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TITLE: Activation of Central Pattern Generator for Respiration Following Complete High Cervical Spinal Cord Interruption

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| 14. ABSTRACT<br>Consistent with the primary goal of the project, we identified the optimal paradigm for epidural stimulation (EDS) to drive paced breathing following complete high cervical (C1) spinal cord injury (SCI) in adult rats. High-frequency EDS of the C4 cervical (HF-C4-EDS) segment (the center of the phrenic motor pool and primary spinal inspiratory control) in combination with pharmacological (intrathecal) disinhibition of GABAa- and glycinergic circuits were able to maintain stable paced breathing and diaphragm muscle contraction in transected animals. Application of GABAa and glycine (inhibitory) receptors blockers (GABAzine and strychnine, respectively) in subthreshold concentrations significantly increased the tidal volume, amplitude of diaphragm electromyography and phrenic nerve activity in response to HF-C4-EDS. Our anatomical immunohistological studies confirmed a presence of distinctive population of GABAa and glycinergic pre-phrenic interneurons located in III-IX laminae of the spinal cord alongside other pre-phrenic interneurons. Electrophysiological experiments with Intraspinal single neuron recordings have shown that application of GABAzine and strychnine increased spiking frequency in late inspiratory interneurons and motoneurons. These neurons could serve as an effective target for HF-C4-EDS and pharmacological neuromodulation for paced breathing following SCI. |                         |                                               |

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## 1. INTRODUCTION:

### INTRODUCTION

Cervical spinal cord injury (SCI) results in a vast range of motor and sensory deficits, among which impaired breathing is one of the most devastating (1). Cervical injuries compromise descending respiratory drive from the brainstem to spinal respiratory motoneurons, including the phrenic motoneurons (PMNs). Impaired phrenic function results in paresis or paralysis of the diaphragm – the primary muscle of respiration. Around 13.3% of tetraplegic patients (~2,300 new cases yearly in USA) with high cervical (C1-C4) spinal cord injury (SCI) require long-term ventilation support (2). Mechanical ventilation, while an important and necessary for life support, contributes to disuse atrophy of the diaphragm, affects venous return, increases the risk of respiratory infections and decreases life expectancy. Thus, weaning from ventilator dependency is the primary clinical goal for these patients.

Electrical stimulation of phrenic nerve and diaphragm are among those methods being increasingly used in clinic (3, 4, 5, 6, 7, 8). Epidural stimulation (EDS) is an alternative neural interfacing strategy that has been explored extensively for non-respiratory functions (9, 10). Effectiveness of EDS for pacing of respiratory muscles was demonstrated by DiMarco et al. (11, 12, 13). Authors showed that Th2-Th3 low frequency (15 – 40 Hz) EDS in dogs was effective for life-supporting breathing, even after bilateral phrenicotomy. However, the first clinical trial with EDS for quadriplegics with bilaterally compromised phrenic nerves yielded little success (14). More successful EDS-pacing of intercostal muscles combined with diaphragm pacing was achieved in patients with at least one phrenic nerve function (15). In recent publications DiMarco and Kowalski (16) described effective high-frequency EDS (HF-EDS) (100-300 Hz) applied at Th2-Th3 level (16, 17).

A spinal origin of respiratory rhythm generation first was proposed in the nineteenth century (18-21), when breathing was observed following experimental spinal transection (Tx) in cats and dogs. Since those initial experiments, several studies have demonstrated persistence of spontaneous respiratory activity following high cervical transection (22-26), although with some variability seen between species and lesion levels. One key finding from these experiments was that preservation of high cervical spinal cord (C1-C2) may be essential for eliciting spinal respiratory rhythm. This conclusion has been reached following in vivo experiments (27), and several studies that have investigated the contribution of upper (C1-C2) cervical respiratory interneurons (or the ‘upper cervical respiratory group’, UCRG) to modulation of respiratory function. It has been proposed that UCRG may provide excitation and inhibition to inspiratory and expiratory MNs respectively (28-29, 30).

Pharmacologically induced respiratory activity has been demonstrated in spinal animals by systemic administration of monoaminergic agonists (5HT, DOPA, Nialamide) and GABA<sub>A</sub>/glycine antagonists (bicuculline & picrotoxin/strychnine) (19-22, 26, 27, 30, 31-33). While GABA<sub>A</sub>- and Glycinergic fast inhibitory mechanisms are known to shape spinal respiratory outputs (34-38), and impact respiratory recovery follow SCI (39-40), no efforts has been made to explore whether inhibitory receptors may serve as therapeutic targets. Taking into account these observations we proposed that intraspinal inhibitory circuits play a key role in control of respiratory MNs under normal and pathological (SCI) conditions, and serve as an ideal target for therapeutically amplifying respiratory activity.

An important consideration is that the majority of previous studies with EDS have been pursued in anesthetized animals (11, 13, 16). However, it is well known that anesthesia suppresses respiratory function (41, 42) and activity of spinal neurons (43, 44) that may affect experimental data and interpretation of results. Therefore, in the present project we have used decerebrate

C1Tx preparation that allowed for experiments in unanesthetized animals. Furthermore, using unanesthetized animals can reveal effects of EDS that might not be seen under anesthesia, such as off target (locomotor) muscle contractions.

While upper-thoracic EDS has been the focus of previous studies, stimulation of cervical segments that correspond to location of the phrenic motor nucleus (C3-C5/C6) innervating the diaphragm has not been investigated in a model of complete SCI. As the diaphragm is the primary inspiratory muscle, contributing about 88% of inspiratory effort during rest and ~ 75% during forced breathing (45, 46, 47), the present project explored whether mid-cervical EDS represents an effective therapeutic target.

In addition, it has been demonstrated that EDS and subdural injection of drugs as neuromodulation tools are widely used in the treatment of pain syndrome and reduction of muscle spasticity in patients with SCI (L).

Therefore, the primary goal of the current project is to build upon these prior works and assess the potential for EDS in combination with pharmacological blocking of spinal fast inhibitory synaptic transmission (blocking of GABA<sub>A</sub> and glycine inhibitory receptors) for more specific pacing of spinal interneurons and motoneurons involved in the shaping of respiratory motor output following C1 transection (C1Tx). To test this hypothesis, we have proposed the following aims:

Specific Aim 1: Identify the location and neurotransmitter profile of spinal inhibitory interneurons related to respiratory motor control and initiating of spinal CPG follow C1-transection.

Specific Aim 2: Identify the optimal combination of intrathecally delivered blockers of fast inhibition (Glycine- and GABA-ergic) and epidural electric stimulation for improving respiratory recovery follow C1-transection

This final report summarizes all the data generated throughout the grant period.

## 2. KEYWORDS:

Spinal cord injury, epidural stimulation, paced breathing, phrenic nucleus, phrenic nerve, diaphragm, disinhibition, GABA, glycine

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

**What were the major goals of the project?**

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

**Aim1: Identify the location and neurotransmitter profile of spinal inhibitory interneurons related to respiratory motor control and initiating of spinal central pattern generator (CPG) follow complete spinal cord transection at C1 cervical level (C1Tx).**

*The Aim1 contains two major tasks:*

**Task 1.1 Characterize C1-C5 respiratory-related neurons and their responses to microinjection of GABA<sub>A</sub>zine, Strychnine, and GABA<sub>A</sub>zine+Strychnine with following juxtacellular labeling and identification of neurotransmitter profile.**

**Task 1.2: Characterize the location neurotransmitter profile (GABA-, Glycine- or Glutamate-ergic) of transsynaptically labeled spinal interneurons connected to phrenic motoneurons.**

**Aim2: Identify the optimal combination of intrathecally delivered blockers of fast inhibition (Glycine- and GABA-ergic) and epidural electric stimulation for improving respiratory recovery follow C1Tx.**

*The Aim2 contains three major tasks:*

**Task 2.1: Identify optimal parameters of epidural stimulation of C1-C5 (C2, C4) ventrolateral aspects with additional bilateral microinjections into phrenic nucleus (C3-C6) and upper cervical respiratory group (C1-C2) of GABA<sub>A</sub>zine, Strychnine, GABA<sub>A</sub>zine+Strychnine in decerebrate artificially ventilated, paralyzed and C1-transected animals.**

**Task 2.2: Identify the optimal combination of epidural stimulation of C1-C5 (C2, C4) ventrolateral aspects and intrathecal application of GABA<sub>A</sub>zine, Strychnine and GABA<sub>A</sub>zine+Strychnine in decerebrate artificially ventilated, paralyzed and C1-transected animals.**

**Task 2.3: Identify the optimal combination of epidural stimulation of C1-C5 (C2, C4) ventrolateral aspects and intrathecal application of GABA<sub>A</sub>zine, Strychnine and GABA<sub>A</sub>zine+Strychnine in decerebrate artificially ventilated, non-paralyzed and C1-transected animals.**

### **What was accomplished under these goals?**

*For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

For **Major Task 1.1**, we have described a heterogenic population of respiratory interneurons at the level of the phrenic nucleus (C3-C5 cervical segments) and upper respiratory group (C1-C2 cervical segments) as a highly-organized, local spinal network innervating the spinal phrenic network.

A total of 295 different respiratory motor- and interneurons were recorded from C1-C5 cervical segments in decerebrated intact (Fig.1-A1-C3) and C1Tx (Fig.1-D1-D3) rats. Motor- and interneurons were identified by the presence or absence of antidromic response to phrenic nerve (PN) stimulation. These neurons were categorized by their firing pattern and location (Fig.2-3).

We have found the higher number of inspiratory augmenting (Insp-Aug, Fig.1-A1) motoneurons (MNs) at the center of phrenic nucleus (PNucl) location (C4, n=13) compared to C3 (n=5) and C5 (n=7) segments (see Fig.2-A for labeled motoneuron [Streptavidin-Alexa-610 was used for neurobiotin detection]). However, the statistical analysis did not detect a difference in the distribution of Insp-Aug MNs between C3-C4-C5 segments (Fig.4-A-dark brown bars). The late inspiratory (late-Insp, Fig.1-A2, Fig.4A, orange bars, n=14) and early inspiratory MNs (early-Insp, Fig.4-A, light green bars, not shown on Fig.1, n=4) were equally distributed throughout the C3-C5 segments. The topographical location of different MNs is shown in Fig. 2 (Insp-Aug – black circles, late-Insp – black diamonds and early-Insp – black crosses). We also identified late-inspiratory MNs, which are distributed slightly dorso-lateral to the tightly clustered PNucl (outlined with red line areas on panels C3-C5 in Fig.2). The bimodal distribution of phrenic motoneurons was confirmed with retrograde monosynaptic tracers (Fast Blue - see Fig.2-B, Texas red and Cholera Toxin subunit B).

We have shown that inspiratory, expiratory and phase spanning interneurons (INs) are widely distributed from C1 to C5 segments (Fig.2-3). The majority of inspiratory INs are present as inspiratory units with tonic (background) activity (Insp+BG) (Fig.1-A3 and Fig.2-3 – black quadrants, n=62). We also found that Insp+BG INs are located mostly dorso-medial to motoneuron pools (outlined with pink line areas on panels C3-C5 in Fig.2). However, some were located in close proximity to phrenic motoneurons. The overall distribution of Insp+BG INs in C1-C5 segments is indicated in Fig.4-B (light green bars). These units are concentrated in C1-C3 (n=15, 17, and 15, respectively) with some in the C4 (n=9) and C5 (n=6) segments. The significant difference in distribution of Insp+BG units was detected between C1-C2-C3 and C5 segments (see Fig.4-B for p-values). Other classes of inspiratory INs (late-Insp and full-inspiratory) were distributed more randomly. Their locations are shown in Fig.2-3 where full-inspiratory (full-Insp are not shown in Fig.1) INs are marked with black stars and late-Insp – with black pentagons, respectively.

Different expiratory INs (n=75) were found among all (C1-C5) segments without significant differences in their distribution (Fig.1-B1-B3, Fig.2-3 and Fig.4-C). We have noted the following tendency in their numbers: Full-Exp>Incrementing-Exp>Decrementing-Exp, which were observed throughout all segments (Fig.4-C). Locations of different expiratory INs are shown in Fig.2-3 where full-Exp INs are marked with red circles, incrementing expiratory (E1) – with red quadrants, and decrementing expiratory (E2) – with red diamond, respectively.

Phase-spanning INs (n=76) firing during transition between two phases of respiratory cycle were found in all segments with greater distribution in C1-C2 versus C3-C5 segments, albeit non-significantly (Fig.4-D). Their patterns are shown in Fig.1-C1-C3 and locations – in Fig. 2-3, where inspiratory-expiratory (I-E) are marked with blue circles, expiratory-inspiratory (E-I) – with blue diamonds, and wide-spanning – with blue quadrants, respectively.

In neuropharmacological experiments, GABAzine (GABAz, selective antagonist of postsynaptic GABA<sub>A</sub> receptors, 4 mM) and Strychnine (STR, antagonist of glycine postsynaptic receptors, 4 mM) were applied to 75 cells with microiontophoresis (+20 nA, 2 min) using multibarrel Carbestar electrode followed by juxtacellular labeling (2% Neurobiotin, +10 nA, 30 min). Late-Insp units with initial frequency rate 10-40 Hz showed the high sensitivity to GABAz or STR application and changed their firing pattern to early-inspiratory type (Fig.5-A-B). Simultaneous administration of GABAz and STR altered late-Insp pattern to tonic (background) activity (Fig.5-C). Insp+BG cells with initial frequency 80-130 Hz showed significant changes in firing rate to application of antagonists while units with frequency more than 130 Hz did not demonstrate altered activity. Fig.6 shows the changes in firing frequency of Late-Insp (dark brown bars, n=7), Insp+BG (light green bars, n=6) and other respiratory (non Late-Insp) units without background activity ('Resp', orange bars, n=10) included Wide-Span (n=4), I-E (n=2), E-I (n=2), and Full-Insp (n=2) in response to application GABAz&STR. The differences in firing rate after antagonist application were the same for all cell types. Relative increase in firing rate (%), however, was significantly higher for Late-Insp units with slowest frequency discharges during control recordings.

In separate experiments, GABAz&STR were applied over C1-C5 segments during PN and single unit recording in C1Tx rats. Spontaneous PN bursting activity was observed starting from 50  $\mu$ M of GABAz&STR and became more regular at 200  $\mu$ M. Different patterns of PN-related neurons (n=19) were recorded at the level of the upper cervical respiratory group (C1-C2) and PNucl (C3-C5) (Fig.1-D). Units that firing simultaneously with PN discharge (9/19), were recorded from the level of the PNucl (ventral C4 segment, 6/9) and VII Rexed layer of the C2 segment (3/9). Two biphasic units, firing at the beginning and after of PN bursts, were found in the middle of V Rexed layer of C1 and C2 segments (Fig.1D-2). Units with long bursts (2.2 $\pm$ 0.47 s) and pre-PN activity were recorded from V layer of C1 (4/8) and C2 (4/8) segments. These neurons with pre-PN (pre-Inspiratory) activity could be candidates for a spinal respiratory rhythm generator.

**Fig.1: Patterns of respiratory-related neurons recorded from C1-C5 cervical segments**

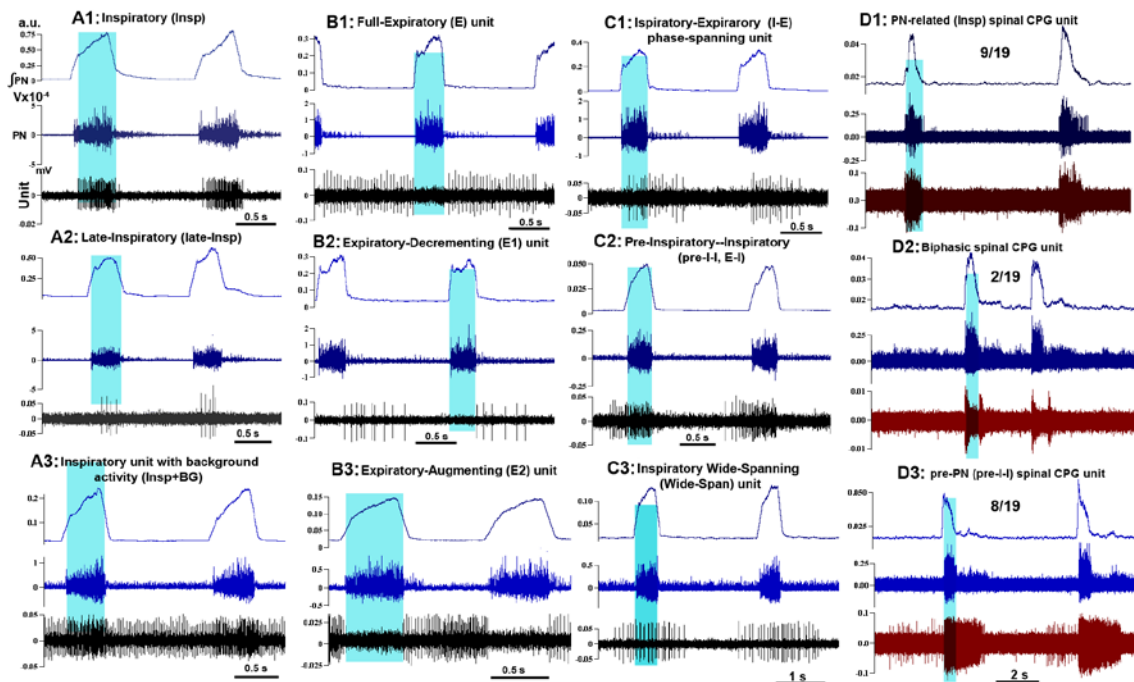


**Fig.1.** Panels A1-A3: inspiratory neurons, B1-B3: expiratory interneurons, C1-C3: phase-spanning interneurons, D1-D3: phrenic-related neurons activated after disinhibition (during application of GABAzine and strychnine over C1-C5 segments) of spinal circuits in C1Tx animals. Each panel from top to bottom:  $\int$ PN (a.u.) – integrated phrenic nerve activity in arbitrary units, PN – phrenic nerve raw activity, Unit – extracellular record of respiratory neurons. Blue shadowed bars are overlapped with inspiratory phase of phrenic nerve activity.

