Effects of serotonergic agents on respiratory recovery after cervical spinal injury

Shih-Hui Hsu (徐詩惠)1 and Kun-Ze Lee (李昆澤)1,2,3,4,5

1Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan; 2Center for Neuroscience, National Sun Yat-sen University, Kaohsiung, Taiwan; 3Institute of Medical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan; 4Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan; and 5Doctoral Degree Program in Marine Biotechnology, National Sun Yat-sen University and Academia Sinica, Kaohsiung, Taiwan

Submitted 23 April 2015; accepted in final form 7 September 2015


Unilateral cervical spinal cord hemisection (i.e., C2Hx) usually interrupts the bulbospinal respiratory pathways and results in respiratory impairment. It has been demonstrated that activation of the serotonin system can promote locomotor recovery after spinal cord injury. The present study was designed to investigate whether serotonergic activation can improve respiratory function during the chronic injury state. Bilateral diaphragm electromyogram and tidal volume were measured in anesthetized and spontaneously breathing adult rats at 8 wk post-C2Hx or C2 laminectomy. A bolus intravenous injection of a serotonin precursor and serotonin receptor agonist (e.g., 5-methoxy-N,N-dimethyltryptamine, quipazine, m-chlorophenylpiperazine) can enhance hindlimb motor function after SCI (2, 4, 25, 32). Modulation of sensory inputs can alter the therapeutic effectiveness of cell-based therapy (35, 36). Spinal hemisection at the second cervical spinal cord (i.e., C2Hx) usually interrupts bulbospinal respiratory pathways and results in respiratory dysfunction (33, 35, 36). However, the tidal volume was not increased after administration of 5-HTP or TCB-2, indicating that the enhancement of ipsilateral diaphragm activity is not associated with improvement of the tidal volume. These results suggest that exogenous activation of the serotonergic system can specifically enhance the ipsilateral diaphragmatic motor outputs, but this approach may not be sufficient to improve respiratory functional recovery following chronic cervical spinal injury.

cervical spinal cord injury; diaphragm; serotonin; respiration

CERVICAL SPINAL CORD INJURY (SCI) usually interrupts bulbospinal pathways and results in respiratory dysfunction (33, 35, 52). Spinal hemisection at the second cervical spinal cord (i.e., C2Hx) is widely used to investigate respiratory function following cervical SCI in rodent models (16, 19, 21). This lesion causes hemidiaphragm paralysis and the cessation of phrenic nerve activity ipsilateral to the injury (23, 44, 45). However, respiratory motor outputs can be induced by robust respiratory challenges (e.g., asphyxia), or they can spontaneously recover over weeks to months postinjury without specific interventions (15, 23, 35, 45). This recovery is proposed to be mediated by activation of the latent crossed spinal pathway (21, 28). Hadley et al. (23) showed that pretreatment with a serotonin [5-hydroxytryptamine (5-HT)]-depleting drug (para-chlorophenylalanine) attenuated asphyxia-induced recovery of phrenic bursting 4 h following C2Hx (23). Spontaneous recovery of phrenic nerve activity during the chronic injury state was also significantly reduced in C2Hx animals that were pretreated with 5-HT neurotoxin (5,7-dihydroxytryptamine) (18). These results suggest that serotonin is a critical neuromodulator involved in the induction of respiratory recovery after cervical SCI.

Serotonin is synthesized from the raphe nucleus in the pons and medulla, which have axonal projections to the phrenic nucleus (6, 10, 29, 48). Accordingly, the modulatory effect of serotonin on spinal motoneuron excitability would be impaired after cervical SCI due to the interruption of raphe-spinal descending pathways. Golder and Mitchell (17) demonstrated that serotonin immunoreactivity within the phrenic nucleus was reduced after C2Hx; however, it can partially recover and correlates with intermittent hypoxia-induced phrenic long-term facilitation. Similarly, the return of serotonergic innervations of the lumbosacral ventral gray matter correlated with locomotor activity, and the treatment with a serotonin antagonist retarded locomotor recovery after thoracic hemisection (49, 50). These findings suggest that serotonin may mediate motor recovery after SCI.

