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Research Paper

Spontaneous respiratory plasticity following unilateral high cervical spinal cord injury in behaving rats

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ABSTRACT

Unilateral cervical C2 hemisection (C2Hx) is a classic model of spinal cord injury (SCI) for studying respiratory dysfunction and plasticity. However, most previous studies were performed under anesthesia, which significantly alters respiratory network. Therefore, the goal of this work was to assess spontaneous diaphragm recovery post-C2Hx in awake, freely behaving animals. Adult rats were chronically implanted with diaphragm EMG electrodes and recorded during 8 weeks post-C2Hx. Our results reveal that ipsilateral diaphragm activity partially recovers within days post-injury and reaches pre-injury amplitude in a few weeks. However, the full extent of spontaneous ipsilateral recovery is significantly attenuated by anesthesia (ketamine/xylazine, isoflurane, and urethane). This suggests that the observed recovery may be attributed in part to activation of NMDA receptors which are suppressed by anesthesia. Despite spontaneous recovery in awake animals, ipsilateral hemidiaphragm dysfunction still persists: i) Inspiratory bursts during basal (slow) breathing exhibit an altered pattern, ii) the amplitude of sighs – or augmented breaths – is significantly decreased, and iii) the injured hemidiaphragm exhibits spontaneous events of hyperexcitation. The results from this study offer an underappreciated insight into spontaneous diaphragm activity and recovery following high cervical spinal cord injury in awake animals.

1. Introduction

High cervical spinal cord injury disrupts suprapspinal input to phrenic motor circuity, resulting in impaired diaphragm function and venitlation. Extensive preclinical research has shown that despite these devastating consequences, there is significant plasticity within the injured respiratory networks. The most commonly used animal model of respiratory dysfunction after spinal cord injury (SCI) is a lateral cervical C2 hemisection (C2Hx) (Bezdudnaya et al., 2017; Dougherty et al., 2012; Fuller et al., 2008; Goshgarian, 1979, 2003, 2009; Lane et al., 2008a; Mantilla et al., 2017; Mantilla et al., 2013; Porter, 1895; Sieck and Mantilla, 2009; Vinit et al., 2006). C2Hx interrupts unilateral projections from the brainstem to ipsilateral phrenic motoneurons resulting in hemidiaphragm paralysis. Immediately after injury, animals adopt a frequent and shallow breathing pattern (Fuller et al., 2008, 2006; Golder et al., 2001b; Hoh et al., 2013). However, acute partial recovery of phrenic and hemidiaphragm activity after C2Hx can be elicited by contralateral phrenicotomy (Goshgarian, 2003; O'Hara Jr and Goshgarian, 1991; Porter, 1895;) or complete termination of ventilator support (asphyxia) in paralyzed animals (Lewis and Brookhart, 1951; O'Hara Jr and Goshgarian, 1991; Zhou et al., 2001). This recovery - referred to as the “crossed-phrenic phenomenon (CPP)” (Rosenbleuth and Ortiz, 1936) – has been attributed to activation of otherwise latent crossed bulbospinal pathways (at C3-C6 level), which become active during increased respiratory drive (Goshgarian, 2003; O'Hara Jr and Goshgarian, 1991). In fact, reducing the respiratory drive abolishes recovery associated with the CPP (Goshgarian, 2009; Lewis and Brookhart, 1951). Spontaneous, long-lasting recovery of phrenic activity, albeit to a limited extent, has also been observed in chronic C2Hx animals and occurs within weeks to months (Fuller et al., 2006, 2008; Mantilla et al., 2013; Nantwi et al., 1999; Sieck and Mantilla, 2009; Vinit et al., 2006). This type of plasticity is known as the “spontaneous crossed-phrenic phenomenon” (sCPP) (Fuller et al., 2008). It is unclear whether the mechanisms underlying the sCPP differ from the CPP, but recovery has been attributed to progressive actiavtion of crossed bulbospinal pathways, axonal sprouting, rerouting of bulbospinal projections (Darlot et al., 2012; Vinit et al., 2005; Vinit et al., 2011) and forming new polysynaptic connections with phrenic motoneurons via cervical spinal interneurons (Darlot et al., 2012; Fuller et al., 2009; Lane et al., 2009; Lane et al., 2008b; Sandhu et al., 2009; Zholudeva et al., 2017).