**Fig.2: Labelling (A&B) and distribution of different patterns of respiratory-related neurons in the C3–C5 cervical segments**

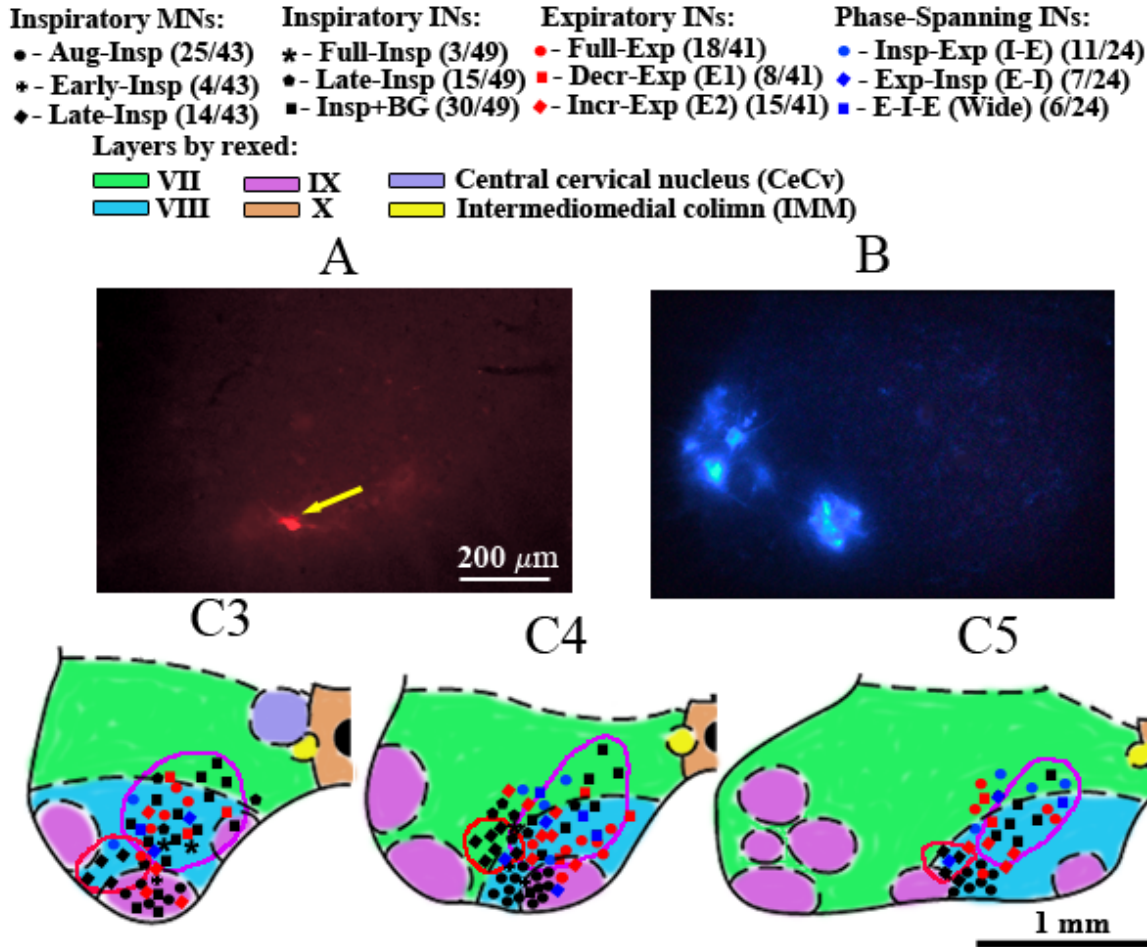


**Fig. 2.** Panel A: labeled motoneuron (Streptavidin-Alexa-610 was used for neurobiotin detection). Panel B: bimodal (ventro-medial and dorso-lateral) distribution of phrenic motoneurons confirmed by different retrograde monosynaptic tracers (Fast Blue). Panels C3-C5: areas outlined with red line contain Late-Insp MNs, which are distributed slightly dorso-lateral to the main PNucL pool; areas outlined with pink line contain Insp+BG Ins, which are located mostly dorso-medial to phrenic motoneuron pools.

**Fig.3: Distribution of different patterns  
of respiratory-related neurons in the C1-C2 segments**

- | Inspiratory:          | Expiratory:          | Phase-Spanning:            |
|-----------------------|----------------------|----------------------------|
| ● - Full-Insp (7/52)  | ● - Full-Exp (15/34) | ● - Insp-Exp (I-E) (19/52) |
| ◆ - Late-Insp (13/52) | ■ - E1(decr) (7/34)  | ◆ - Exp-Insp (E-I) (22/52) |
| ■ - Insp+BG (32/52)   | ◆ - E2 (aug) (12/34) | ■ - E-I-E (Wide) (11/52)   |

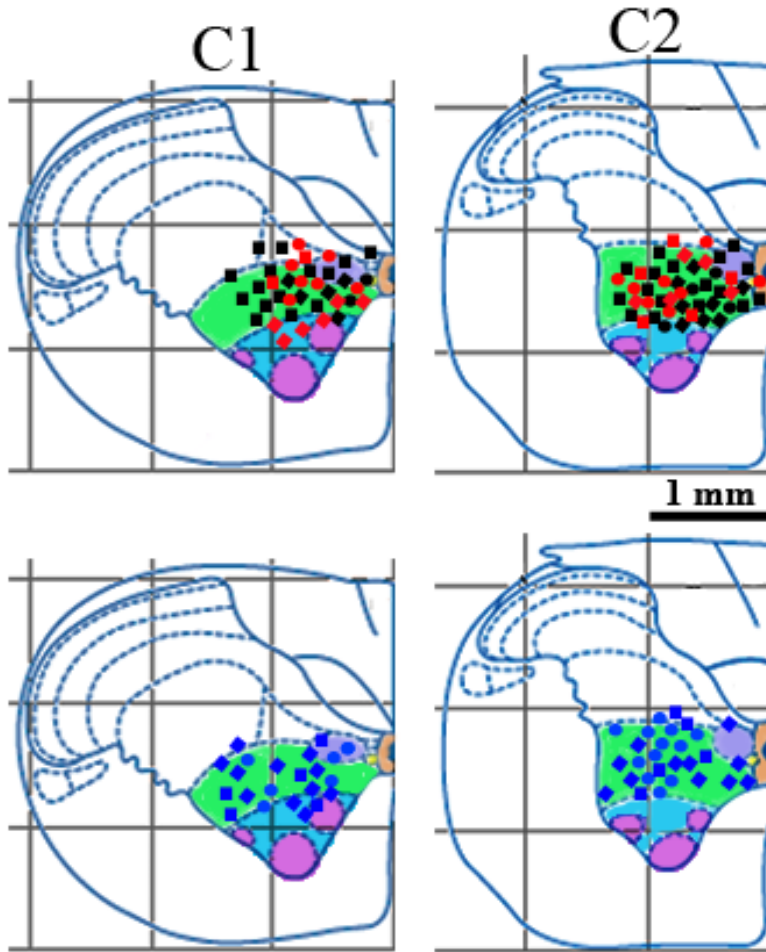


**Fig.4: Graphs of distribution of different patterns of respiratory-related neurons in the cervical C1-C5 segments**

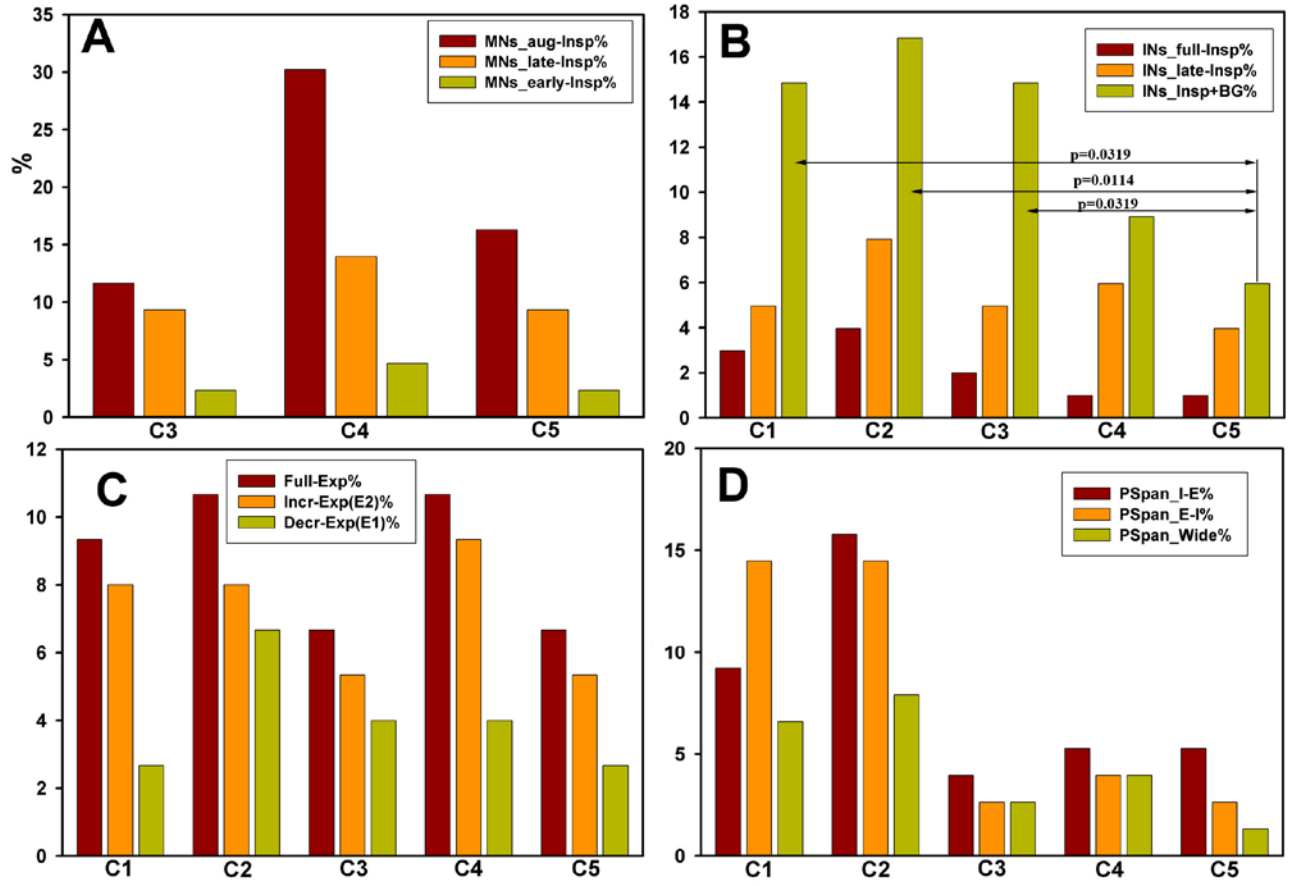


**Fig.4.** Panel A: Distribution of phrenic motoneurons, B: Distribution of inspiratory interneurons, C: Distribution of expiratory interneurons, D: Distribution of phase-spanning interneurons.

**Fig.5: Responses of late-Inspiratory spinal neuron to GABazine (A), strychnine (B), and GABazine+Strychnine application (C)**

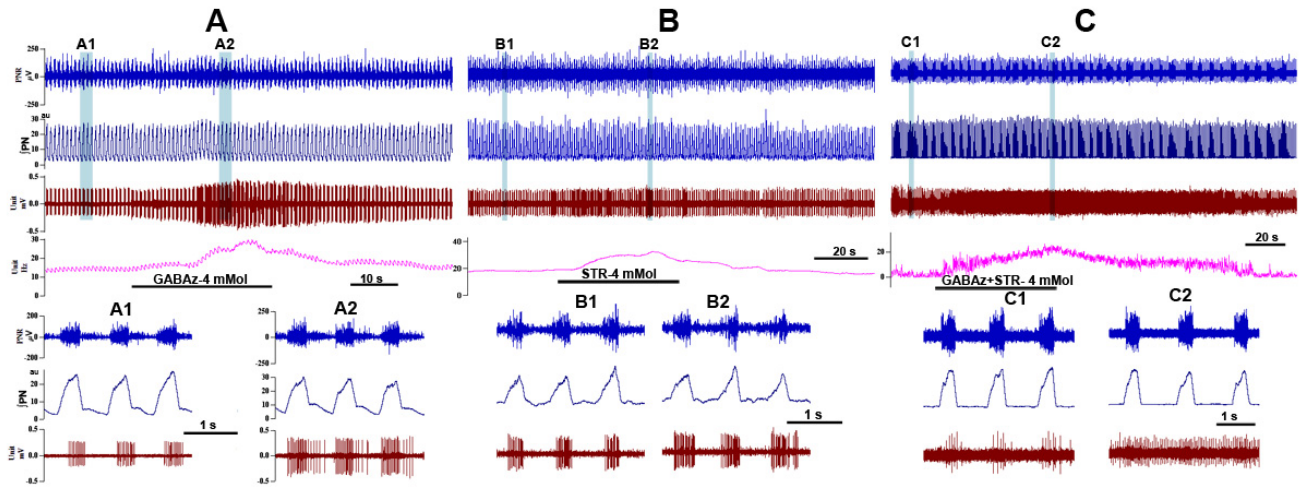


**Fig.5.** Panel A –GABazine application; Panel B-strychnine application; Panel C- GABazine&Strychnine application; Blue shadowed segments (extended records): A1-C1 –before application of antagonists, A2-C2 – during application of antagonists. Each panel from top to

bottom: PN – phrenic nerve raw activity,  $\int$ PN (a.u.) – integrated phrenic nerve activity in arbitrary units, Unit – extracellular record of respiratory neurons; Changes in frequency rate (Hz, pink lines).

**Fig.6. Responses of respiratory neurons to GABAzine+strychnine application**

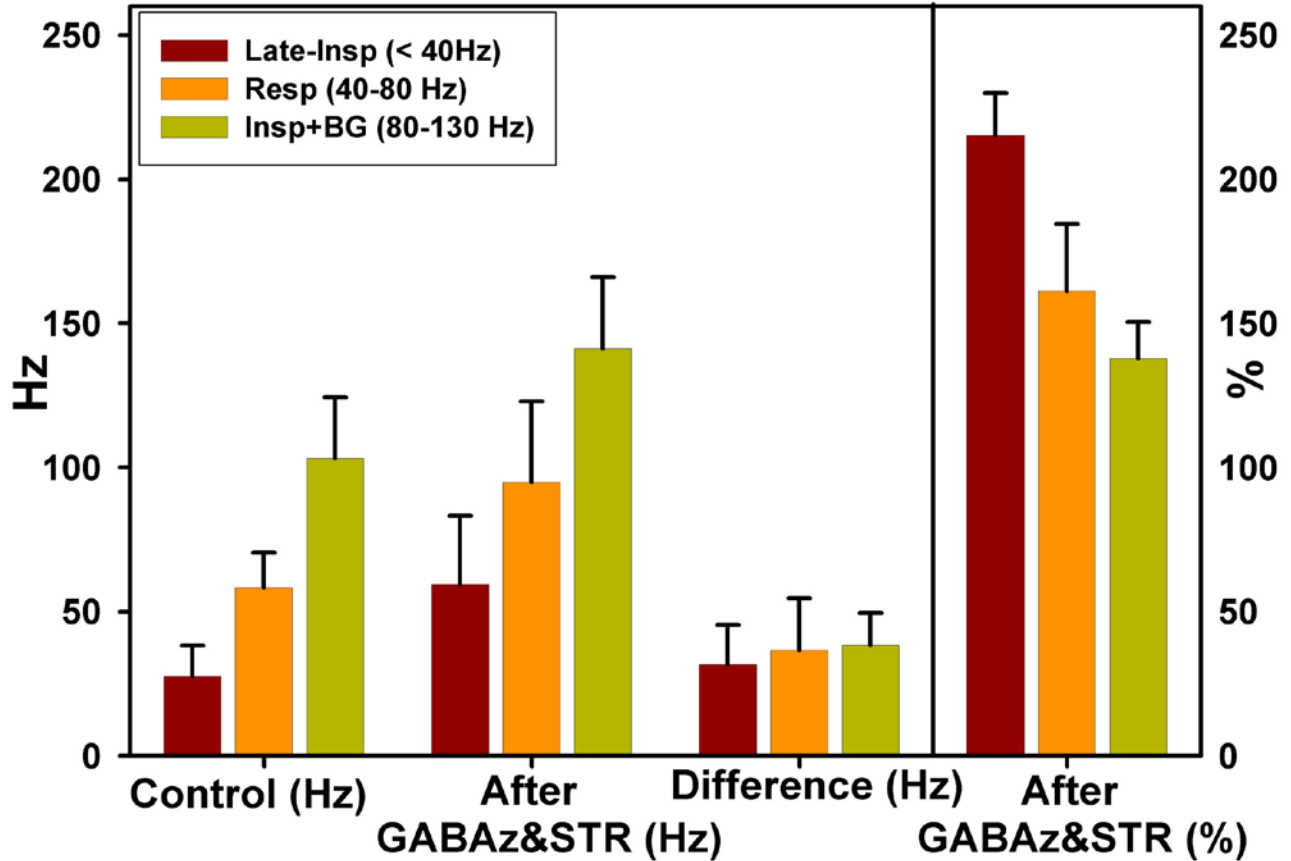


**Fig.6.** Dark brown bars: Late-Insp units with initial frequency rate 10-40 Hz. Orange bars: The respiratory group ('Resp') includes different respiratory (non Late-Insp) units with initial firing rate 40-80 Hz without background activity (n=10, Wide-Spanning (n=4), I-E (n=2), E-I (n=2), and Full-Insp (n=2)). Light green bars: Insp+BG cells with initial frequency 80-130 Hz. The percentage (%) changes of frequency rate for all groups of neurons after GABAz&STR application are shown on the right panel.