Many studies have shown that pharmacological activation of the serotonin system can improve motor function after SCI. For example, administration of a serotonin reuptake inhibitor improves the recovery of forelimb function and skilled motor performance in rats that underwent bilateral crushing of the C4 spinal cord (51). Moreover, application of a serotonin precursor [i.e., 5-hydroxytryptophan (5-HTP)] or serotonin receptor agonist (e.g., 5-methoxy-N,N-dimethyltryptamine, quipazine, m-chlorophenylpiperazine) can enhance hindlimb motor function after SCI (2, 4, 25, 32). Modulation of sensory inputs can also regulate respiratory motor function after cervical SCI (13, 22). Zimmer and Goshgarian (57) and Choi et al. (8) demonstrated that activation of the serotonin 1A receptor to reduce hyperexcitability of sensory afferent inputs can enhance respiratory motor recovery following unilateral cervical SCI during both acute and chronic injury states. Zhou and colleagues (55, 56) demonstrated that phrenic bursting ipsilateral to C2Hx can be induced by treatment with a serotonin precursor and serotonin 2 receptor agonist at 1 day postinjury. However, the degree of glial scar formation and macrophage infiltration were differentially expressed between the subchronic and chronic injury states, suggesting the microenvironment of the injured spinal cord is time-dependently changed after injury (47). This alteration may influence the therapeutic effectiveness of cellu-
lar replacement and/or pharmacological therapies. In addition, respiratory motor neuroplasticity is differentially expressed across different time points postinjury (17). It remains unclear whether manipulation of the serotonin system during the chronic injury state can still improve respiratory function. Previous studies have shown that serotonin type 2A (5-HT2A) receptors within the cervical ventral horn were upregulated after cervical SCI (14), suggesting sensitivity of phrenic motoneurons to 5-HT may be enhanced during a chronic injury state. Accordingly, the present study hypothesized that acute serotonergic activation can improve respiratory functional recovery in chronic C2Hx animals. Three different approaches to activate the serotonin system were used: 1) increasing serotonin synthesis via application of a serotonin precursor (5-HTP); 2) prolonging endogenous serotonin effects via blocking the serotonin reuptake transporter using a serotonin reuptake inhibitor (i.e., fluoxetine); and 3) directly activating 5-HT2A receptors via administration of a 5-HT2A agonist [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2)].

**MATERIALS AND METHODS**

**Animals**

All experimental protocols were approved by the Institutional Animal Care and Use Committee at National Sun Yat-sen University. A total of 85 male adult Sprague-Dawley rats were purchased from the BioLasco Taiwan and were divided into the sham (C2 laminectomy only) (n = 40) and C2Hx (n = 45) groups.

**SCI**

All animals were anesthetized with xylazine (10 mg/kg sc, Rompun, Bayer) and ketamine (140 mg/kg ip, Ketalar, Pfizer) at 9-10 wk of age. After absence of a toe-pinch withdrawal reflex, the animals received an incision of dorsal skin and muscles followed by a C2 laminectomy. The left C2 spinal cord of C2Hx animals was then incised by the microscalpel, and a lesion cavity was created by the gentle aspiration using a micropipette connected to a suction pump (TOMIN). The dura was sutured with 10-0 nylon (UNIK) sutures, and the overlying muscles and skin were closed with 4-0 chromic (UNIK) and 4-0 nylon sutures (UNIK), respectively. Animals were given subcutaneous injections of yohimbine (1.2 mg/kg, Tocris) to reverse the action of xylazine, lactated Ringer solution (5 ml, Nang Kuang Pharmaceutical) to prevent dehydration, and buprenorphine (0.03 mg/kg, Shilin Sinseng Pharmaceutical) for analgesia. Manual expression of the bladder was performed until the animal recovered to urinate voluntarily. Oral supply of Nutri-cal (1–3 ml, EVSCO pharmaceuticals) and lactated Ringer solution injection (5 ml sc) were applied daily until recovery of volitional eating and drinking.

**Measurements of Cardiorespiratory Patterns**

Animals were anesthetized with urethane (1.6 g/kg ip, Sigma) at 8 wk (55.8 ± 0.2 days) after C2 laminectomy or C2Hx surgery to measure cardiorespiratory patterns before and after serotonergic agent administration. After an absence of the toe-pinch withdrawal reflex, the animal was maintained in a supine position, and the rectal temperature was monitored by an electrical thermometer and maintained at 37 ± 1°C by a servo-controlled heating pad (model T-1000, CWE). Animals were then tracheotomized, and an endotracheal tube (PE-240, Clay Adams) was connected to a respiratory flow head (MLT1L, ADInstruments) coupled with a spirometer (FE141, ADInstruments) for respiratory flow measurement. The femoral vein and artery were catheterized for serotonergic agent administration and blood pressure measurement (transducer: DTX-1; amplifier: BPM-1700, A-M Systems). Signals were processed with the rectified and bandpass filtered (0.3–10 kHz) by a differential A/C amplifier (Cambridge Electronic Design). All physiological signals were digitized using CED Power 1401 (Cambridge Electronic Design) at a sampling rate of 100 Hz (e.g., airflow, blood pressure, PETCO2) or 10 kHz (e.g., diaphragm EMG activity) and recorded in a computer by Spike 2 software.