However, diaphragm recovery via sCPP has been shown to be

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dependent on plasticity of glutamatergic (Alilain and Goshgarian, 2007, 2008; Gransee et al., 2017; Mantilla et al., 2012, 2017), serotonergic (Basura et al., 2001; Fuller et al., 2005; Lee and Gonzalez-Rothi, 2017; Mantilla et al., 2012, 2017) and adenosinergic (Golder et al., 2008; Minic et al., 2017; Nantwi, 2009; Nantwi and Goshgarian, 2002) inputs to phrenic motoneurons. Glutamate is the primary excitatory neurotransmitter in the CNS and mediates respiratory synaptic inputs to phrenic motoneurons from the medulla (Alheid and McCrimmon, 2008; Chitravanshi and Sapru, 1996; Liu et al., 1990; McCrimmon et al., 1989). Different types of glutamate receptors were identified within phrenic motoneurons including ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) (Alilain and Goshgarian, 2008; Gransee et al., 2017; Mantilla et al., 2012; Robinson and Ellenberger, 1997), and metabotropic (mGluRs) (Dong and Feldman, 1999; Gransee et al., 2017) receptors. Previous studies have reported increased expression of NMDA receptors (Alilain and Goshgarian, 2008) in spinal cord tissue containing the phrenic nucleus and directly in the phrenic motoneurons (Gransee et al., 2017; Mantilla et al., 2012) following spontaneous recovery post-C2Hx. Pharmacological blocking of NMDA receptors decreased the amplitude of recovered diaphragm (Mantilla et al., 2017) with no effect on contralateral side and abolished the long-term facilitation of phrenic activity following intermittent hypoxia (McGuire et al., 2005).

A caveat with the majority of experimental studies using the C2Hx is that plasticity and recovery of phrenic and diaphragm function were evaluated under various types of anesthesia. Anesthesia suppresses respiratory drive and activity of spinal respiratory neurons by affecting multiple neurotransmitter systems, including blockade of NMDA receptors (Dickinson et al., 2007; Hara and Harris, 2002; Zorumski et al., 2016). Such adverse effects of anesthesia can significantly alter the obtained results. Therefore, the goal of the current study was to assess spontaneous phrenic motor system recovery using chronic diaphragm electromyography (EMG) recordings in awake, behaving animals following C2Hx up to 8 weeks. The results of the present work demonstrate for the first time that the extent of phrenic recovery following C2Hx may be much greater than previously appreciated.

2. Materials and methods

Female, Sprague-Dawley rats (Envigo; 230–260 g) were used for this study. 7 rats total were used for chronic recording of diaphragm EMG activity. All experiments were performed with approval from the Institutional Animal Care and Use Committee at Drexel University, and following the National Research Council's Guidelines (USA).

2.1. Diaphragm EMG electrode implantation

Diaphragm EMG electrode implantation was modified from that previously described by Mantilla (Mantilla et al., 2011). The electrodes are comprised of insulated, stranded stainless steel wires (CoonerWire, part AS631), ~20 cm long, with a small 1–2 mm uninsulated area and anchor at one end (ball of dental cement – Grip Cement, *Dentsply International Inc., #675571 and liquid #675572*). The opposite end of the wire was also uninsulated (3 mm), inserted into the cut end of a 26 5/8 gauge needle (0.45 mm × 16 mm). The proximal (unbeveled) end was crimped to fix the wire inside, and the needle was manually bent into a curve (Supplemental Fig. 1A).