**Fig.7: Examples of double (A) and triple (B) labeling, and distribution of Phrenic-related interneurons (A4, C1-C5) with different (GABA & Glycine-ergic) neurotransmitter profiles in the cervical C1-C5 segments**

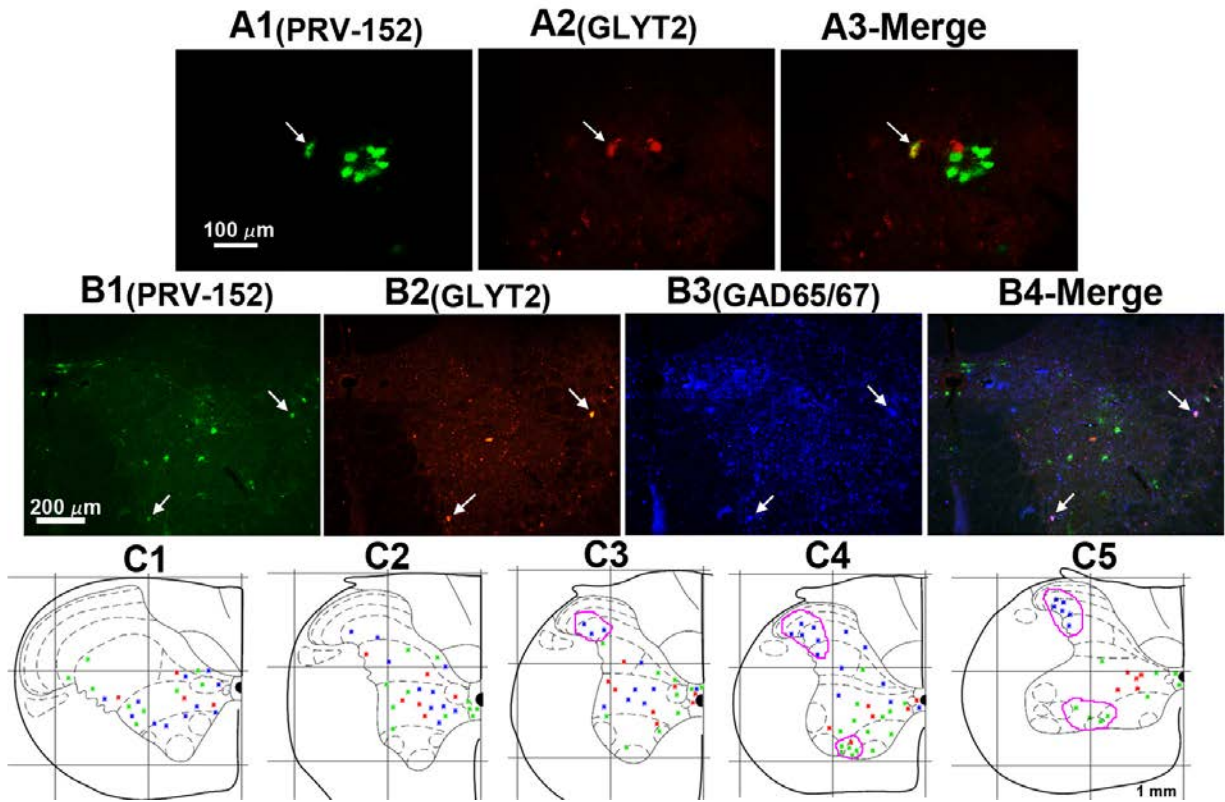
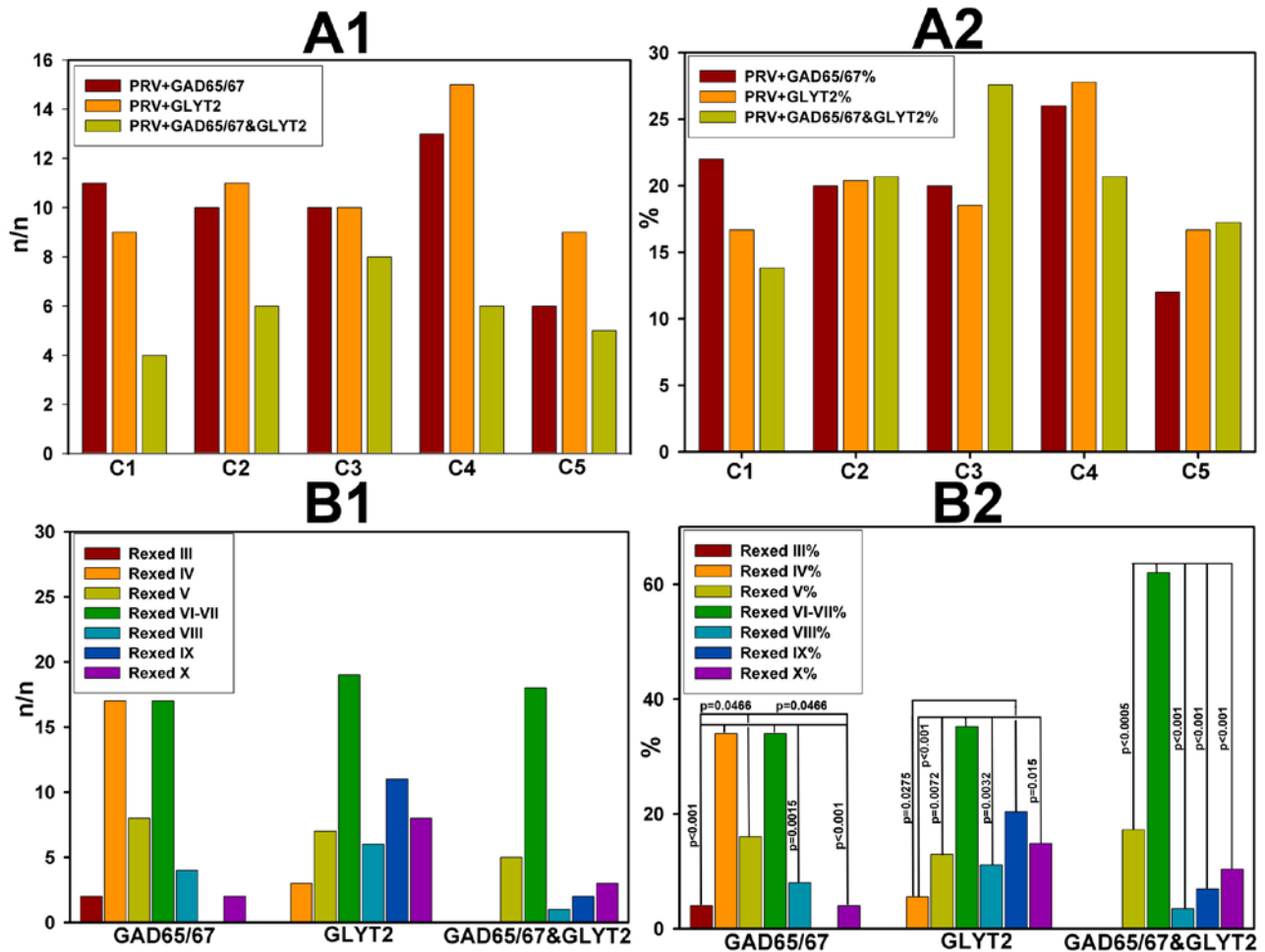


**Fig.7.** A1-A3 panels: double-labeling (PRV+GLYT2) of phrenic related glycinergic (red) interneuron near phrenic motoneurons (green). B1-B3: triple-labeling (PRV+GAD65/67+GLYT2) of phrenic-related interneurons (green) containing both GABA (blue) and glycine (red) neurotransmitters. C1-C5 Panels: blue stars - GABA-ergic neurons (dense groups of GABA-ergic neurons are outlined with pink line in dorsal horn III-IV layers), green stars - glycinergic neurons (compact groups of glycinergic neurons are outlined with red line in IX layer -close to phrenic motoneurons), red stars - neurons containing both GABA and glycine.

**Fig.8. Distribution of labeled GABA- and glycinergic phrenic-related spinal interneurons in C1-C5 segments (A1-A2) and Rexed layers (B1-B2) by numbers (A1, B1) and % (A2, B2)**



In summary of **Major Task 1.1**:

1) The phrenic nucleus represents a heterogenic population of respiratory spinal motoneurons and interneurons that may be a part of a highly-organized, local network subserving inspiratory output during eupnea and different respiratory disorders including spinal cord injury:

1a) We have mapped and quantified late-inspiratory motoneurons located slightly dorso-lateral to the inspiratory-augmenting motoneurons, which may play an important role (by recruitment) in the compensation of breathing after spinal cord injury.

1b) Topographical analyses of the most representative populations of recorded interneurons revealed that Insp+BG units are located mostly dorso-medial to motoneuron pools. However, some of them were found in vicinity of motoneurons. These neurons may play an important role in regulation of phrenic motoneuron excitability during all phases of the respiratory cycle and may be a source for excitatory spinal respiratory drive post-SCI.

2) Expiratory interneurons were found in all segments of the phrenic motoneuron pool (C3-C5) and upper cervical respiratory group (C1-C2). The wide distribution of expiratory spinal interneurons could suggest that they play a role in inhibitory mechanisms described previously (L). After complete spinal cord injury, these units can be a source of tonic inhibition, which suppress respiratory motoneuron activity.

3) Phase-spanning interneurons, the majority of which are located at C1-C2 level, may play an important role in smooth transition between respiratory phases. We noted, that the firing pattern of expiratory-inspiratory- units (E-I) is very close to medullary pre-Bötzinger neurons – the main kernel of respiratory rhythm generation. These neurons may relay/amplify supraspinal drive to respiratory motoneurons during normal respiratory behavior and contribute to initiation of a spinal CPG for breathing after high cervical SCI. Consistent with this idea, we recorded long-bursting units with pre-phrenic activity in C1-C2 segments after disinhibition of spinal circuits in C1Tx animals.

4) Biphasic units recorded during activation of spinal CPG by GABA<sub>A</sub>&STR application may provide evidence for active inhibition within the spinal CPG network. The firing pattern of these units is very close to biphasic medullary neurons participating in respiratory rhythm and pattern formation (Homma and Onimaru- L). These authors have demonstrated that biphasic medullary neurons are inhibited by GABA-ergic mechanisms during inspiration. Our results could suggest that the threshold concentration of GABA<sub>A</sub>&STR (50  $\mu$ M) for initiation of spinal CPG in C1Tx animals may target mainly tonic but not phasic inhibition widely presented in spinal oscillatory networks (for e.g., locomotion and bladder voiding).

5) We also have found that late-Insp interneurons are the most sensitive spinal units to GABA<sub>A</sub> and Glycine-receptor blockers (GABAzine and Strychnine, respectively). Low-frequency Late-Insp neurons showed the same increase in frequency rate after GABA<sub>A</sub>&STR microinjections as higher-frequency units (Insp+BG, for e.g.). We conclude that these neurons are strongly inhibited during normal breathing and can be a target for neuropharmacological recruiting to improve inspiratory motor output during different pathological conditions including SCI.

For **Major Task 1.2**, transsynaptic tracing with pseudorabies viruses (PRV-152 and PRV-614) and immunohistochemical identification of glutamate decarboxylase isoforms (GAD65/67) and glycine transporter 2 (GLYT2), identified neurotransmitter profiles (GABA- or glycinergic) of phrenic-related spinal interneurons. Interneurons containing both GABA and glycine (GABA/Gly) were studied in triple-labeling experiments, an example of which is shown in Fig. 7 (B1-B4). The total number of GABA- (n=50), glycine- (n=54) and GABA/Gly (n=29) phrenic-related interneurons was distributed along C1-C5 segments with a non-significant increase in labeled cells within the C4 segment (Fig.8-A1-A2). Topographical analysis of the representative populations of transsynaptically labeled interneurons reveals the most dense concentration of GABA/Gly, GABA- and glycinergic units in Rexed area VII (Fig.7-C1-C5: blue and green stars and Fig. 8-B1-B2: green bars). Besides this, the greatest number of GABA-ergic neurons was found in IV layer of the dorsal horn (Fig.7-C1-C5: blue: blue stars and Fig. 8-B1-B2: orange bars at 'GAD5/67'). About 20% of glycinergic neurons were detected in IX layer – close to PNucl MNs (Fig.7-C1-C5: green stars

outlined with red line areas and Fig.8-B1-B2: blue bar at 'GLYT2'). In general, the distribution of GABAergic INs was observed dorsolaterally, toward to dorsal horn (Fig.7-C1-C5: blue stars outlined with pink line areas), and glycinergic cells were seen ventromedially, toward lamina X (around the central canal). An example of double-labeled (PRV-152+GLYT2) glycinergic interneurons in the vicinity of phrenic motoneurons is shown in Fig.7 (A1-A3). These neurons comprise the pre-phrenic Renshaw cells that prevent overexcitation of phrenic MNs during inspiration.

In summary of **Major Task 1.2**:

- 1) GABA-ergic phrenic-related interneurons are distributed within the the dorsolateral spinal cord with the greaetest density within laminae IV and VII. These neurons may play an important role in cross-phrenic phenomen observed following high cervical hemisection (L).
- 2) Glycine-ergic phrenic-related interneurons were detected within the ventromedial spinal cord. Dense clusters of these cells were found near phrenic motoneurones (VIII) and lamina X surrounding the central canal. We propose that those cells nearest motoneurons belong to Renshaw cells providing recurrent inhibition.
- 3) Phrenic-related interneurons containing both GABA and glycine inhibitory neurotransmitters were distributed primarily within lamina VII. The co-existence of GABA and glycine has already been demonstrated in the brainstem, but remains under-explored in the spinal cord (Todd et al., 1996).

For **Major Tasks 2.1, 2.2 and 2.3**, we identified the minimal current of EDS (threshold) affecting tracheal pressure (TP) using a single biphasic pulse of different lengths (0.1, 0.2 and 0.5 ms) applied to C1-C5 segments. Fig. 9B shows an example of a recording during stimulation where changes in TP to a single pulse are indicated by black dots. The average threshold values for single pulse EDS at different segments are presented in graph D (Fig. 9D). While no significant differences were detected between threshold currents applied to C3 and C5 segments, stimulation at the C4 level resulted in significantly lower thresholds for all pulse lengths. We have shown that pulse duration of 0.1 ms resulted in significantly higher thresholds for all segments (Fig. 9D), while 0.2 and 0.5 ms were more optimal. Furthermore, 0.2 ms pulse duration produced much smaller artifacts than 0.5 ms pulses and therefore decreasing unnecessary disturbance of nerve tissue by electrical current, thus perhaps more optimal for EDS. The results of this experimental group also indicate that C4 EDS yields more optimal effects on TP and PN responses than stimulation at C3, C5 or even C1-C2. While stimulation at 3 x threshold (3T) current did not produce any visible limb muscle contractions, more than 5T (>400  $\mu$ A) resulted in contraction of upper- and lower limb muscles and was therefore avoided in the present work. 3T was considered optimal for the present studies,

Single pulse EDS at 3T(~238  $\mu$ A,) elicited two component PN responses: high-amplitude N1 (direct) and lower amplitude N2 (synaptic) (Fig. 9C, middle panel). Intrathecal administration of GABA<sub>A</sub> & STR at subthreshold dose over C4 segment significantly affected the amplitude of N2 component. The amplitude of N2 components increased by 1.89 times (Fig. 9C, right panel with dark red trace).

In non-paralyzed animals, trains of EDS were applied to the C4 segment (C4-EDS) once per second (1 Hz) during 0.33 s with an optimal stimulus length (0.2 ms) and different frequencies (100, 200 and 300 Hz) (stimulation paradigm outlined in Fig. 9A) that were in the physiological range of phrenic nerve frequency bands – MFO (~110-150 Hz, middle-frequency oscillations), HFO (~155-220 Hz, high-frequency oscillations) and UHFO (~225-285 Hz, upper HFO) in rats (43, 44, 45). The train duration (0.33 s) was chosen based on average inspiratory time in adult rats (Connely et al., 1992). EDS with frequencies of 200-300 Hz successfully paced stable breathing at 3T (220-240  $\mu$ A) for more than 30 min (see an example of record in Fig. 10). The tidal volume maintained with EDS train frequency 200 Hz ( $1.34 \pm 0.1$  ml) and 300 Hz ( $1.37 \pm 0.12$  ml) showed significant difference vs 100 Hz ( $1.22 \pm 0.07$  ml) ( $p=0.03$  [100 vs 200 Hz] and  $p=0.0016$  [100 vs 300 Hz]). A current intensity of 1.5T- (~100-120  $\mu$ A) failed to support paced breathing, with a progressive decrease of tidal volume and unstable end-tidal CO<sub>2</sub> in almost all trials (11 of 13) (published in Bezdudnaya et al., 2018).

The combinatory effects of GABA<sub>A</sub>&STR intrathecal application and HF-EDS on PN responses were initially studied in decerebrate paralyzed C1Tx rats. Trains of EDS (100, 200 and 300 Hz) were applied to C4 cervical segment as described above. Our data demonstrated the time and frequency depending reciprocal changes in PN direct (early) N1 (~ 1 ms of latency) and synaptic (late) N2 (~ 3-4 ms of latency) components in responses to different frequencies of EDS trains applied in combination with intrathecal delivery of GABA<sub>A</sub>&STR (Fig. 11 and graph in Fig.12A). Fig. 11 shows an example of changes of PN components during HF-EDS before (blue traces) and after (dark red traces) of GABA<sub>A</sub>&STR administration. On the right side of Fig. 11, averaged PN responses are shown for the first (1/2) and second (2/2) half of stimulus trains. In the presence of GABA<sub>A</sub>&STR, N1 amplitudes were significantly increased at 100-200 Hz (Fig.11 and pink line in Fig. 12A) and then declined at 300 Hz,. The N2 component progressively increased from 100 Hz and reached maximum at 200 Hz (Fig. 11 and red line in Fig. 12A). In the absence of GABA<sub>A</sub>&STR, the maximum for N2 component was observed at higher frequency (300 Hz) of EDS (Fig. 11 and blue line on Fig. 12A). The latencies of PN components were not significantly different to all frequencies of HF-EDS applied before and after GABA<sub>A</sub>&STR application.