**Serotonergic Agents**

On the day of the terminal experiment, the baseline cardiorespiratory parameters (e.g., blood pressure, heart rate, and tidal volume) and bilateral diaphragm EMG activity were recorded at least for 10 min, and a single bolus intravenous injection of serotonergic agent (5-HTP) (Sigma), 10 mg/kg (sham, n = 15; C2Hx, n = 23); fluoxetine (Matrix Scientific), 10 mg/kg (sham, n = 10; C2Hx, n = 9); TCB-2 (Tocris), 0.05 mg/kg (sham, n = 15; C2Hx, n = 13) was then applied. The cardiorespiratory and diaphragm EMG response to serotonergic agent administration was recorded for at least 60 min.

**Table 1. The cardiorespiratory pattern of sham and C2Hx animals at baseline**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>C2Hx</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Frequency, breaths/min</td>
<td>125 ± 4</td>
<td>138 ± 3*</td>
</tr>
<tr>
<td>Tidal volume, ml</td>
<td>2.2 ± 0.0</td>
<td>1.7 ± 0.0*</td>
</tr>
<tr>
<td>PETCO2, Torr</td>
<td>52.5 ± 1.1</td>
<td>56.7 ± 1.2*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>83 ± 2</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>348 ± 6</td>
<td>345 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. C2Hx, spinal hemisection at the second cervical spinal cord; PETCO2, end-tidal partial pressure of CO2. The sample size for PETCO2 measurement is 39 sham and 43 C2Hx animals. *p = 0.01, †p < 0.01 compared with the sham animal.
Spinal Cord Histology

Spinal cord histology was conducted as previously described (34, 52). The C2 spinal cord was cryoprotected and transversely sectioned into 40-μm slices (CM 1850, Leica) and mounted on glass slides, stained with the cresyl violet (Acros Organics), and examined under a microscope (DM750, Leica). The photograph of spinal cord slices was taken with a digital camera (EOS 600D, Canon) connected to the microscope. The area of spared dorsal and ventral lateral white matter tissue at the lesion epicenter was manually outlined using Image J software and expressed as a percentage of the contralateral [i.e., uninjured side, percent contralateral (%CL)], dorsal, and ventral lateral white matter area, respectively (34). A representative example of cervical spinal cord hemisection is presented in Fig. 1.

Data Analysis and Statistics

The cardiorespiratory and bilateral diaphragm EMG parameters before and after the administration of serotonergic agents were calculated by Spike 2 script. The tidal volume and respiratory cycle duration of individual breath were calculated from respiratory airflow trace data. Specifically, the inspiratory duration (TI) is defined as the period when the flow is below the zero, while the expiratory duration (TE) is defined as the period when the flow is above the zero. The respiratory frequency was calculated as 60/(TI + TE). The tidal volume is derived from the integration of inspiratory flow using Spike 2 script. The inspiratory diaphragmatic activity was defined as the difference between the maximal and minimal value of the rectified and smoothed EMG signals within an individual breath. The EMG data were expressed in microvolt (mV) and normalized as a percentage of the baseline (%BL). The background activity of the diaphragm was defined as the minimum value of the rectified and smoothed EMG signals during TE and was expressed in mV. All physiological parameters were averaged over 30 s at 1, 3, and 5 min before (i.e., baseline), and 30 s, and 5, 15, 30, and 60 min after drug administration. The baseline cardiorespiratory patterns and diaphragm EMG activity were obtained by averaging three data points before drug administration.
The baseline cardiorespiratory parameters between sham and C2Hx animals were compared using a two-tail unpaired t-test. Bilateral diaphragm EMG activity during the baseline was analyzed by a two-way mixed-design analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc test [factor one: animal group (sham vs. C2Hx); factor two: right vs. left diaphragm]. The cardiorespiratory responses and diaphragm EMG activity before and after serotonergic agent administration were analyzed by a two-way mixed-design ANOVA followed by Student-Newman-Keuls post hoc test [factor one: animal group (sham vs. C2Hx); factor two: time points]. To further demonstrate differential effects of different 5-HT agents, a series of box-and-whisker plots was used to illustrate the response of tidal volume and bilateral diaphragm EMG activity at 5 and 15 min postdrug administration.

All data are expressed as means ± SE. A P value < 0.05 was considered statistically significant.

RESULTS

Spinal Cord Histology

A representative example of hemisected spinal cord section was provided in Fig. 1. The extent of the spared ventral lateral spinal white matter tissue ipsilateral to the lesion was only 1.0 ± 0.4% CL, suggesting nearly completely lesioned. However, a moderate level of the dorsal column remained preserved (50.4 ± 4.4% CL) in our C2Hx model.

