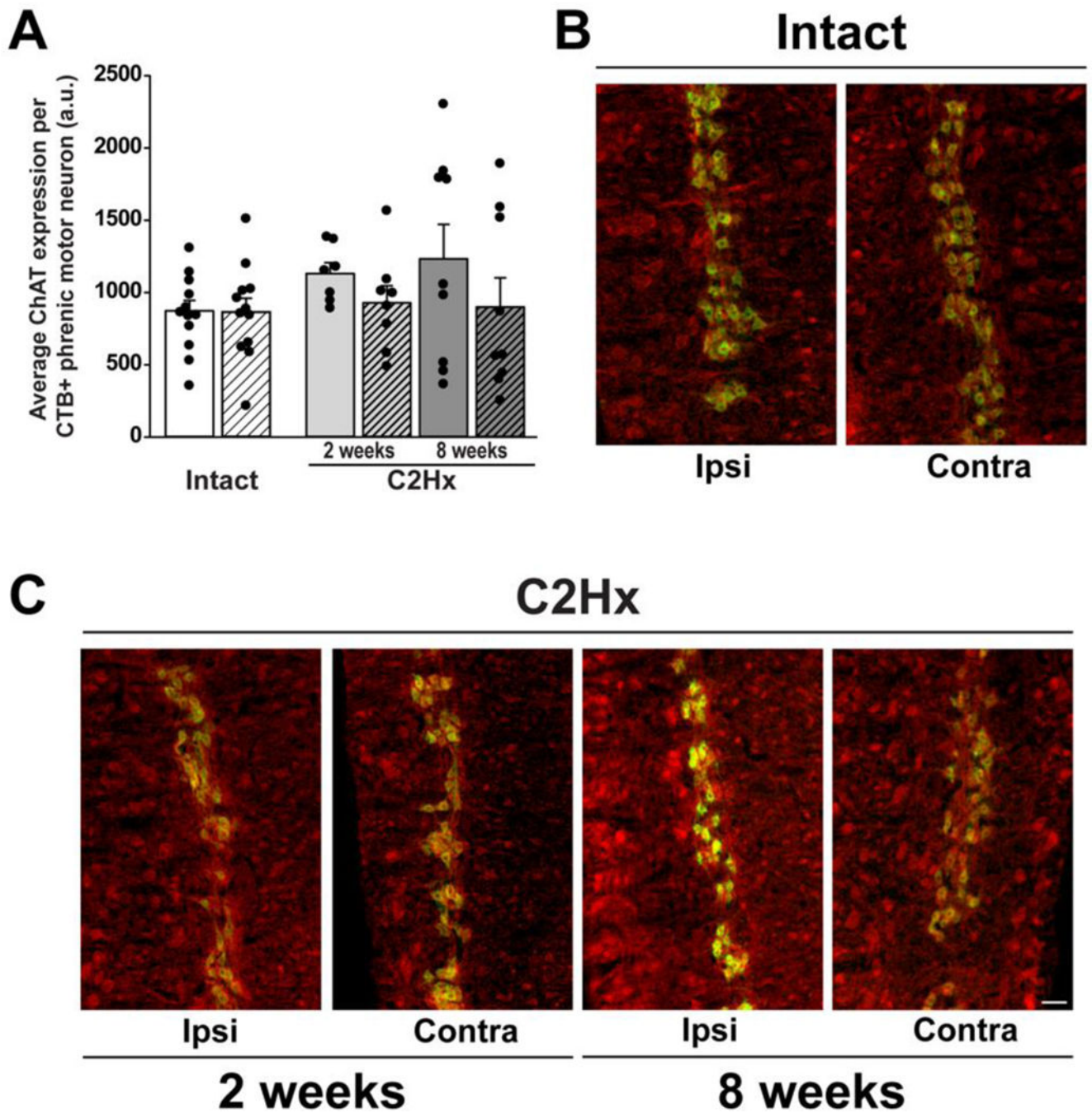
Figure 5: Choline acetyltransferase (ChAT) expression in CtB-labelled phrenic motor neurons in spinal intact and C2Hx rats, 2- and 8-weeks post-injury.

A: Quantification of ChAT expression within CtB-labelled phrenic motor neurons in spinal intact (white bars) and C2Hx rats at 2- and 8-week time-points (light and dark grey bars, respectively). Left/ipsilateral and right/contralateral sides of the cervical spinal cord are indicated by solid and hashed bars, respectively. Data were analyzed with repeated measures ANOVA with "group" (intact vs. 2wk C2Hx vs. 8wk C2Hx) as the between subjects variable and "side" (left/ipsilateral vs. right/contralateral) as the within subject repeated measure. For this analysis, intact groups (2 vs. 8 weeks) were combined to form a single control

group ("intact"), as there were no differences between the two time points in intact rats (time: p=0.25, side: p=0.85). ChAT expression in CtB-labelled phrenic motor neurons was not affected by C2Hx at 2- or 8-weeks post-injury compared to spinal intact rats (group: p=0.39), however there was an effect of side (side: p=0.04). B-D: Representative images illustrating ChAT expression (red) in CtB-labelled phrenic motor neurons (green) in spinal intact (B) and C2Hx rats at 2- (C) and 8-week (D) time-points (10x). Scale bar: 100μm. A.U.: arbitrary units. Differences were considered significant if p<0.05. Data are displayed as mean ± SEM.