During intraspinal administration of GABA<sub>A</sub>&STR into PN<sub>ucl</sub> (graph in 12C), N1 component (direct activation of phrenic motoneurons) was significantly increased in the range of 100-200 Hz, the N2 (synaptic) component shown a weak elevation at 100 Hz with decay at 200-300 Hz. These results confirm the presence of GABA<sub>A</sub> and glycine receptors in phrenic motoneurons (L). The generation of N2 synaptic component can be depended on excitatory neurons located in more dorsal layers (V-VII).

Intrathecal or intraspinal administration of antagonists at the upper cervical respiratory group (C1-C2) demonstrated increase only N2 synaptic component (red lined on graphs of Fig.12-B-D). It may evidence that C1-C2 segments are the source of descending inhibition targeting mainly pre-phrenic interneurons.

In a study by the Edgerton Lab (Lavrov et al., 2006) performed on low transected (Th6-L4) chronic rats, three components (direct, monosynaptic and polysynaptic) of tibial muscle EMG were

detected in response to EDS applied to 1st sacral segment. Consistent with this, the N1-component with short latency ( $1.08 \pm 0.02$  ms) may reflect direct activation of phrenic motoneurons, while N2 with longer latency ( $2.54 \pm 0.05$  ms) can be related to synaptic origin.

Next, we performed combined C4-EDS and intrathecal delivery of antagonists (GABAzine and strychnine) of inhibitory GABA<sub>A</sub> and glycine receptors to elicit paced breathing during diaphragm EMG recording in C1Tx non-paralyzed animals. Previously, we identified that 200 Hz is the optimal train frequency of EDS combined with intrathecal administration of GABAz&STR that produced the biggest PN responses. Therefore, we used these stimulation parameters to pace respiratory activity in C1Tx non-paralyzed animals before, during and after pharmacological disinhibition of spinal circuits by sub-threshold doses of GABAz&STR (25  $\mu$ M). Fig. 13 shows an example of a paced breathing (~22 min record) during C4-EDS (200 Hz) before (extended panel B), during (extended panel C) and after GABAz&STR intrathecal administration (extended panel D). Our results show that  $3 \pm 0.79$  min after GABAz&STR administration tidal volume and DiEMG amplitudes were significantly increased (Tv: from  $1.38 \pm 0.12$  to  $2.19 \pm 0.21$  ml,  $P=0.00167$ ; DiEMG amplitudes increased by  $89.48 \pm 26.83$  %,  $P=0.012$ , Fig. 14). However, 17.34 $\pm$ 2.51 min after starting of GABAz&STR administration these parameters returned to pre-application levels, indicating the washing out of drug effect.

In summary for **Major Task 2.1.-2.3:**

- 1) Spinal GABA- and glycinergic inhibitory mechanisms play an essential role in regulation of phrenic MNs pre-phrenic INs.
- 2) The drastic increase of synaptic N2 component during C4-HF-EDS in combination with intrathecal delivery of antagonists may suggest that the major portion of pre-phrenic interneurons at C4 level is inhibited following SCI. These pre-phrenic interneurons are becoming a key target in research devoted to restoration of breathing following SCI (L).
- 3) The present work highlights the advantage of EDS-paced breathing at the level of the phrenic following complete SCI.
- 4) A combined strategy of simultaneous using of C4-EDS and neuropharmacological disinhibition leads to:
  - 2a) shift of effective frequency stimulation from 300 to 200 Hz that require less amount of electrical energy applied to spinal cord during EDS,
  - 2b) increase of vital parameters such as paced tidal volume in parallel with diaphragm EMG and PN responses.
- 5) Taken together, our experimental data can be used for developing an alternative or an additional treatment to those traditionally employed (direct phrenic nerve and diaphragm pacing) for life-supporting paced breathing in tetraplegic patients. These results will guide translation of clinically relevant treatments and improve the quality of life in people with serious respiratory disorders, including SCI.

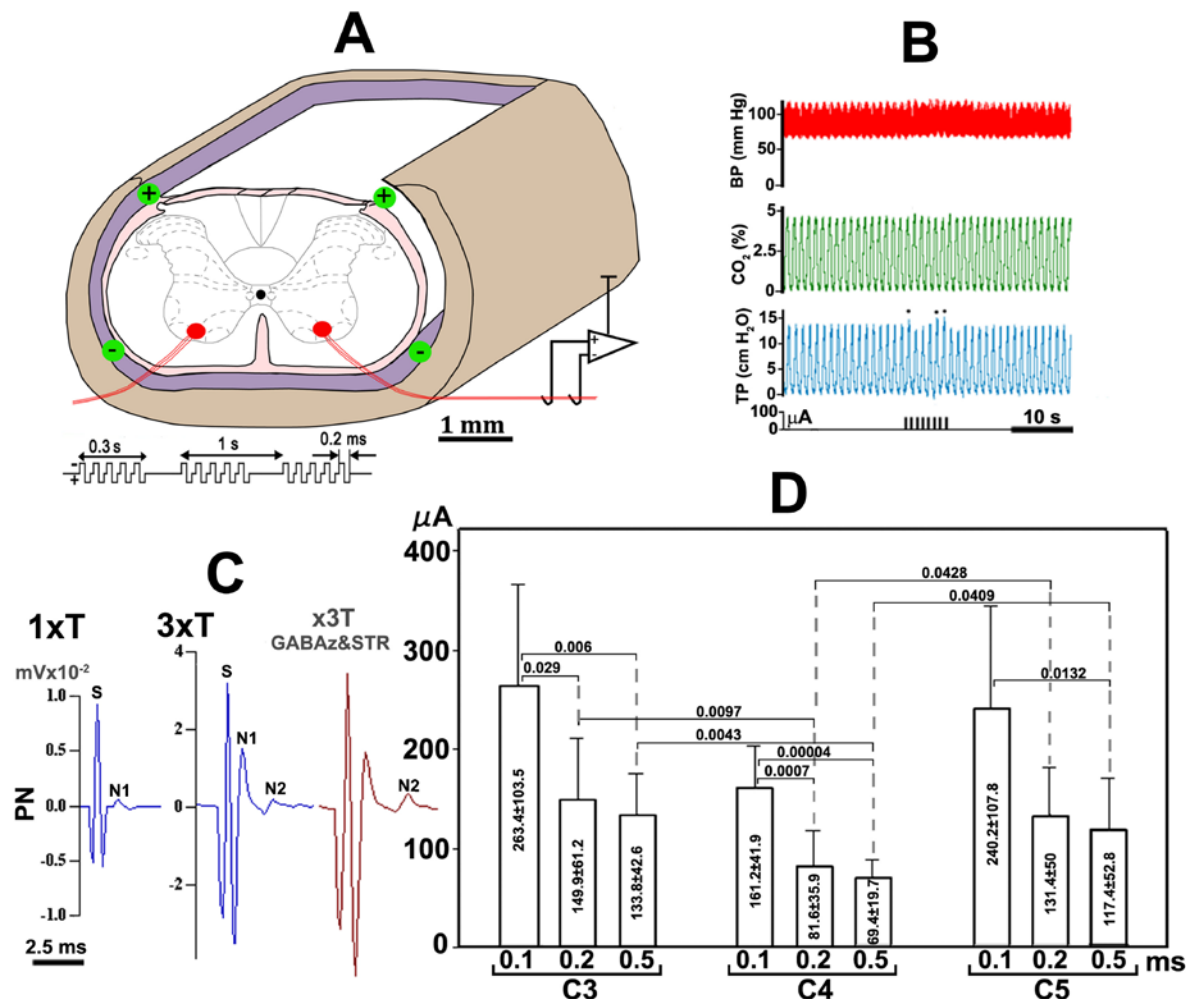


**Fig. 9.** P A: Schematic diaphragm of EDS electrode locations near the ventral and dorsolateral surface of C4 cervical segment. Phrenic motor nucleus and PN (red) are shown relative to the electrodes for recording of phrenic nerve activity (and input to operation amplifier (black)). The details of stimulation paradigm are presented at the bottom of panel. B: an example of detecting the minimal EDS-current affecting tracheal pressure (marked with dark dots). C: PN threshold (x1T) and supra-threshold (x3T) responses to single C4-EDS pulse (0.2 ms) before (blue middle trace) and after (dark red right trace) administration of GABA<sub>A</sub>z&STR (25  $\mu$ Mol). N1 - direct and N2 - synaptic PN components. Stimulus artifacts are marked with asterisks. D: Average minimal thresholds of EDS-current affecting tracheal pressure (see Fig.1B) applied with different pulse lengths (0.1, 0.2 and 0.5 ms) to C3-C5 segments (C3, C4, and C5). The mean ( $\pm$ SD) threshold values are shown inside of bars.

**Fig. 10. An example of the stable breathing during high-frequency (300 Hz) C4-EDS-pacing with 3x thresholds of current intensity.**

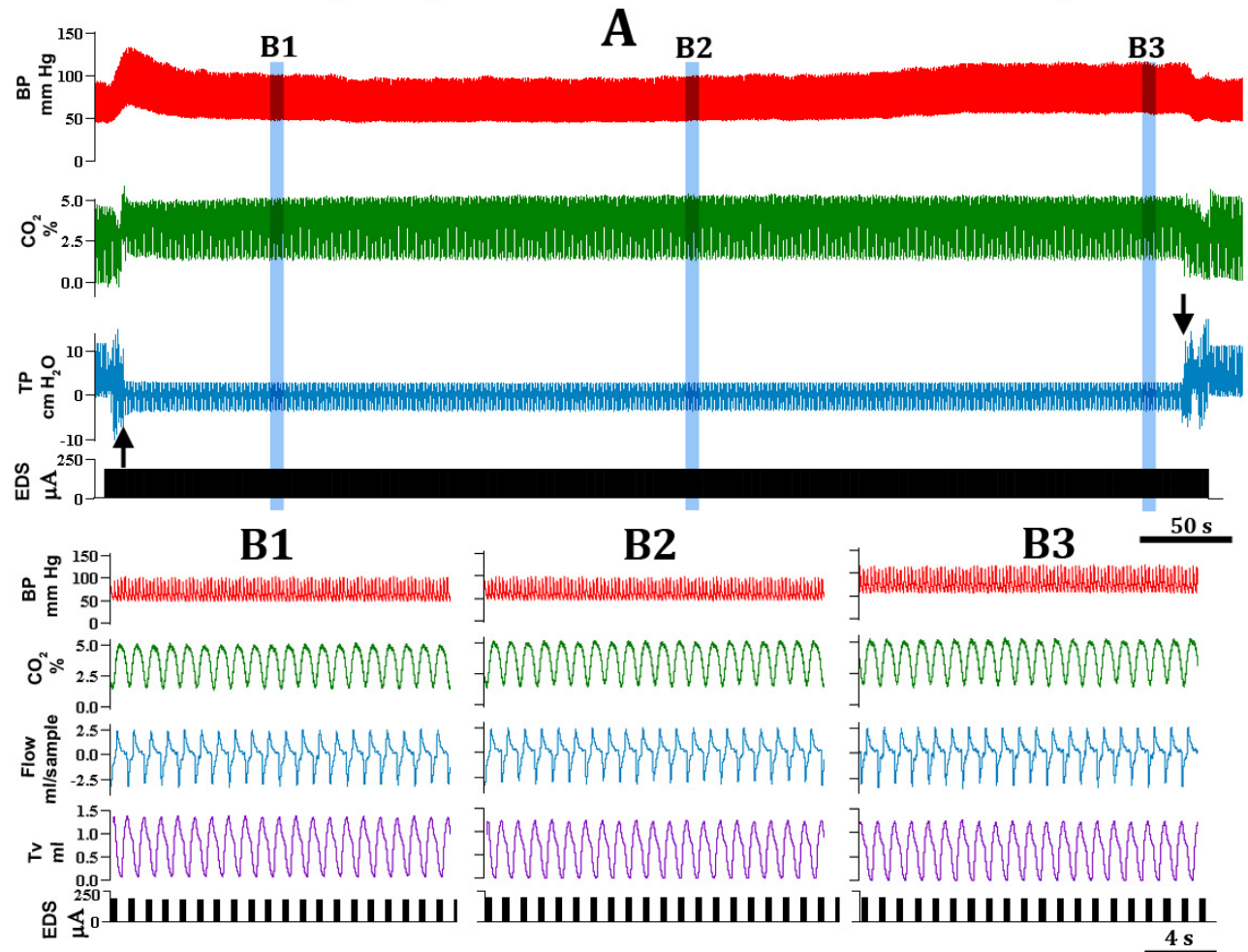


**Fig. 10. An example of the stable breathing during high-frequency (300 Hz) C4-EDS-pacing with 3x threshold of current intensity.**

Black arrows indicate turning assisted ventilation off or on (up and down arrows, respectively). B1-B3 highlighted segments demonstrate stable changes of paced breathing in expanded time scale during EDS with respiratory flow (cyan) and tidal volume (Tv, purple). Labels and legends for three top traces on panels A and B1-B3 (from the top to bottom): blood pressure (BP in mm Hg, red), end-tidal carbon dioxide concentration (CO<sub>2</sub> in %, green), tracheal pressure (TP in cm H<sub>2</sub>O, blue) was recorded before and after C4-EDS, respiratory flow in milliliters per sample rate (Flow, ml/sr, blue) was recorded during C4-EDS. The bottom black trace on panels A and B1-B3 indicates the current of EDS ( $\mu$ A) applied to C4 cervical segment.

**Fig.11. Examples of phrenic nerve responses to C4-HF-EDS before and after intrathecal application of GABAz&STR**

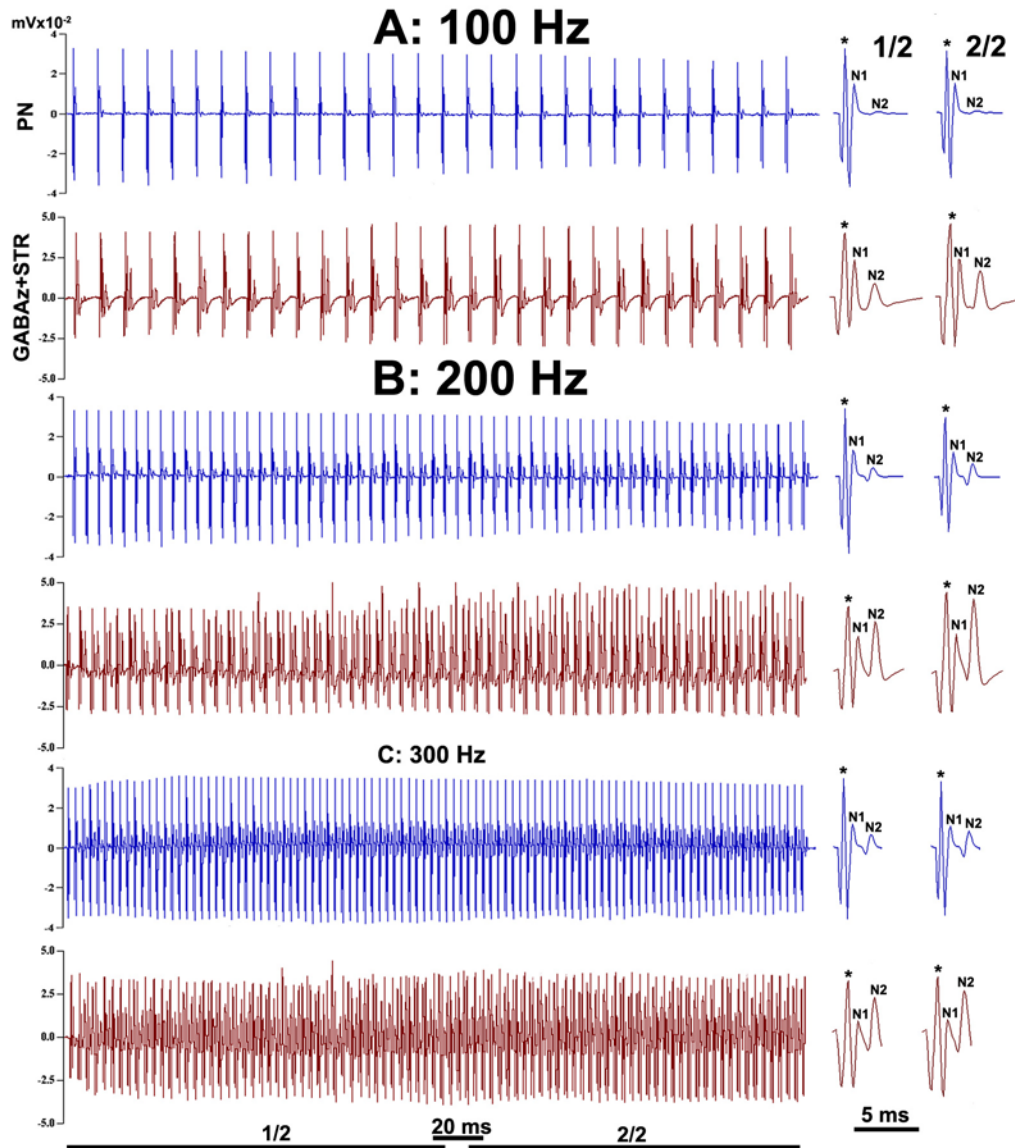


**Fig. 11 . Examples of phrenic nerve responses to C4-HF-EDS before and after intrathecal application of GABAz&STR.**

Right Panel: 1/2 and 2/2 are stimulus-triggered averaging of phrenic nerve responses during first and second half time period of stimulation at different frequencies (each half is marked with solid line below panel C. Asterisks, N1 and N2 identify stimulus artifact, the first (N1) and second (N2) phrenic nerve components, respectively.