Respiratory Motor Output following Chronic Cervical SCI

At chronic injury state (i.e., 8 wk postinjury), C2Hx animals had a higher respiratory frequency and lower tidal volume than sham animals (P = 0.01, Table 1). Moreover, PetCO2 was higher in C2Hx animals compared with sham animals (P = 0.01, Table 1). Mean arterial blood pressure and heart rate were similar between two groups (P > 0.05, Table 1). The bilateral diaphragm EMG activity of sham animals exhibited robust respiratory activity during baseline (Fig. 2A). The burst amplitude of EMG signals was similar between the contralateral (i.e., right, 0.60 ± 0.05 mV) and ipsilateral (i.e., left, 0.61 ± 0.06 mV) diaphragm in sham animals. C2Hx animals also demonstrated a pronounced inspiratory bursting in the contralateral diaphragm (0.75 ± 0.07 mV), which was slightly higher than that in sham animals (0.60 ± 0.05 mV) (P = 0.045). However, inspiratory activity of ipsilateral diaphragm EMG was only detected in 51% (19/37) of C2Hx animals, and the rest of the animals (18/37, 49%) did not exhibit stable and obvious inspiratory bursting during baseline. The amplitude of ipsilateral diaphragm EMG signals was significantly reduced compared with the contralateral diaphragm in C2Hx animals (ipsilateral: 0.02 ± 0.01 mV; contralateral: 0.75 ± 0.07 mV, P < 0.01) and the ipsilateral diaphragm in sham animals (0.61 ± 0.06 mV, P < 0.01).

Influence of Serotonergic Agents on Cardiorespiratory Response

5-HTP. Representative examples of cardiorespiratory pattern and diaphragm EMG in response to intravenous injection of 5-HTP are shown in Fig. 2. 5-HTP administration did not induce significant changes in the respiratory frequency (Fig. 3A, open circles) and tidal volume (Fig. 3B, open circles) of sham animals; however, respiratory frequency of C2Hx animals significantly decreased from 134 ± 6 breaths/min during baseline to 120 ± 5 breaths/min at 5 min and 123 ± 4 breaths/min at 60 min after 5-HTP treatment (P < 0.01, Fig. 3A, shaded circles). This reduction in respiratory frequency was not associated with an alteration of tidal volume (Fig. 3B,

![Image](http://jap.physiology.org/) by 10.220.33.6 on July 9, 2017)
shaded circles). Injection of 5-HTP caused a transient reduction in blood pressure from 84 ± 4 to 69 ± 4 mmHg in sham animals (P < 0.01, Fig. 3C, open circles), but induced a persistent hypotension in C2Hx animals (P < 0.01, Fig. 3C, shaded circles). Specifically, the mean arterial blood pressure decreased from 77 ± 3 mmHg during baseline to 51 ± 3 mmHg at 60 min after 5-HTP administration. The heart rate is similar between sham and C2Hx animals during baseline, but sham animals showed a persistent tachycardia from 5 min (357 ± 13 beats/min) to 60 min (364 ± 9 beats/min) in response to 5-HTP administration (P < 0.01, Fig. 3D, open circles).

Bilateral diaphragm EMG activity of sham animals did not show significant changes after intravenous 5-HTP administration (Fig. 4, open circles). However, 5-HTP injection resulted in a substantial increase in inspiratory activity of the ipsilateral diaphragm in C2Hx animals (P < 0.01, Fig. 4B, shaded circles). Specifically, ipsilateral diaphragm EMG activity increased from 0.02 ± 0.01 mV (i.e., 100% BL) to 0.16 ± 0.04 mV (i.e., 826 ± 302% BL) immediately and was maintained at 0.07 ± 0.02 mV (i.e., 532 ± 216% BL) at 60 min after 5-HTP treatment. In addition, the background activity of ipsilateral diaphragm was recruited following 5-HTP injection in C2Hx animals (Fig. 4D, shaded circles).

**Fluoxetine.** Figure 5 demonstrates the cardiorespiratory pattern and diaphragm EMG before and after the intravenous injection of fluoxetine. Respiratory frequency (Fig. 6A) was not significantly altered after fluoxetine treatment, but the tidal volume was immediately increased from 2.2 ± 0.0 to 2.5 ± 0.0 ml in sham animals (P < 0.01, Fig. 6B, open circles) and from 1.9 ± 0.1 to 2.2 ± 0.1 ml in C2Hx animals (P < 0.01, Fig. 6B, shaded circles). The tidal volume returned to the baseline level within 15 min postinjection in both groups. Fluoxetine also caused initial hypotension (sham: 61 ± 4 mmHg; C2Hx: 59 ± 3 mmHg), followed by rebound hypertension in sham and C2Hx animals (Fig. 6C). Significant bradycardia was also observed in C2Hx animals at 30 s (323 ± 9 beats/min) and 5 min (316 ± 9 beats/min) following fluoxetine administration (P < 0.05, Fig. 6D, shaded circles).