For electrode implantation, all animals were anesthetized with xylazine (10 mg/kg, s.q.) and ketamine (120 mg/kg, i. p.), and received analgesia (buprenorphine; 0.03 mg/kg, s.q.). The skin overlying the rat's abdomen and skull were shaved and disinfected with antiseptics (betadine and 70% ethanol wipes) for surgery. A midline longitudinal incision was made on the head, and the skin retracted to expose the skull. Three holes were made in the skull (Supplemental Fig. 1B) using a drill (Saeyang Co). Surgical bone screws (Component Supply Co, part# SHCX-080-03) were inserted into each hole (Supplemental Fig. 1B). Silver wire was attached to one screw, which served as a ground. Then dental cement was applied over the skull and screws to create a platform for electrode connectors.

A skin incision was made along the midline of the animal's abdomen and chest to expose the abdominal and caudal-most external intercostal muscles. A median incision was made through the abdominal wall along to the linea alba to expose the abdominal surface of the diaphragm. Two electrodes (~1 cm apart) were sutured (dorsal-ventral direction) through the medial costal part of hemidiaphragm on each side for bipolar recording. The cement anchor ball at the end of each wire held the electrode in place in the diaphragm (Supplemental Fig. 1C). The needles and attached electrodes were pulled through the lateral space of xiphoideus to externalize the wires, and the abdominal muscles were sutured (4-0 Vicryl). The wires on each side of sternum were gently braided and needles removed. A loop was created from each pair of wires to avoid tension during chest movements. The free ends of the electrodes were then guided under the skin towards the head, and retrieved in front of each ear. This left the wires lateral to the neck musculature which would be later surgically exposed for C2Hx. The muscle and skin on the abdomen were sutured and closed with sterile wound clips, respectively. Wires from the EMG electrodes were connected to a 4-pin connector (Digi-key, #A104980-ND), and the silver wire that was attached to one of the three skull screws was connected to a separate 2 pin-connector. All connectors for recording electrodes and ground were then fixed to the head with dental cement (Supplemental Fig. 1D). After surgery, animals received yohimbine (1.2 mg/kg, s.q.) to reverse effect of xylazine and lactated Ringers solution (5 ml, s.q.) to prevent dehydration. Additional analgesia (buprenorphine), lactated Ringers injections (5 ml/day, s.q.) and oral dietary supplement (Nutrical; 1–3 ml) were given daily as needed.

2.2. C2Hx surgery

One week after diaphragm EMG electrode implantation, all animals received a C2Hx. The skin on the dorsal side of neck was shaved and cleaned as described above. An incision was made from the base of the skull to the shoulder blades through the skin and muscle overlying the cervical vertebrae. A partial laminectomy was done at the C2 vertebral level. An incision (~2 mm) was made in the dura mater rostrocaudally along to the midline of the dorsal spinal cord. A C2Hx was made immediately caudal to the C2 rootlets, from the midline to lateral edge of the spinal cord using a No. 11 scalpel blade. To visually confirm the extent of the injury, a fine tipped glass pipette connected to a vacuum pump was used to gently aspirate spinal tissue at the lesion site. Dura and surrounding muscles were sutured (10–0 and 4–0 sterile suture, respectively) and skin was closed using wound clips. Yohimbine (1.2 mg/kg, s.q.), lactated Ringers solution (5 ml, s.q.), and buprenorphine (0.03 mg/kg, s.q.) were given postoperatively, as described above.

2.3. Diaphragm EMG recordings

Awake diaphragm EMG recordings were made in standard rodent cages over a 10–20 min period of exploration/rest, using a CED digitizer (Cambridge Electronic Design LTD (CED), model 1401), differential AC amplifier (A-M System, Model 1700), and digital video camera (Logitech) with Spike2 (CED) software. EMG signal (10,000 samples/s) was amplified by ×1000 and filtered 100–5000 Hz. Long, flexible, insulated wires were connected to the head stage (Supplemental Fig. 1D), and the animal was allowed to move freely within the cage. These recordings allowed us to monitor diaphragm muscle activity pre-, acutely post- (< 1 h) and weekly post-C2Hx for up to 8 weeks.

2.4. Terminal phrenic nerve recordings

For terminal recordings of phrenic nerve activity, animals were