**Fig.12. Statistical graphs of Phrenic nerve responses to HF-EDS after intrathecal (A&B) and intraspinal (C&D) application of GABA<sub>A</sub>&STR at C4 (A&C) and C1-C2 (B&D) cervical levels**

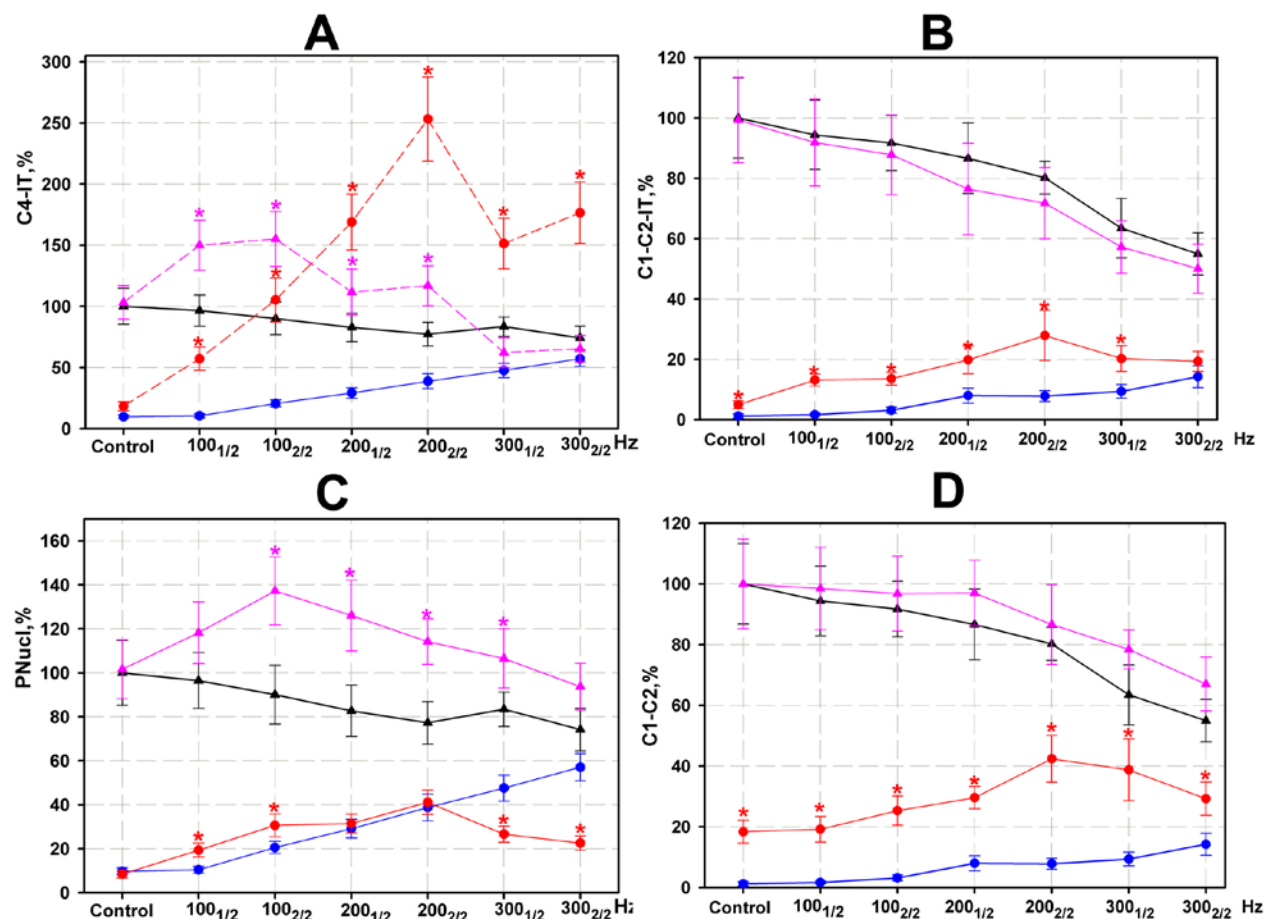


**Fig. 12. Statistical graphs of phrenic nerve responses to HF-EDS after intrathecal (A&B) and intraspinal (C&D) application of GABA<sub>A</sub>&STR at C4 (A&C) and C1-C2 (B&D) cervical levels.**

Stimulus-triggered averaging of phrenic nerve components during the first (1/1) and second (2/2) half of 100, 200, and 300 Hz EDS. The peak values of the N1 and N2 components of the first pulse of each frequency train were selected as the control amplitudes. The changes in amplitudes of N1 (black triangles – before and pink – after GABA&STR administration) and N2 (blue dots – before and red – after GABA<sub>A</sub>&STR application) components were normalized (in %) to the first N1 component (was chosen as the base control level - 100%). Significant differences between means ( $p < 0.05$ ) are marked by asterisk (\*).

**Fig. 13. An example of stable paced breathing during 200 Hz EDS combined with intrathecal delivery of GABA<sub>A</sub>&STR in C1Tx animals without ventilatory support.**

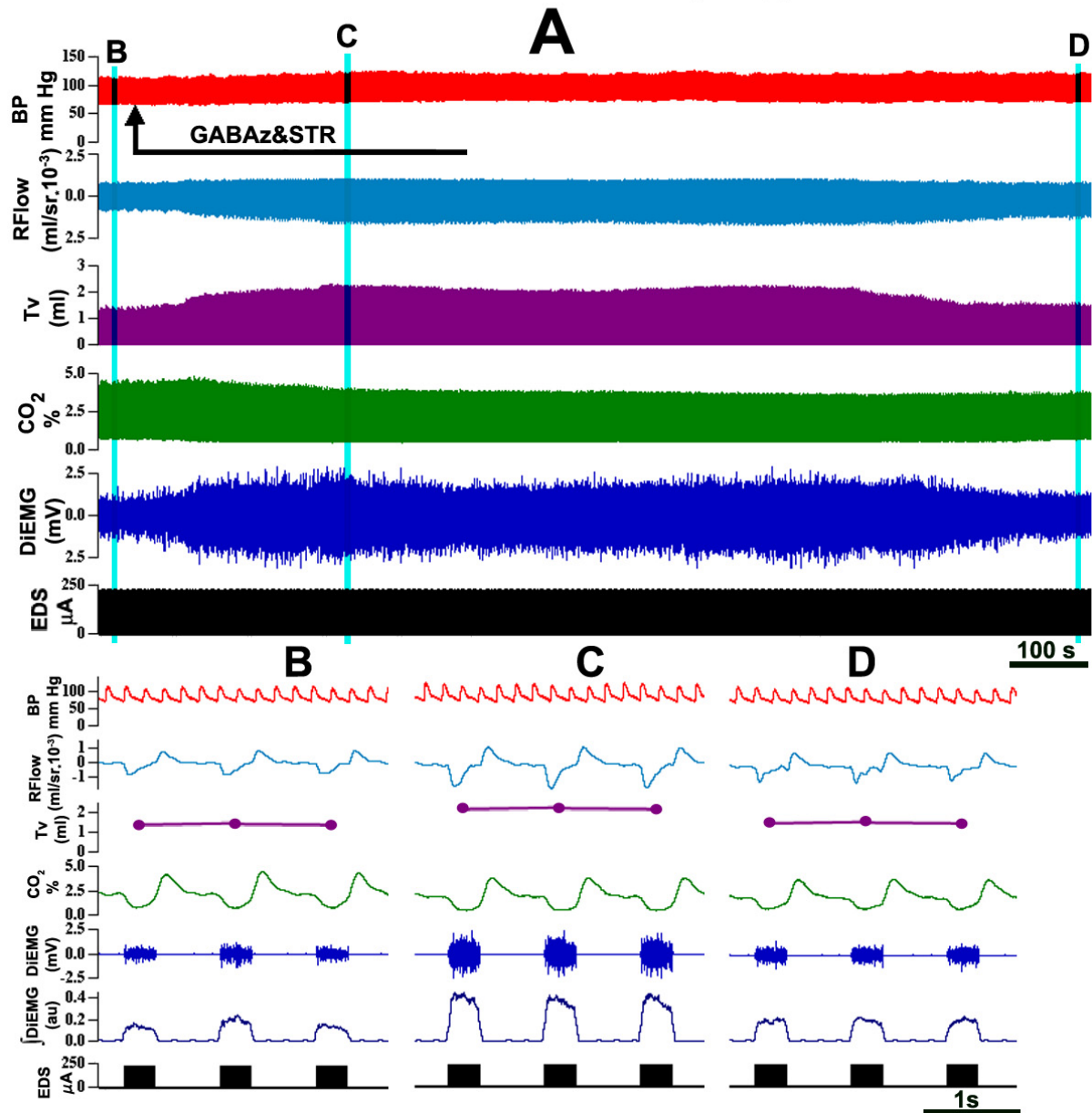


**Fig. 13. An example of stable paced breathing during 200 Hz EDS combined with intrathecal delivery of GABA<sub>A</sub>&STR in C1Tx animals without ventilatory support.**

A: Black arrows indicate the starting of GABA<sub>A</sub>&STR (25  $\mu$ Mol, 10  $\mu$ L) injection. B, C and D highlighted segments demonstrate periods before, during and after GABA<sub>A</sub>&STR application. Labels and legends (from top to bottom): blood pressure (BP in mmHg, red), respiratory flow (Flow in ml/sample rate, cyan), tidal volume (Tv in ml, purple), end-tidal carbon dioxide concentration

(CO<sub>2</sub> in %, green), diaphragm EMG (DiEMG, blue) and C4-EDS current ( $\mu$ A, black).  $\int$ DiEMG - integrated DiEMG.

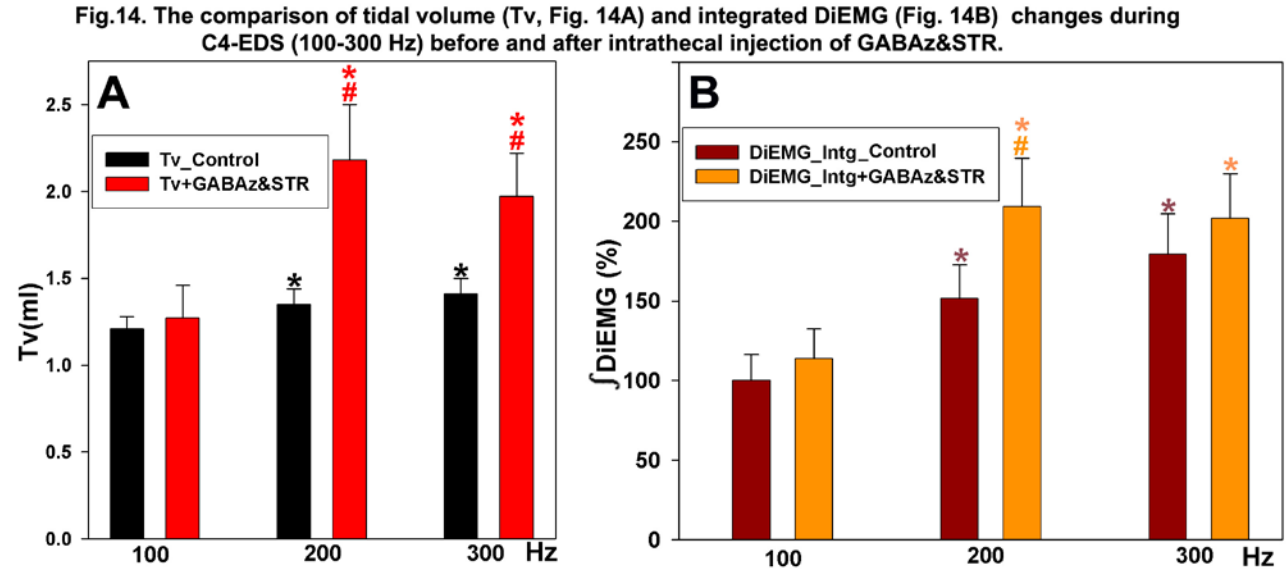


**Fig.14. The comparison of tidal volume (Tv, A) and integrated DiEMG (B) amplitudes changes during C4-EDS (100-300 Hz) before and after intrathecal injection of GABAz&STR.**

Significant differences between means of Tv and diEMG amplitudes at 100-300 Hz EDS are marked by asterisks (\*). Significant differences ( $p < 0.05$ ) between mean values obtained before and after intrathecal administration of GABAz&STR are marked with number signs (#).  $\int$ DiEMG - integrated DiEMG.

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**What opportunities for training and professional development has the project provided?**

*If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

Nothing to report

**How were the results disseminated to communities of interest?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

Nothing to Report.

*If this is the final report, state “Nothing to Report.”*

*Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

- 4. IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

**What was the impact on the development of the principal discipline(s) of the project?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

In the majority of cases people with tetraplegia are not able to breathe voluntarily, thus artificial ventilation is required. Our project is devoted to the development of treatments that harness the neuroplastic potential of spinal circuits (interneurons and motoneurons) that are capable of generating rhythmic breathing-like activity in respiratory muscles in an animal model of complete high cervical spinal cord injury. To date the role of spinal respiratory interneurons in respiratory motor pattern formation is almost ignored. The present work addresses this gap in research, bringing this clinically relevant concept to the forefront of this field of SCI research by proving the importance of intraspinal circuits in respiratory motor pattern formation.

**What was the impact on other disciplines?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

**What was the impact on technology transfer?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

**What was the impact on society beyond science and technology?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

**Changes in approach and reasons for change**

*Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

**Actual or anticipated problems or delays and actions or plans to resolve them**

*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

**Changes that had a significant impact on expenditures**

*Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

*Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

**Significant changes in use or care of human subjects**

**Significant changes in use or care of vertebrate animals**

**Significant changes in use of biohazards and/or select agents**

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

*Report only the major publication(s) resulting from the work under this award.*

**Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Ghali Michael George Zaki and Marchenko Vitaliy\*. Patterns of Phrenic Nerve Discharge after Complete High Cervical Spinal Cord Injury in the Decerebrate Rat. Journal of Neurotrauma. June 2016, 33(12): 1115-1127. acknowledgement of federal support: yes.
2. Hormigo KM, Zholudeva LV, Spruance VM, **Marchenko V**, Cote MP, Vinit S, Giszter S, Bezdudnaya T, Lane MA. Enhancing neural activity to drive respiratory plasticity following cervical spinal cord injuryExp Neurol. 2017 Jan; 287(Pt 2):276-287. *Acknowledgement of federal support: yes.*
3. Bezdudnaya T, Lane M.A., Marchenko V.\*  
Paced breathing and phrenic nerve responses evoked by epidural stimulation following complete high cervical spinal cord injury. J Appl Physiol 125: 687–696, 2018.  
*Acknowledgement of federal support: yes.*
4. Lyandysha V. Zholudeva, Liang Qiang, Vitaliy Marchenko, Kimberly J. Dougherty, Shelly E. Sakiyama-Elbert, and Michael A. Lane. The Neuroplastic and Therapeutic Potential of Spinal Interneurons in the Injured Spinal Cord. Trends in Neurosciences, September 2018, Vol. 41, No. 9, 625-639.

**Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

**Other publications, conference papers and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (\*) if presentation produced a manuscript.*

1. Abstract: Neuroscience Society annual meeting (November, 2018, San Diego, CA):  
 \*V. MARCHENKO, T. BEZDUDNAYA, M. A. LANE.  
 ‘The role of fast inhibition in facilitation of phrenic nerve and diaphragm activity during epidural stimulation following complete cervical spinal cord injury in rats’  
 Control/Tracking Number: 2018-S-9685-SfN. Acknowledgement of federal support: yes.  
 1. Abstract: \*V. MARCHENKO, T. BEZDUDNAYA, M. A. LANE.  
 Epidural stimulation and pharmacological blockade of fast inhibition improve respiratory pacing following complete spinal cord injury Abstract was submitted for SFN-2017 Symposia. Control/Tracking Number: 2016-S-13226-SfN. Acknowledgement of federal support: yes.

- **Website(s) or other Internet site(s)**  
*List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*
  
- **Technologies or techniques**  
*Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.*

- **Inventions, patent applications, and/or licenses**  
*Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.*
  
- **Other Products**  
*Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:*
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### **What individuals have worked on the project?**

*Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of*

compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith  
Project Role: Graduate Student  
Researcher Identifier (e.g. ORCID ID): 1234567  
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.  
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name: Vitaliy Marchenko – no changes  
Project Role: PI

Name: Michael Lane – no changes  
Project Role: collaborator

Name: Tatiana Bezdudnaya  
Project Role: Research-Instructor  
Nearest person month worked: 100% (or 12 months per year), \$ 60,000 yearly salary (from Dec, 1<sup>st</sup>, 2017)  
Researcher Identifier: n/a  
Contribution to Project: Dr. Bezdudnaya, PhD, works at our department as instructor and has a many year strong experiences in electrophysiology and programming. She was hired on part time (35% of salary support) from Sept 1<sup>st</sup>/2015 and transferred to full time (100% of salary support) from December 1<sup>st</sup> 2017. She performs work in electrophysiological experiments and data analyses.

Name: Kristiina Hormigo  
Project Role: Research Assistant II  
Nearest person month worked: 100% (or 12 months per year), \$ 37,500 yearly salary (from Aug 1<sup>st</sup> 2016)  
Researcher Identifier: n/a  
Contribution to Project: Kristiina Hormigo, MS, works at our department as Research Assistant II and has a strong experience in immunohistochemistry and maintaining the lab. She was hired on part time (50% of salary support) from May 1st/2016 and transferred to full time (100% of salary support) from August, 1<sup>st</sup> 2016. She performs lab management experiments, and data analyses.