The intravenous administration of fluoxetine did not significantly influence bilateral diaphragm EMG activity in sham animals (Figs. 7, open circles). Despite the fact that tidal volume was transiently increased in C2Hx animals (Fig. 6B, shaded circles), the inspiratory activity of the contralateral diaphragm was significantly attenuated following fluoxetine treatment (Fig. 7A, shaded circles). Unlike the excitatory effect of 5-HTP on ipsilateral diaphragm EMG activity, fluoxetine did not significantly modulate the inspiratory or background activity of the ipsilateral diaphragm in C2Hx animals (Figs. 7, B and D, shaded circles).

**TCB-2.** Representative examples depicting the influence of TCB-2 administration on the cardiorespiratory pattern and diaphragm EMG are shown in Fig. 8. Respiratory frequency was immediately reduced from 140 ± 5 to 126 ± 6 breaths/min in C2Hx animals after TCB-2 injection (P < 0.01; Fig. 9A, shaded circles). Tidal volume was transiently reduced from 2.3 ± 0.1 to 1.9 ± 0.1 ml in sham animals (P < 0.01; Fig. 9B, open circles) and from 1.8 ± 0.0 to 1.4 ± 0.1 ml in C2Hx animals (P < 0.01; Fig. 9B, shaded circles) following TCB-2 administration. TCB-2 injection also caused a significant increase in blood pressure and heart rate in both groups of animals (Figs. 9, C and D). Specifically, the mean arterial blood pressure immediately increased from 83 ± 4 to 113 ± 4 mmHg in sham animals (P < 0.01; Fig.
9C, open circles) and from 80 ± 3 to 110 ± 4 mmHg in C2Hx animals (P < 0.01; Fig. 9C, shaded circles). The heart rate also significantly increased from 364 ± 12 beats/min during baseline to 404 ± 12 beats/min at 5 min after TCB-2 administration in sham animals (P < 0.01; Fig. 9D, open circles). Similarly, C2Hx animals also showed an increase in heart rate from 334 ± 11 beats/min during baseline to 379 ± 11 beats/min at 5 min post-drug administration (P < 0.01; Fig. 9D, shaded circles). The tachycardiac effect of TCB-2 can persist for at least 60 min (sham: 390 ± 9 beats/min; C2Hx: 360 ± 9 beats/min) after drug administration (P < 0.01, Fig. 9D).

The inspiratory activity of the bilateral diaphragm EMG in sham animals was not altered within 30 min post-TCB-2 administration (Figs. 10, A and B, open circles). The background activity of bilateral diaphragm EMG activity in sham animals was very weak [contralateral (right): 0.01 ± 0.00 mV; ipsilateral (left): 0.02 ± 0.00 mV] during baseline. However, it significantly increased to 0.04 ± 0.01 mV in contralateral diaphragm EMG and 0.05 ± 0.01 mV in ipsilateral diaphragm EMG at 15 min postinjection (P < 0.01, Figs. 10, C and D, open circles) and remained higher than the baseline value at 60 min postinjection (contralateral: 0.03 ± 0.01 mV; ipsilateral: 0.03 ± 0.01 mV) (P < 0.05, Figs. 10, C and D).

Both the inspiratory and background activity of the ipsilateral diaphragm were robustly enhanced after TCB-2 treatment in C2Hx animals. Specifically, inspiratory activity was increased from 0.02 ± 0.01 mV during baseline to 0.20 ± 0.04 mV (i.e., 524 ± 199% BL) at 5 min postinjection (P < 0.01, Fig. 10B, shaded circles). The augmented inspiratory diaphragm activity can persist for up 30 min (i.e., 0.18 ± 0.03 mV; 500 ± 132% BL) after injection of TCB-2 (P < 0.01, Fig. 10B, shaded circles). Background activity was recruited and reached to a significant level (0.03 ± 0.01 mV) at 30 min postinjection (P < 0.05, Fig. 10D, shaded circles).
To demonstrate effects of different 5-HT agents on the diaphragm EMG activity and tidal volume, a series of box-and-whisker plots was illustrated in Fig. 11. Contralateral diaphragm EMG activity was not substantially influenced by three types of 5-HT agents in both sham and C2Hx groups (Fig. 11A). However, ipsilateral diaphragm EMG activity was robustly enhanced by 5-HTP and TCB-2 in C2Hx animals (Fig. 11B). Animals from both sham and C2Hx groups have a stable tidal volume after administration of 5-HT agent (Fig. 11C). These data indicated that activation of serotonin system by 5-HTP and TCB-2 has a greater impact on the ipsilateral diaphragm EMG activity than the effect of endogenous serotonergic activation via fluoxetine.