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

**What other organizations were involved as partners?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

*Provide the following information for each partnership:*

*Organization Name:*

*Location of Organization: (if foreign location list country)*

*Partner’s contribution to the project (identify one or more)*

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

## **8. SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:**

**QUAD CHARTS:**

## **9. APPENDICES:.**

# Patterns of Phrenic Nerve Discharge after Complete High Cervical Spinal Cord Injury in the Decerebrate Rat

Michael George Zaki Ghali and Vitaliy Marchenko

## Abstract

Studies conducted since the second half of the 19th century have revealed spontaneous as well as pharmacologically induced phasic/rhythmic discharge in spinal respiratory motor outputs of cats, dogs, rabbits, and neonatal rats following high cervical transection (Tx). The extent to which these various studies validate the existence of a true spinal respiratory rhythm generator remains debated. In this set of studies, we seek to characterize patterns of spontaneous phasic/rhythmic, asphyxia-induced, and pharmacologically induced activity occurring in phrenic nerve (PhN) discharge after complete high cervical (C<sub>1</sub>-C<sub>2</sub>) spinal cord transection. Experiments were performed on 20 unanesthetized decerebrate Sprague-Dawley adult male rats. Patterns of spontaneous activity after spinalization included tonic, phasic, slow oscillatory, and long-lasting tonic discharges. Topical application of antagonists of GABA<sub>A</sub> and glycine receptors to C<sub>1</sub>- and C<sub>2</sub>- spinal segments induced left-right synchronized phasic decrementing activity in PhN discharge that was abolished by an additional C<sub>2</sub>Tx. Asphyxia elicited increases in tonic activity and left-right synchronized gasp-like bursts in PhN discharge, demonstrating the presence of spinal circuits that may underlie a spinal gasping-like mechanism. We conclude that intrinsic slow oscillators and a phasic burst/rhythm generator exist in the spinal cord of the adult rat. If present in humans, this mechanism may be exploited to recover respiratory function in patients sustaining severe spinal cord injury.

**Key words:** breathing; gasping; respiration; spinal CPG; spinal cord transection

## Introduction

CERVICAL SPINAL CORD INJURY (SCI) results in a vast range of motor and sensory deficits, among which impaired breathing is one of the most devastating. While respiratory dysfunction is a leading contributor to morbidity and mortality among persons sustaining spinal (especially cervical) cord damage, there is mounting experimental and clinical evidence for spontaneous neuroplasticity and improvements in respiratory function.<sup>1</sup> Unfortunately, the extent of long-term recovery attributable to plasticity is limited to an extent corresponding to the level and degree of damage sustained.

While the spinal central pattern generator (CPG) for locomotion is well characterized, with its role in locomotor recovery after SCI also described in several studies, the anatomical and functional properties of the spinal CPG for respiratory-related activity remain poorly understood.<sup>2,3</sup> A spinal origin for respiration was proposed as early as the 19th century by Brown-Séquard (1860),<sup>4</sup> Langerdorff (1880),<sup>5</sup> and Wertheimer (1886),<sup>6</sup> who reported observing breathing after spinal transection (Tx). The proponents of a spinal respiratory rhythm generator therefore concluded that hemisection or complete Tx transiently inhibits breathing via spinal shock or “inhibition.”

In an elegant set of studies, Porter (1895)<sup>7</sup> sought to disprove the hypothesis of his contemporaries and helped to confirm a supraspinal origin for resting respiration (eupnea). He performed an additional Tx at the C<sub>5</sub> level, however, that could directly compromise phrenic motoneurons (PhMNs). It is now widely acknowledged that respiratory drive and pattern formation arise from neurons in the brainstem.<sup>8–11</sup> Accordingly, the existence of a spinal respiratory CPG became largely dismissed.

In the latter half of the 20th century, spontaneous recovery of respiratory activity was demonstrated after complete C<sub>1</sub>Tx in unanesthetized, decerebrate adult cats and dogs, the arterially perfused *in situ* preparation of the juvenile rat, and the *in vitro* preparation of the neonatal rat and mouse, providing evidence for the existence of a spinal mechanism underlying breathing.<sup>12–20</sup> St. John and associates<sup>21</sup> and Huang and St. John<sup>22</sup> (1988), however, failed to observe recovery of regular spontaneous respiratory activity after C<sub>1</sub>Tx in unanesthetized decerebrate cats. Hypoxia-induced gasping-like PhN activity with decrementing shape was recorded in some cats after C<sub>1</sub>Tx transection.<sup>21</sup> In the anesthetized state, spontaneous phasic activity in PhN discharge was not observed after Tx in numerous publications, but has been shown in dogs.<sup>12,13,23–27</sup>

Previous works have shown that the upper cervical (C<sub>1</sub>-C<sub>2</sub>) segments contain only inspiratory neurons in cats and monkeys and both inspiratory (~90%) and expiratory (~10%) units in rats.<sup>28-30</sup> It has been proposed that this group may provide excitation to inspiratory and inhibition to expiratory MNs via spinal interneurons.<sup>28-30</sup> According to the hypothesis of Aoki and colleagues,<sup>14,15</sup> the CPG for spinal breathing is located at the C<sub>1</sub>-C<sub>2</sub> (upper cervical respiratory group; UCRG) level. Ablation of UCRG after C<sub>2</sub>-C<sub>3</sub> Tx abolished more caudal respiratory discharges in cats,<sup>13,22</sup> but not in dogs, however.<sup>13</sup>

Discrepancies in these observations may be the result of the use of different species, preparations, and/or variability in the SCI proper regarding the level of Tx (C<sub>1</sub>-C<sub>2</sub> or C<sub>2</sub>-C<sub>3</sub>) and spread of descending edema and inflammation as a function of spinal cord size. Given this, the question about the critical importance of UCRG for the spinal respiratory CPG is still unresolved and requires further experimental elucidation.

Pharmacologically induced (e.g., by 5-HT, DOPA, strychnine, bicuculline, picrotoxin, and doxapram) rhythmic respiratory-related activity in unanesthetized decerebrate and intact anesthetized spinal dogs, cats, and rabbits has been demonstrated in a number of publications.<sup>5,6,12,13,31-35</sup> In all of these studies, pharmacological agents were injected intravenously, and only unilateral inspiratory activity was recorded. Unilateral phasic inspiratory activity, however, may be out of phase with the contralateral side, as was shown after midsagittal section of the medulla.<sup>36</sup>

Over the last several decades, the rat has become increasingly used as a model to study traumatic SCI, a trend favored not only by their availability, but also on account of similarity of morphological, biochemical, and functional changes that occur after SCI with those observed in humans.<sup>37</sup> The existence of a spinal CPG for respiration has been primarily investigated and shown in isolated (*in vitro*) brainstem-spinal cord preparations and slices from neonatal rats.<sup>17-20</sup> In anesthetized adult rats, however, spontaneous recovery of the spinal CPG for respiration following high cervical Tx has not been observed.<sup>24-26</sup> It is possible that anesthesia may suppress the spinal network responsible for recovery of inspiratory phasic discharges after high cervical Tx.

In this report, we seek to test the hypotheses that spontaneous, as well as asphyxia- and pharmacologically induced, phasic and/or rhythmic left-right synchronized phrenic nerve (PhN) activity can be generated by spinal mechanisms and become manifest after high cervical SCI in the unanesthetized decerebrate adult rat, as well as characterize these patterns. Also, the UCRG can be an important structure in recovery of inspiratory phasic activity after high cervical C<sub>1</sub> Tx. To evaluate this hypothesis, UCRG will be disinhibited by local (over C<sub>1</sub>-C<sub>2</sub> cervical segments) application of blockers of GABA<sub>A</sub> (gabazine; GABAz) and glycine (strychnine; STR) receptors and an additional Tx at the lower border of C<sub>2</sub> rootlets for ablation of UCRG will be performed.

## Methods

### Animal preparation

**General surgical preparation.** All procedures were approved by the Drexel University Institutional Animal Care and Use Committee, which oversees Drexel University's AAALAC International-accredited animal program. Twenty spontaneously breathing, Sprague-Dawley adult male rats (380-420 g) were initially anesthetized with isoflurane vaporized in O<sub>2</sub> (Matrix; 4-5% induction, 2.0-2.5% maintenance) via a snout mask. Anesthetic depth was maintained at a level such that withdrawal reflexes and changes

in heart rate and blood pressure in response to pinches of the distal hind limbs were absent. After tracheotomy with an atraumatic glass tube, animals were artificially ventilated with the same gas mixture (45-65 cycles\*min<sup>-1</sup>, 2.5-3.0 mL tidal volume; Columbus Apparatus). The electrocardiogram was measured via three small subcutaneous electrodes using conventional amplification and filtering (Neurolog; Digitimer, Hertfordshire, UK) and monitored using an audio amplifier (model AM10; Grass Instruments) and oscilloscope. One femoral artery and vein were cannulated for measurement of arterial pressure and infusion of drugs/fluids, respectively.

During initial surgical preparation and recordings, rectal temperature was maintained at 37.0±0.1°C via a servocontrolled heating blanket coupled to a rectal thermometer (Harvard Apparatus). Using a ventral approach, the PhNs were dissected free from the surrounding tissue and transected immediately superior to the subclavian vessels. The internal carotid arteries were gently dissected from surrounding neurovascular elements and ligated above the carotid bodies to prevent bleeding after decerebration.

After the ventral dissection, animals were placed prone and an incision was made from theinion to the spinous process of C7. Dorsal neck muscles (trapezius, suboccipital, semispinalis, and splenius capitis) were removed and soft tissue dissected off the cervical vertebrae in preparation for subsequent laminectomy. The previously ventrally dissected and transected PhNs were retrieved dorsally. Animals were then given a subcutaneous injection of dexamethasone (1 mg\*kg<sup>-1</sup>) to prevent/attenuate cerebral and spinal edema after decerebration and cervical Tx, respectively.<sup>38</sup> The dorsal cervical incision was closed using suture in preparation for animal transfer to a stereotaxic device.

**Decerebration.** After the initial surgical preparation, animals were placed prone in a stereotaxic device and arterial and tracheal cannulae were connected to pressure transducers (CDXII; Argon Medical) for monitoring tracheal and arterial blood pressure, respectively, using conventional amplifiers (Gould). The dorsal cervical incision was reopened and extended rostrally 2 mm anterior to bregma and the cranial convexity cleared of soft tissue.

Biparietal craniotomies were made using a variable-speed surgical drill (Foredom Electric), the dura was opened widely using iridectomy scissors, and the superior sagittal sinus ligated proximally and distally using suture and divided. The neuraxis was carefully transected at the rostral border of the superior colliculus using a blunt-edged microspatula to avoid severing the posterior communicating arteries at the cranial base. Brain tissue rostral to the Tx was removed by suction, and residual bleeding was prevented/arrested by packing the anterior and middle cranial fossae with small pieces of gelfoam (USP; Pharmacia) soaked in cold thrombin solution (50 U\*<sup>-1</sup> USP, dissolved in physiological saline).

**Laminectomy.** Immediately after decerebration, the skin over the craniotomy site was sutured and the head ventroflexed 20 degrees to improve access to the craniocervical junction. Rongeurs were then used to perform atlantoaxial (C<sub>1</sub>-C<sub>2</sub>) laminectomy. The dura was opened in the midline using iridectomy scissors from the caudal medulla to the lower border of the C<sub>2</sub> vertebra.

**Post-surgical stabilization.** Fifteen to 20 min after laminectomy, anesthesia was slowly withdrawn and animals were paralyzed by an intravenous bolus injection (2 mg\*kg<sup>-1</sup>) followed by continuous infusion (3-4 mg\*kg<sup>-1</sup>h<sup>-1</sup>) of vecuronium bromide (Abbott Labs) dissolved (0.4 mg\*<sup>-1</sup> mL<sup>-1</sup>) in Ringer-Locke solution to maintain a stable mean arterial pressure (MAP) of 90-100 mm Hg pre-spinalization and 70-80 mm Hg after cervical Tx. End-tidal CO<sub>2</sub> was maintained between 5.0-5.2% (Capstar CWE) by adjusting ventilation rate. PhN recordings were initiated ≥1 h after decerebration (approximately 40 min after laminectomy). The

central ends of the PhN were placed on bipolar silver electrodes (AM Systems; Cat# 787000, diameter 0.01"; interelectrode distance 5 mm) and immersed in a mineral oil pool formed by skin flaps.

**Cervical Tx.** After control recordings before cervical Tx in the unanesthetized decerebrate state, isoflurane (2–3%) was added to the ventilated gas mixture to minimize excitotoxic damage and spinal shock from subsequent spinal cord lesioning. When eupneic PhN discharge became suppressed in amplitude by ~50%, high cervical C<sub>1</sub> (2.5 mm caudal to the calamus scriptorius) or C<sub>2</sub> (immediately caudal to C<sub>2</sub> rootlets) Tx was performed using a sharp microscalpel blade or iridectomy scissors with (n = 11) or without (n = 9) aspiration of the caudal medulla (Fig. 1). The location of C<sub>1</sub> dorsal rootlets in rats is 2.5–3.0 mm below the calamus scriptorius or 0.5–1 mm below the upper edge of the posterior arch of the first cervical vertebra.

In experiments without aspiration of the caudal medulla, hypoglossal nerve (XII) activity was additionally recorded as a motor output of medullary respiratory rhythm generation that was not phase-locked to PhN bursting activity below C<sub>1</sub>- (C<sub>1</sub>Tx) or C<sub>2</sub>-transections (C<sub>2</sub>Tx). For ablation of UCRG, all C<sub>2</sub>Tx were performed at the lower border of C<sub>2</sub> rootlets. Anesthesia was withdrawn immediately thereafter (Fig. 1). Completeness of decerebration (i.e., level) and spinalization was confirmed macrohistologically after experiments. Tx was performed at the C<sub>1</sub> (n = 12 [8/12 with aspiration of caudal medulla]) or C<sub>2</sub> (n = 8 [3/8 with aspiration of caudal medulla]) levels.

**GABAz/STR.** Six C<sub>1</sub>Tx animals received local treatment of C<sub>1</sub>-C<sub>2</sub> spinal segments with GABAz (SR95531, Sigma-Aldrich) and STR (Sigma-Aldrich), 3 h after cervical Tx, using topical application (technique adapted from Lu and colleagues<sup>39</sup>), in animals exhibiting no recovery of spontaneous activity after myelotomy. A cocktail of GABAz/STR (final concentration of 4 mM each) in artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 1.25 mM NaHCO<sub>3</sub>, 4 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.25 mM MgSO<sub>4</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, pH = 7.4) was micropipetted onto a filter paper pledget (3 × 2 mm) and placed on the dorsal surface of the C<sub>1</sub> and upper C<sub>2</sub> spinal segments for 10 min. Other investigators have also used this approach of combined (i.e., GABA<sub>A</sub> and glycinergic) blockade of fast inhibitory synaptic transmission.<sup>40</sup>

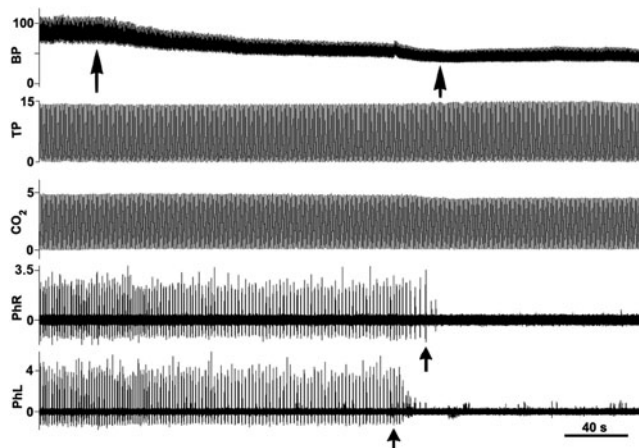


**FIG. 1.** Compressed record of C<sub>1</sub> transection. Start of lesioning is indicated by the arrow in the left phrenic nerve (PhL) neurogram and end of lesioning by the arrow in the right phrenic nerve (PhR) neurogram. Isoflurane (2%) is administered during the period of injury:—on (arrow on left side of blood pressure [BP] trace), off (arrow on right side of BP trace). TP, tracheal pressure (cm H<sub>2</sub>O); CO<sub>2</sub> (%), end-tidal carbon dioxide.

Cottonoids soaked in aCSF placed immediately after durotomy to protect the spinal cord (and replaced several times during the recovery period) served as the control treatment (n = 6), because the active treatment solution used (GABAz/STR 4 mM each in aCSF) is essentially isotonic to plasma (in all animals not treated with GABAz/STR, cottonoids soaked in aCSF were placed on the dorsal surface of the spinal cord for protection from parenchymal dehydration). These were subsequently removed, the area irrigated with aCSF, and a cottonoid soaked in the same placed to protect the spinal cord from drying. We eschewed the use of the commonly used GABA<sub>A</sub> receptor antagonist bicuculline, because its blockade of Ca<sup>2+</sup>-activated K<sup>+</sup> channels may cause nonspecific excitation.<sup>41</sup>

Investigators using topical application of a drug have typically used a concentration at least an order of magnitude that of the standard microinjection concentration to achieve adequate concentration at the intended site of action (e.g., 1 M L-glutamate).<sup>39</sup> In seeking to avoid nonspecific effects resulting from hypertonicity or excessive drug concentration, we used the standard microinjection concentration of GABAz and STR for topical application, resulting in a correspondingly lower concentration of drug at the intended site of action (C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons) and a negligible concentration at distant sites.<sup>42</sup> A longer duration of application was used to allow a slow steady infusion of drug to C<sub>1</sub>-C<sub>2</sub> spinal neurons.