**DISCUSSION**

The present study demonstrated that supplementation with a serotonin precursor or direct activation of 5-HT2A receptors can significantly enhance diaphragm activity ipsilaterally to hemisection during the chronic injury state. However, augmentation of endogenous serotonin by a serotonin reuptake inhibitor did not improve recovery of the diaphragmatic motor output. In addition, enhancement of ipsilateral diaphragm activity following administration of 5-HTP or TCB-2 was not accompanied by a recovery of tidal volume. These results suggested that acute effects of serotonergic activation by a single bolus injection of 5-HTP and TCB-2 can restore partial diaphragm activity, but was not sufficient to provide functional improvement in tidal volume at the chronic stage of cervical SCI.

**Critique of Methods**

Several concerns regarding our experimental approach should be discussed. First, the present experiment was performed in anesthetized animals. Although urethane has been widely used as an anesthetic for physiopharmacological studies (42), a previous report indicated that urethane can influence several neurotransmitter-gated ion channels (24). Therefore, 5-HT agent-induced alterations in the cardiorespiratory pattern and diaphragm EMG activity may be complicated by urethane anesthesia. Second, serotonergic agents were delivered through the systemic injection in the present study. Because the serotonin system is widely distributed in the central and peripheral nervous system, we suspected that alteration of the cardiorespiratory pattern and diaphragm EMG following serotonergic activation could be due to the action of 5-HT agents on the supraspinal, spinal level, sensory afferents, and peripheral tissue (26). Since a moderate portion of dorsal funiculus was preserved in the present study, the modulatory effect of 5-HT agents on the cardiorespiratory function may be partially mediated by alteration of sensory inputs following drug administration (8, 57). Third, there are at least seven subfamilies of serotonin receptors (26, 31). Effects of 5-HTP and fluoxetine on respiratory motor outputs and cardiovascular responses could result from combinatorial activation of multiple serotonin receptor subtypes. Fourth, respiratory motor outputs were measured in the spontaneously breathing animal model. Therefore, changes of breathing pattern following drug administration may in turn indirectly influenced the cardiorespiratory pattern and diaphragm EMG activity. Fifth, most animals received the laparotomic surgery for diaphragm EMG record-
ing, which can change the respiratory mechanics (58). Accordingly, we cannot exclude the possibility that the laparotomy may affect the breathing pattern in response to serotonergic agent administration.

**Respiratory Motor Outputs following Chronic Cervical Spinal Cord Hemisection**

C2Hx animals exhibited a lower tidal volume compared with sham animals in previous studies (12, 19, 34–35). The decreased tidal volume can attenuate the Hering-Breuer inflation reflex and induce a disinhibitory effect on respiratory frequency. In addition, the lower tidal volume also reduced the efficiency of gas exchange and then caused a slight elevation of PETCO2, which in turn activated central and/or peripheral chemoreceptors. These secondary effects of reduced tidal volume may induce a compensatory increase in the respiratory frequency and cause a rapid shallow breathing pattern in C2Hx animals.

Previous studies have demonstrated that unilateral hemisection at high cervical spinal cord causes hemidiaphragm paralysis (7, 44, 53). During the subchronic to chronic injury state, the ipsilateral diaphragm or phrenic nerve activity can spontaneously and gradually recover due to activation of a latent crossed phrenic pathway (7, 35, 54). This concept is supported by the current experiment showing that a subset of C2Hx animals demonstrated a weak inspiratory activity during baseline conditions. However, some animals did not exhibit detectable inspiratory bursting in the ipsilateral diaphragm. We speculated that this phenomenon could be due to use of a vagal-intact animal model. Our laboratory’s previous study revealed that bilateral vagotomy significantly enhanced the ipsilateral phrenic nerve activity in chronic C2Hx animals, suggesting that vagal afferent inputs constrains activation of the crossed phrenic pathway (37). We also noticed that there is a compensatory increase in contralateral diaphragm activity in C2Hx animals. The augmentation of contralateral diaphragm activity is considered a type of compensatory respiratory plasticity (30), which may be able to sustain essential ventilation after cervical spinal injury.