**Interventions.** After Tx, hypercapnic challenges (10% CO<sub>2</sub>, 90% O<sub>2</sub>) were performed in all animals to physiologically confirm completeness of the Tx by failure to elicit eupneic activity. Lung inflation (a classic test for the Hering-Breuer reflex that strongly inhibits inspiration via activation of lung stretch receptors) was performed via expiratory occlusion to a peak tracheal pressure at least 1.5 times control peak tracheal pressure to physiologically confirm a nonpontomedullary (e.g., spinal) origin of activity for spontaneous and GABAz/STR-induced activity in PhN discharge after cervical Tx. Ventilator shutdown was also performed in animals to exclude that spontaneous and GABA/STR-induced activity in PhN discharge is maintained by entrainment to the ventilator rhythm.

**Recordings.** Recordings (10<sup>2</sup> gain; 10–5,000 Hz band pass filter; Neurolog, Digitimer, UK) of efferent activity were obtained from the peripheral ends of each PhN before, during, and (up to 4–6 h) after high cervical Tx in all animals. The electrical activity of the two PhNs, expiratory CO<sub>2</sub> level, arterial blood pressure, and tracheal pressure were recorded onto the hard disk of a personal computer at 10,000 samples/sec, using a 16-bit A/D converter with visualization software (ADInstruments). “ADI”-files were transferred into MATLAB and Spike-2 (CED, UK) software format for data analyses. Integrated ( $\int$ ) PhN activity was estimated off line with  $\tau = 50$  msec (time constant). PhN neurogram amplitude is shown in millivolts (mV) with gain coefficient 10<sup>-2</sup> for all figures.

**Asphyxia testing, perfusion, and fixation.** At the end of the experiments, asphyxia tests were performed in some animals (n = 8; 3/12 animals after C<sub>1</sub>Tx and 5/8 animals after C<sub>2</sub>Tx) by turning off ventilation. This group in which asphyxia-induced activity was systematically analyzed did not receive previous GABAz/STR treatment, although all animals treated with GABAz/STR received asphyxia, and this was a separate analysis. To reverse anoxia-induced edema of neural tissues, when phasic and/or tonic phrenic activity approached an isoelectric baseline, animals were immediately resuscitated by reinstituting artificial ventilation followed by slow infusion of 2 mL of 5% dextrose in Ringer-Locke (over 10–20 sec) to recover hemodynamic status to a MAP  $\geq 70$  mm Hg.

Subsequently, animals were transcatheterially perfused with 500 mL of aCSF (20°C, pH 7.4) with heparin (1000 U·L<sup>-1</sup>) followed by 500 mL 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The craniospinal neuraxis was removed and post-fixed in

the same fixative for 48 h at 4°C for assessment of level of decerebration and completeness of spinal Tx.

### Data analysis

In analyzing spontaneous recovery patterns after high cervical Tx, animals were segregated into groups according to the type of spinal cord lesion sustained, as described earlier. Patterns of spontaneous discharge were then classified as tonic (no discernible phasic or oscillatory activity), phasic (discernible bursting <10 sec in duration), slow oscillatory (regular oscillatory activity with a cycle length >10 sec), or long-lasting (>1 min, irregular cycle length) tonic discharges. Mean and standard error (SE) of burst duration ( $T_b$ ) and cycle length ( $T_c$ ) were then determined using MATLAB. Values for the population are given as mean  $\pm$  SE. For small-sample categorical comparisons, Fisher's exact test was used. The qualitative presence or absence of bursting in response to asphyxia was determined and burst durations calculated accordingly.

In analyzing the GABAz/STR-induced activity, PhN output variables including burst duration and cycle length were determined post-spinalization using  $\geq 30$  cycles (normal breathing; control recording taken under unanesthetized decerebrate conditions during a period of stable arterial and tracheal pressure and end-tidal  $\text{CO}_2$ ) of GABAz/STR-induced phrenic bursting (activity was analyzed starting in the second half of the drug application interval at which time bursting frequency had risen to a stable plateau), respectively. Spike2 (version 6, Cambridge Electronic Design), MATLAB (version R2012a, The MathWorks), IBM SPSS STATISTICS 20, and custom-written scripts for measurement of parameters from PhN were used for data analysis. Event markers for onset and offset of integrated ( $\tau = 50$  ms) PhN activity were derived from the raw recordings.

## Results

### General

After high cervical ( $\text{C}_1\text{-C}_2$ ) Tx, activity in phrenic discharge was silenced in all animals during 1.5–2.5 h after high cervical Tx. We observed four general patterns of subsequent spontaneous phrenic activity: (1) exclusive tonic activity (Fig. 2A, lower panel), (2) phasic discharge (Fig. 2B–D and 3), (3) slow oscillations—phrenic slow oscillations (PSOs) (Fig. 4B), and long-lasting tonic discharges (Fig. 4C).

In  $\text{C}_1$ -transected animals, application of GABAz/STR over  $\text{C}_1\text{-C}_2$  segments induced left-right synchronized PhN bursting (Fig. 5). After an additional Tx performed at the  $\text{C}_2$  level, GABAz/STR-induced bursts were completely abolished (Fig. 5C). Asphyxia-induced bursting after  $\text{C}_1$ - or  $\text{C}_2$ -Tx was characteristically decrementing, as occurs in terminal gasping in spinal-intact animals<sup>21</sup> (Fig. 6–8). Responses to asphyxia in GABAz/STR pre-treated animals were characterized mostly by an increase in tonic PhN discharge (Fig. 9). Three hours after cervical Tx, hypercapnia (10%  $\text{CO}_2$ ,  $\text{O}_2$  balance) tests failed to elicit eupnea or any activity in PhN discharge in all animals.

### Spontaneous recovery

The most common pattern of spontaneous phrenic discharge after Tx was tonic activity (10/20 animals; 6/12  $\text{C}_1$ Tx and 4/8  $\text{C}_2$ Tx) with no statistical difference of occurrence incidence in  $\text{C}_1$ Tx vs.  $\text{C}_2$ Tx groups ( $p > 0.05$ ; Fisher exact test). This pattern was marked by the absence of discernible phasic discharges or variations in the baseline tonic activity (Fig. 2A, lower panel).

We observed nonrhythmic asynchronous phasic discharge in  $\text{C}_1$ Tx and  $\text{C}_2$ Tx (7/20 animals): 4/12  $\text{C}_1$ Tx (2 bilateral, 2 unilateral

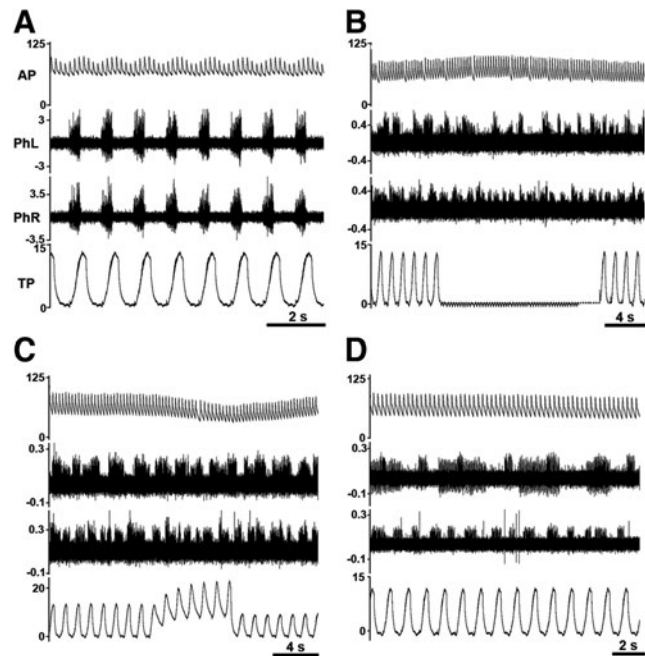


**FIG. 2.** Phasic discharge in a  $\text{C}_1$ Tx (transection) animal. (A) Eupnea. (B) Phasic discharge emerges in both phrenic nerves after Tx and persists after turning off the ventilator. (C) Nonsuppression by lung inflation test. (D) Differential evolution of rhythmic discharge in left and right phrenic nerves. Rhythm in left phrenic nerve (PhL) becomes biphasic—short and intermediate duration bursts are intermingled. AP, arterial pressure (mm Hg); PhR, right phrenic nerve; TP, tracheal pressure (cmH<sub>2</sub>O).

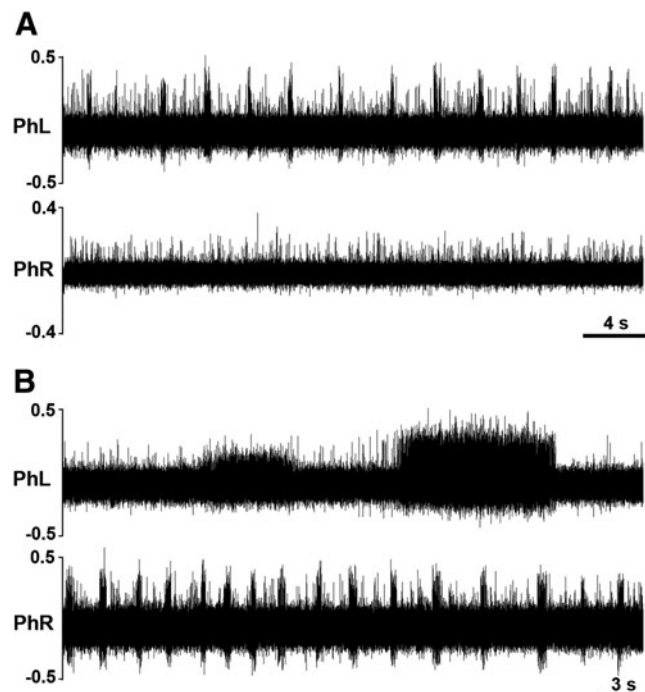


**FIG. 3.** Examples of unilateral phasic discharges in  $\text{C}_1$ Tx (transection) animals. Unilateral phasic discharge in two animals with the additional presence of tonic (A) and tonic with irregular phrenic slow oscillations (B). PhL, left phrenic nerve; PhR, right phrenic nerve.

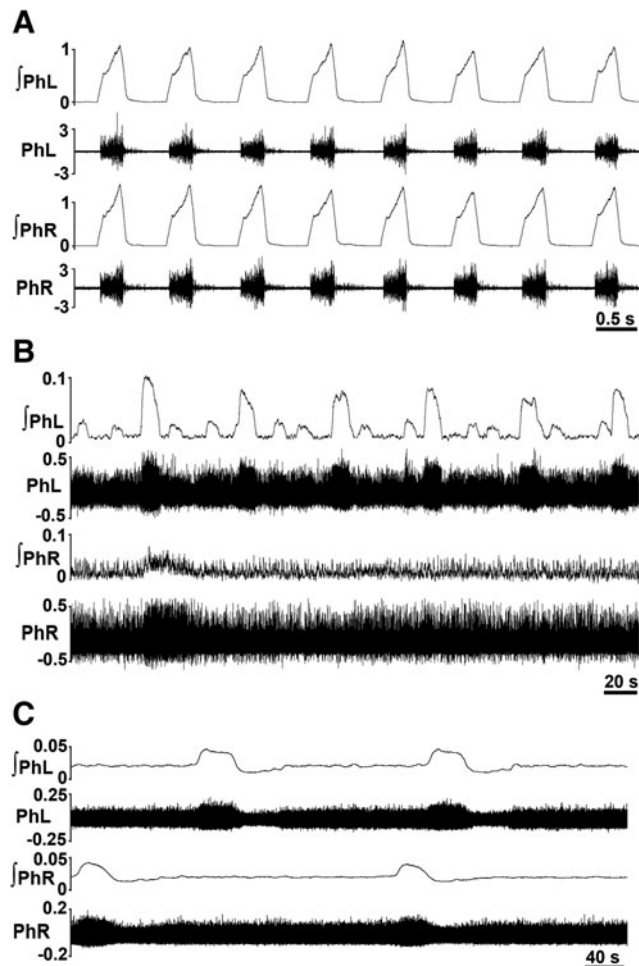


**FIG. 4.** Phrenic slow oscillations in a  $C_2$ Tx (transection) animal. (A) Eupnea. (B) Rhythmic slow oscillations appear in left phrenic nerve (PhL) and short duration phasic activity in right phrenic nerve (PhR). (C) Long-lasting tonic discharge with increasing amplitude at the end of the tonic epoch in both PhNs.

left), 3/8  $C_2$ Tx (1 bilateral, 2 unilateral left) (Fig. 2B–D and Fig. 3). Burst durations ( $m \pm SE$ ) averaged across all animals was  $0.90 \pm 0.36$  sec, for the  $C_1$ Tx group –  $1.59 \pm 0.68$  sec, and for the  $C_2$ Tx group –  $0.38 \pm 0.11$  sec. Cycle lengths averaged across all animals were  $2.06 \pm 0.25$  sec, for the  $C_1$ Tx group –  $2.48 \pm 0.41$  sec, and for the  $C_2$ Tx group –  $1.70 \pm 0.33$  sec. There were no statistical differences in the occurrence of phasic activity between  $C_1$ Tx and  $C_2$ Tx groups ( $p > 0.05$ ; Fisher exact test).

Phasic discharges had a peaked profile on the raw neurogram or were plateau in shape, the latter with action potentials of nearly uniform height, suggesting the firing of a single unit or a few cells contributing to the discharge. A noneupneic origin for phasic discharge in spinal PhN activity was confirmed by nonsuppression in response to lung inflation (Fig. 2C); maintenance of rhythmic-like activity by the ventilator was excluded by persistence of the rhythm after turning off the respiratory pump (Fig. 2B).

Irregular slow oscillatory discharge (PSOs) occurred relatively infrequently (3/20 animals: 2/12  $C_1$ Tx [bilateral] and  $C_2$ Tx [1 unilateral left]) (Fig. 4B). Cycle length averaged across all animals was  $33.75 \pm 7.96$  sec. Long-lasting irregular asynchronous tonic discharges with increasing amplitude at the end of the tonic discharge epoch were observed in two animals (Fig. 4C). The average

duration of tonic activity was  $247 \pm 54$  sec, and length of diminished amplitude between tonic discharges was  $38 \pm 12$  sec.

#### GABAz/STR-induced phrenic bursting

Local treatment of  $C_1$ – $C_2$  spinal segments with GABAz/STR 3 h after  $C_1$ Tx transection induced left-right synchronized phrenic bursting that was significantly less regular than eupnea with respect to both rhythm and amplitude (Fig. 5). Time from drug application to the first burst ( $T_{onset}$ ) was  $107.17 \pm 30.25$  sec. GABAz/STR-induced phasic activity in PhN discharge evolved from a slow regular rhythmic-like activity with occasional doublets (Fig. 5D1) to a pattern exhibiting clusterization (Fig. 5D2) and finally to diminishing clusterization (Fig. 5D3) and a return to more regular rhythmic like-activity (Fig. 5B). Lung inflation tests suppressed phasic eupneic activity pre-Tx and failed to suppress post-Tx GABAz/STR-induced bursting (all GABAz/STR-treated animals). Completeness of spinal Tx was subsequently confirmed histologically in all cases.

Burst duration of GABAz/STR-induced phrenic bursting and bursting frequency averaged  $1.74 \pm 0.19$  sec and  $6.99 \pm 0.51 \text{ min}^{-1}$ , respectively. It should be noted that while onsets were tightly coupled, offsets were not. The functional and mechanistic implications of rhythm variability and stochasticity will be described in another article. After an additional  $C_2$ Tx, GABAz/STR-induced bursts were completely abolished.

#### Asphyxia-induced bursting and increase in tonic discharge

Asphyxia tests induced bursting in PhN discharge in six of eight transected animals (2/3  $C_1$ Tx and 4/5  $C_2$ Tx). This group did not receive previous GABAz/STR treatment. Responses to asphyxia included a terminal increase in tonic discharge (mono- or multiphasic) with (6/8 animals) or without (2/8 animals) regular decrementing (a rapid rise to maximal integrated activity occurring before half of the normalized inspiratory epoch, followed by a slower descent of the integrated activity) bursts superimposed on the increase in tonic activity (Fig. 6–8).

In two of three asphyxia-tested  $C_1$ Tx animals, decrementing bursts prominently discernible from the baseline increase in tonic discharge were observed in response to asphyxia. In one animal, this was regular and rhythmic (Fig. 6) and in another, only a few bursts occurred (Fig. 7). In the latter animal, left-right synchronized decrementing bursts were seen in both PhN and hypoglossal discharge, but phrenic and hypoglossal bursts were out of phase with each other. In the third  $C_1$ -transected animal, there was only an increase in tonic discharge without superimposed phasic activity.

In the  $C_2$ Tx group, superimposed phasic bursting occurred in four of five animals (Fig. 8). In  $C_1$ -transected animals, asphyxia-induced bursting was characteristically uniformly decrementing, as occurs in terminal gasping in spinal-intact animals<sup>21</sup> (Fig. 6), while in  $C_2$ -transected animals, they were more polymorphic by shape, duration, and amplitude (Fig. 8). In transected animals, duration of asphyxia-induced bursts averaged  $0.88 \pm 0.18$  sec over all animals,  $0.67 \pm 0.29$  sec in  $C_1$ Tx animals, and  $0.99 \pm 0.23$  sec in  $C_2$ Tx animals.