**Influence of Serotonergic Agents on Respiratory Motor Outputs following Cervical SCI**

5-HTP. Our current results demonstrated that the intravenous injection of 5-HTP did not produce substantial effects on the cardiorespiratory pattern or bilateral diaphragm EMG in sham animals. However, the same dose of 5-HTP induced a significant reduction in respiratory frequency and blood pressure, as well as a robust increase in ipsilateral diaphragm bursting in C2Hx animals, suggesting that sensitivity to 5-HTP is elevated following chronic spinal injury. It is well-established that 5-HTP is converted to 5-HT via the action of aromatic l-amino acid decarboxylase (AADC). A recent report demonstrated that SCI induced an upregulation of AADC in spinal neurons, vessel endothelial cells, and pericytes (38), which can synthesize 5-HT from exogenously applied 5-HTP. Furthermore, the expression of spinal 5-HT2 receptors is increased after spinal injury (14, 43). Taken together, hypersensitivity to 5-HTP following SCI could result from the upregulation of AADC and/or 5-HT receptors.

The inhibitory influence of 5-HTP on respiratory frequency in C2Hx animals could result from activation of 5-HT receptors in supraspinal regions. This concept was supported by

![Fig. 7. Effects of fluoxetine on the bilateral Dia activity in sham and C2Hx animals. Inspiratory (A and B) and background (C and D) activity of the Dia (CL (A and C), IL (B and D)) were expressed in mV. Open circles, sham animals; shaded circles, C2Hx animals. Values are means ± SE. ††P < 0.01, significant difference in the averaged value of three time points during the BL between sham and C2Hx animals. *P < 0.05 vs. BL. ##P < 0.01: significant difference between sham and C2Hx animals after drug administration.](image-url)
previous studies showing that 5-HTP-induced respiratory depression was blocked by central inhibition of AADC (3), but it was not attenuated by a peripheral inhibitor of AADC or section of vagus nerves and glossopharyngeal nerves (39). Despite the fact that respiratory frequency was reduced following 5-HTP injection, both the inspiratory and background activity of the ipsilateral diaphragm were robustly enhanced. Because contralateral diaphragm activity did not show detectable changes, we proposed that the excitatory effect of 5-HTP on ipsilateral phrenic motor outputs is specifically at the spinal level, but is not due to an increase in central respiratory drives. It has been shown that phrenic motoneurons express 5-HT2A, 5-HT2B, and 5-HT7 receptors (27, 40). Accordingly, the excitatory effect of 5-HTP on ipsilateral phrenic outputs could be mediated by the activation of these receptors. However, we cannot exclude the possibility that 5-HTP may activate 5-HT receptors in the central respiratory networks (46), which may specifically enhance crossed phrenic pathways to increase phrenic outputs ipsilateral to the lesion.

Fluoxetine. Although 5-HTP induced a significant increase in ipsilateral diaphragm activity in C2Hx animals, this effect was not observed following fluoxetine treatment. Golder and Mitchell (17) demonstrated that serotonergic innervations of the phrenic nucleus were reduced by cervical spinal hemisection. Although it can gradually recover during the chronic injury state, the innervation intensity remained lower than that in the sham animals (17). In addition, a serotonin reuptake transporter was depleted after SCI (25). Accordingly, the less pronounced effect of fluoxetine on the diaphragm EMG in the present study could be due to insufficient endogenous 5-HT and/or the downregulation of serotonin reuptake transporter following cervical SCI.

TCB-2. TCB-2 is a highly potent 5-HT2A receptor agonist; therefore, changes in the cardiorespiratory response following

Fig. 8. Representative examples of the cardiorespiratory pattern and Dia EMG activity before and after intravenous injection of TCB-2 in a sham (A) and C2Hx (B) rat. TCB-2 recruited bursting in the IL Dia EMG in C2Hx animal but did not significantly influence inspiratory bursting of the bilateral Dia EMG in sham rat. The abbreviations are the same as those defined in Fig. 2 legend. The unit for the Dia EMG is mV. Please note that the axes scales of IL Dia are different between A and B.
TCB-2 administration primarily result from the activation of 5-HT\textsubscript{2A} receptors, which are widely distributed in the respiratory circuit, including brain stem, pons, and spinal cord (46). Prior studies have shown that 5-HT\textsubscript{2A} receptor expression on phrenic motoneurons was upregulated after cervical spinal hemisection (14, 43). As a result, the effect of TCB-2 on phrenic bursting may be potentiated in spinal-injured animals. Current data supported this concept by demonstrating that the inspiratory activity of the ipsilateral diaphragm was robustly enhanced after TCB administration. In addition, the background activity of the bilateral diaphragm was elevated by TCB-2 treatment in both groups of animals. These findings suggest that activation of 5-HT\textsubscript{2A} receptors can generally enhance motoneuron excitability and in turn augment excitatory synaptic inputs.

Unlike the hypotensive effect of 5-HTP and fluoxetine, administration of TCB-2 caused significant hypertension and tachycardia in both groups. Maeshima et al. (41) demonstrated that 5-HT\textsubscript{2A} receptors are expressed in sympathetic preganglionic neurons in the intermediolateral nucleus of the thoracic spinal cord. Systemic administration of a 5-HT\textsubscript{2A} receptor agonist caused a sympathetically mediated vasoconstriction (5). Moreover, intrathecal administration of a 5-HT\textsubscript{2A} agonist caused a pressor response (9). Accordingly, the excitatory effect of TCB-2 on the blood pressure and heart rate in our study may be attributed to sympathetic excitation.

**Physiological Significance and Clinical Implications**

Zhu and colleagues (55, 56) demonstrated that serotonergic activation by administration of a serotonin precursor and 5-HT\textsubscript{2A} receptor agonist can recruit previously quiescent phrenic nerve activity ipsilateral to C2Hx at 1 day postinjury in female rats under vagotomized conditions. In addition, systemic activation of 5-HT\textsubscript{1A} receptor can improve phrenic motor outputs in vagotomized spinal-injured rats at 16 wk post-C2Hx (57). The present study extended these observations showing that serotonergic activation improved ipsilateral diaphragm activity in vagal-intact male rats during the chronic injury state. Although these studies indicated that serotonergic activation contributes to respiratory recovery, several different aspects of physiological significance should be discussed. First, Zhou et al. (55, 56) indicated that activation of the serotonin system can improve ipsilateral phrenic bursting during the acute injury state; however, most patients with SCI are in subchronic to chronic stages, so it is essential to develop a therapy that has beneficial effects on respiratory function during this time window. The present study demonstrated that administration of 5-HTP or TCB-2 can enhance diaphragm activity or recruit previously silent diaphragm EMG at 8 wk postinjury, suggesting that activation of the serotonin system during the chronic injury state is still valid. Second, female hormones can contribute to the respiratory recovery following cervical spinal injury (11). Therefore, influence of serotonergic activation on respiratory recovery may be distinct between female vs. male animals. Since most patients with SCI are males (1), it is valuable to evaluate the effects of serotonergic agents on male animals. Our experiments showed that the diaphragm activity of male animals can respond to 5-HTP and TCB-2 treatment, suggesting that serotonergic activation can improve phrenic outputs, regardless of sex. Third, a prior report indicated that vagal inputs restricts the expression of crossed phrenic pathways (37); thus the excitatory effects of serotonergic agents on ipsilateral phrenic motor outputs of C2Hx animals may be overestimated in the vagotomized ani-
Serotonin Activation Restores Diaphragm Activity • Hsu SH et al.

A

B

C

D

Fig. 10. Impact of TCB-2 on the bilateral Dia activity in sham and C2Hx animals. Inspiratory (A and B) and background (C and D) activity of the Dia [CL (A and C), IL (B and D)] were expressed in mV. Open circles, sham animals; shaded circles, C2Hx animals. *P < 0.05; **P < 0.01 vs. BL. #P < 0.05; ##P < 0.01: significant difference between sham and C2Hx animals after drug administration.

mal model. The present study observed that the diaphragm activity of vagal-intact C2Hx animals can still be augmented following injection of 5-HP and TCB-2, indicating that the excitatory effects of serotonergic activation on respiratory outputs can override the inhibitory influence of vagal inputs. Taken together, our results suggest that modulation of the serotonergic system during the chronic injury state may be considered a potential therapeutic strategy to improve respiratory function following cervical spinal injury.

Indicator of Therapeutic Effectiveness on Respiratory Functional Recovery

Although the present study demonstrated that ipsilateral diaphragm activity could be increased, the tidal volume did not significantly improve after administration of 5-HTP and TCB-2. This finding suggests that serotonergic activation-induced enhancement of diaphragm activity is not sufficient to contribute to tidal volume recovery. The long-term administration of serotonergic agents or their combination with other therapies (e.g., intermittent hypoxia, cellular transplantation) (20, 36) may be necessary to induce significant improvements in respiratory function. In addition, it may be warranted for future studies to measure multiple indicators of respiratory motor outputs (e.g., phrenic nerve, diaphragm and tidal volume) to evaluate the therapeutic effectiveness of potential treatments.

GRANTS

Support for this work was provided by grants from the National Health Research Institutes (NHRI-EX104-10223NC), Ministry of Science and Technology (Most 102–2320-B-110-004-MY3), and National Sun Yat-sen University–Kaohsiung Medical University Joint Research Project (2014-I006).

REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Fig. 11. The box-and-whisker plot demonstrating alteration of CL (A) and IL (B) Dia EMG activity and VT (C) following administration of 5-HT agents. Data were expressed as a percentage of BL. The solid dot and band in the box represent the mean and median value, respectively. The top and bottom of the box are the third and first quartiles, respectively. The end of the top and bottom whisker are the 90th and 10th percentiles, respectively.