Asphyxia testing was performed in all GABAz/STR-treated animals. Responses to asphyxia in GABAz/STR pre-treated animals were characterized by an increase in tonic discharge after ventilation withdrawal (Fig. 9). In four of six animals, GABAz/STR-induced bursting persisted into the period of asphyxic challenge, overlapping the increase in tonic discharge; in the remaining

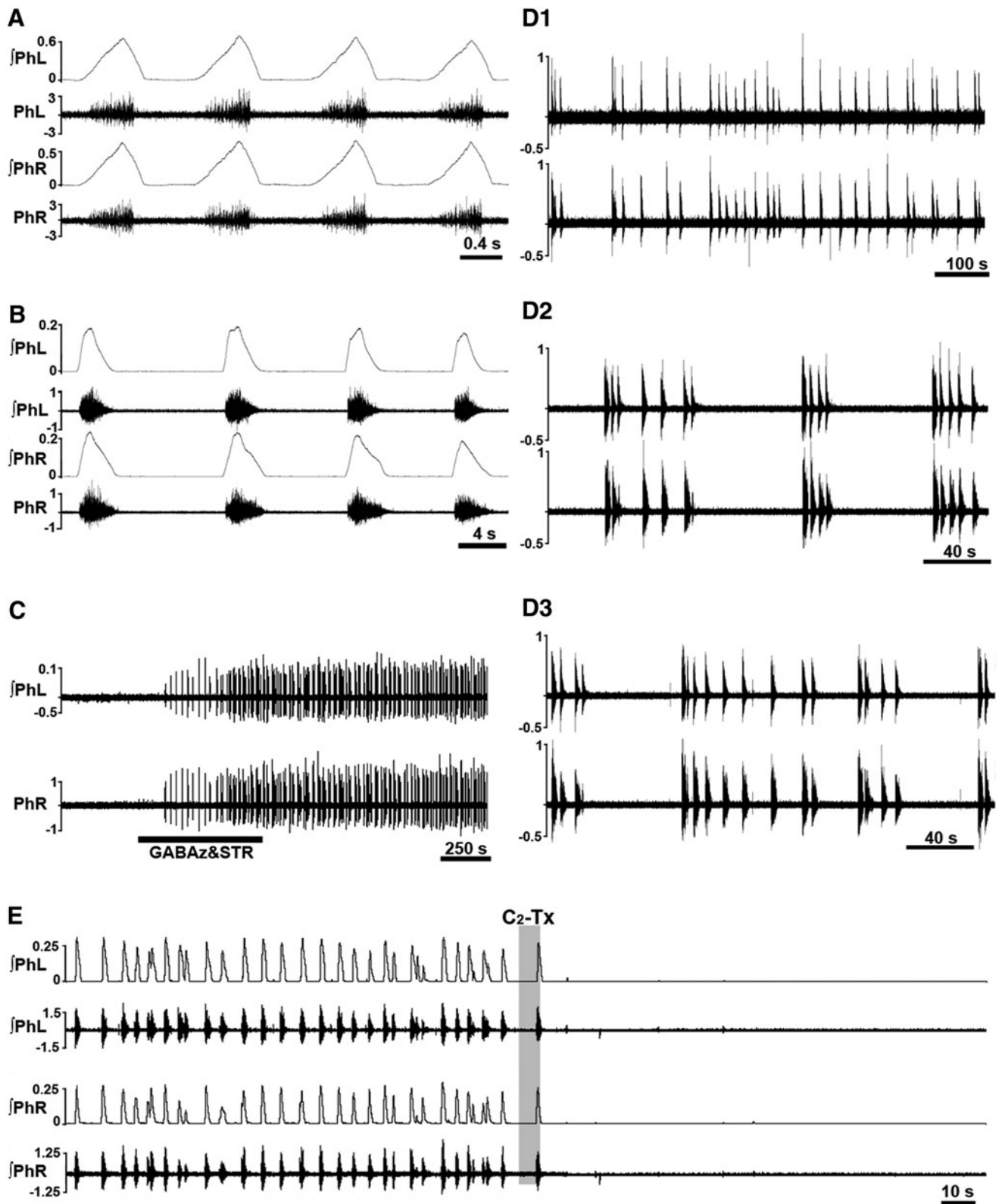


**FIG. 5.** Gabazine and strychnine (GABAz/STR)-induced phasic activity in phrenic nerve (PhN) discharge after  $C_1$ -spinalization compared with eupneic bursting. **(A)** Eupnea. **(B)** GABAz/STR-induced PhN activity. **(C)** Compressed record of GABAz/STR application. **(D)** Evolution of GABAz/STR-induced phasic PhN activity from slow regular rhythmic-like activity with occasional doublets (D1), to a pattern exhibiting clusterization pattern (D2), to diminishing cluster bursting and a return to regular rhythmic-like activity (D3). **(E)** Abolition of GABAz/STR-induced phasic PhN activity in  $C_1$ Tx (transection) animal after additional Tx at the  $C_2$  level. Note the inconsistency of cycle length and burst amplitude during GABAz/STR-induced phrenic bursting compared with the normal eupneic rhythm, as well as the difference in burst shape of the former (decrementing profile) compared with the latter (augmenting or ramp-like pattern). Integrated PhN activity ( $\int$ PhN) is shown in arbitrary units.

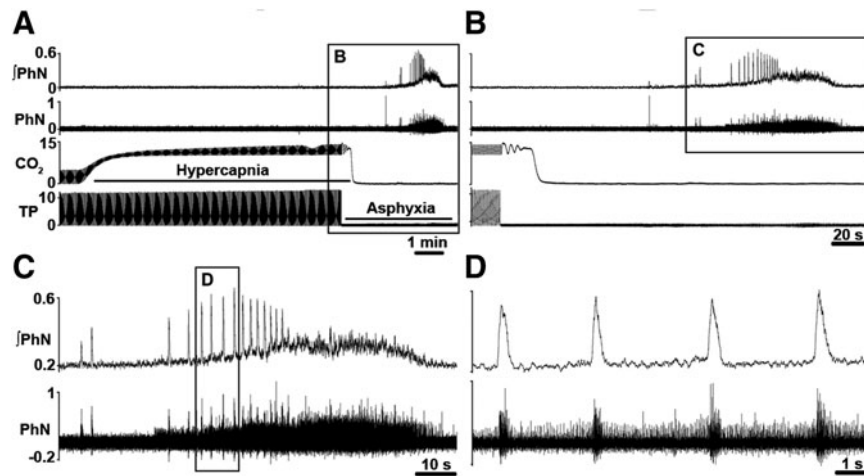


**FIG. 6.** Decrementing pattern of asphyxia-induced phrenic bursting in a  $C_1$ Tx (transection) animal. (A) 10% hypercapnia tests fail to elicit eupneic or any other activity in phrenic nerve (PhN) discharge. An asphyxia test performed immediately thereafter reveals the appearance of phasic PhN activity superimposed on an increase in tonic discharge. (B–D) Magnified views of insets in A, B, and C, respectively. Asphyxia-induced bursts have a decrementing pattern highly reminiscent of classic brainstem gasping (lower right subpanel D). TP, tracheal pressure;  $CO_2$ , end-tidal carbon dioxide.

two animals, a cessation of bursting occurred with only an asphyxia-induced increase in tonic discharge observed.

## Discussion

We report on the existence of multiple patterns of phrenic nerve activity occurring after complete, high cervical SCI spontaneously and in response to asphyxia and pharmacological blockade of fast inhibitory synaptic transmission in decerebrate adult rats. There are five principal findings of this study. First, we report that in the majority of experimental observations, tonic activity is the most typical spontaneous pattern observed after high cervical Tx and may represent emergence of spasticity as a consequence of associated loss of descending tonic inhibition.<sup>43,44</sup> Second, irregular asynchronous phasic activity in PhN discharge after high cervical Tx occurring spontaneously is an occasionally observed pattern in adult rats.

Third, we report on the infrequent occurrence of slow oscillations in PhN discharge in adult rats sustaining high cervical SCI. Fourth, in both  $C_1$ - and  $C_2$ Tx animals, asphyxia elicits tonic activity and left-right synchronized gasp-like bursting in PhN discharge.

Last, in  $C_1$ -transected animals, GABAz/STR treatment of  $C_1$ - $C_2$  spinal segments induces phasic synchronous activity in PhN discharge bilaterally that is abolished after an additional  $C_2$ Tx.

Our results extend the findings of other investigators indicating the presence of phasic activity in respiratory neural/muscle output after high cervical Tx to the adult rat and validate the hypothesis for the existence of a spinal respiratory generator. In addition, we propose the existence of spinal mechanisms that may be involved in the generation of gasp-like bursting in concert with or independently of medullary regions classically described to underlie the same.

## Technical considerations

Although we cannot definitively exclude drug diffusion to the phrenic nucleus (i.e., via systemic routes), measures were taken to prevent subdural diffusion, and it is unlikely that a sufficient concentration reached the phrenic nucleus for this to account for observed effects. First, the GABAz/STR-soaked pledget was placed over  $C_1$  and upper  $C_2$  spinal segments, maintaining a gap between the location of drug application and phrenic nucleus levels. Second,

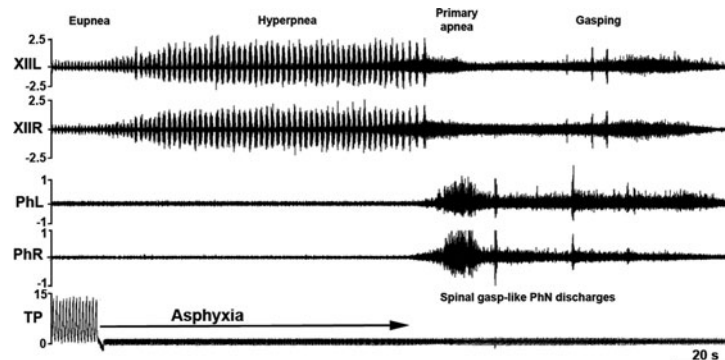


**FIG. 7.** Out-of-phase asphyxia-induced phrenic and hypoglossal bursting in a  $C_1$ Tx (transection) animal. Asphyxia induces brainstem gasping in hypoglossal nerve (XII) discharge that is left-right synchronized but uncoupled from left-right synchronized phrenic nerve (PhN) bursts occurring in response to the same asphyxia test. PhL, left phrenic nerve; PhR, right phrenic nerve; XIIIL, left hypoglossal nerve; XIIR, right hypoglossal nerve; TP, tracheal pressure.

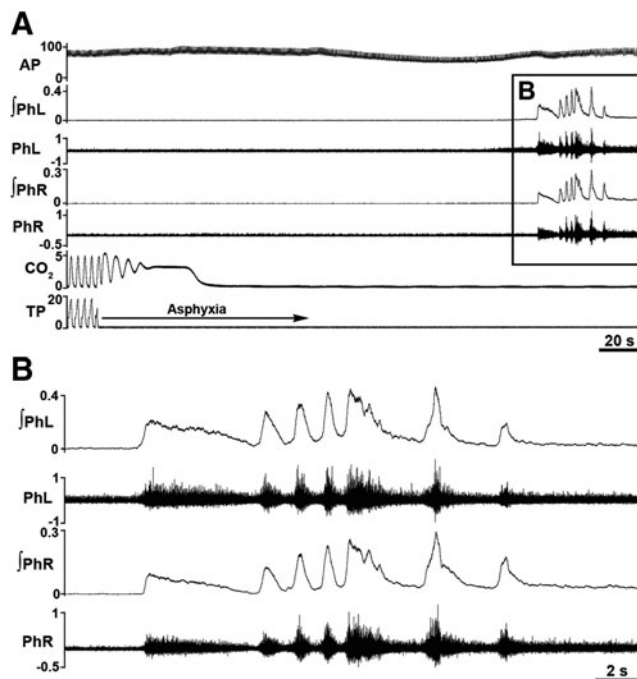


**FIG. 8.** Asphyxia-induced phrenic bursting in a C<sub>2</sub>Tx (transection) animal. (A) Hemodynamic and phrenic output dynamics in response to asphyxia. (B) magnified view of inset in A. AP, arterial pressure; PhL, left phrenic nerve; PhR, right phrenic nerve; CO<sub>2</sub>, end-tidal carbon dioxide; TP, tracheal pressure.

laminectomy was not performed over C<sub>3</sub>–C<sub>5</sub>. Third, a thin oil-soaked cottonoid was placed on the dorsal surface of the caudal half of the C<sub>2</sub> spinal segment to prevent subdural diffusion. Fourth, the cavity created by aspiration of the medulla was packed with a small oil-soaked cottonoid. Fifth, the cervical spinal cord was maintained in a slight incline from rostral to caudal levels with the C<sub>7</sub> vertebra fixed at a level higher than C<sub>1</sub>–C<sub>2</sub>. Last, GABA<sub>A</sub>/STR-induced phasic activity in PhN discharge was abolished by C<sub>2</sub>Tx.

#### *Patterns of spontaneous activity in phrenic discharge after high cervical Tx*

Phasic activity in PhN discharge was either bilateral and asynchronous or unilateral. The bilateral asynchronous (3/20) or

unilateral (4/20) activity recorded after C<sub>1</sub>- or C<sub>2</sub>Tx may be the result of independent half-center spinal CPGs, which become merged into one coordinated block by (1) supraspinal drive in intact animals or (2) asphyxic challenges or pharmacological disinhibition in spinalized animals (see below). Unilateral PhN phasic discharges (4/20) may result from either the activity of a half-center spinal CPG with reciprocal inhibitory connections silencing the contralateral half-center spinal CPG or functional asymmetry of left and right spinal circuitry underlying these discharges; further studies are needed to resolve the mechanistic underpinnings of this phenomenon.

In this report, we confirm that these discharges are of spinal origin and may have a source caudal to the putative upper cervical respiratory group, as they occurred in both C<sub>1</sub>- and C<sub>2</sub>Tx animals. Moreover, they are unrelated to entrained respiratory pump activity, because discharges are (1) observed during ventilator inspiratory and expiratory epochs (Fig. 2) and (2) persist after turning off the pump (Fig. 2B). Our results, however, did not confirm the recovery of synchronous rhythmic spontaneous respiratory activity, as observed in unanesthetized and anesthetized cats, rabbits, and dogs.<sup>4-6,12-15</sup>

Phasic PhN discharges may putatively represent a rudimentary respiratory activity, which can become incorporated in a more complicated spinal network along with the locomotor CPG. There exist some analogies and coupling between breathing and locomotor activity in cats.<sup>31-35</sup> It has been demonstrated that phase-locked rhythmic discharges of phrenic and hindlimb muscle nerves can be elicited in spinalized rabbits treated with DOPA (precursor to catecholamine neurotransmitters: dopamine, norepinephrine [noradrenaline], and epinephrine [adrenaline]) or nialamide (non-selective, irreversible inhibitor of monoamine oxidase—enzyme responsible for catecholamine degradation).<sup>31-35</sup>

Authors observed that rhythmic bursts usually developed first in the hindlimb nerves and the frequency of phrenic bursting was reduced after a second spinal Tx at the lower thoracic (T<sub>12</sub>) level.<sup>31</sup> They suggested that a spinal phrenic rhythm may be driven by hindlimb locomotor generators. In the experiments of Aoki and coworkers<sup>15</sup> in 1980, however, diaphragmatic activity occurred usually without hindlimb muscle activity, and even when hindlimb stepping rhythm developed simultaneously, the phrenic rhythm was not locked to it. These findings may support the view that spinal respiratory and locomotor generators exist independently.

Because the increase in breathing frequency occurred during the hindlimb stepping movements, possibly from proprioceptive limb

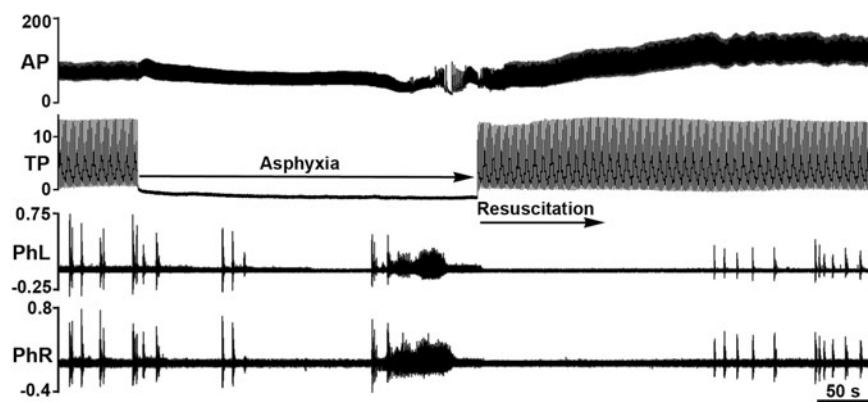


**FIG. 9.** Response to asphyxia of gabazine and strychnine (GABA<sub>A</sub>/STR)-induced PhN phasic activity. AP, arterial pressure (mm Hg); TP, tracheal pressure; PhL, left phrenic nerve; PhR, right phrenic nerve.

afferents, some spinal interconnections may exist between respiratory and stepping generators. It is considered that under certain pharmacological conditions, respiratory and stepping rhythm generators may be more tightly coupled.<sup>32,45–47</sup> A more thorough understanding of integration and synchronization of spinal CPGs mediating different behaviors needs further investigation.

PhN slow rhythmic oscillations were observed in a relatively small number of animals (3/20) (Fig. 4B). These discharges may be reminiscent of what was reported as a spinal pattern of activity in a spinal-intact *in vitro* preparation of the neonatal rat in response to pharmacological activation using L-glutamate, amphetamine, 5-hydroxytryptophan, and a low potassium solution, persisting in only one animal after a C<sub>2</sub>Tx in that study.<sup>19</sup> That a pharmacologically induced pattern in neonatal animals is analogous to a spontaneously occurring spinal pattern in an adult animal lends credence to the proposition that drug-induced discharge after cervical Tx may represent native patterns coded for by intrinsic spinal networks.

In two animals, we observed irregular asynchronous long-lasting tonic discharges with increasing amplitude at the end of the tonic discharge epoch. We also observed diminished PhN activity between

tonic discharge epochs, which may result from initialization of inhibitory mechanisms after phasic-like increases in amplitude toward the end of tonic discharges. This may represent a “transitional” (metastable) pattern between tonic and slow oscillatory activity.

#### GABAz/STR-induced phrenic bursting after cervical Tx

Phasic activity was induced in PhN discharge after C<sub>1</sub>-transection by treatment of the dorsal surface of the upper cervical (C<sub>1</sub>-C<sub>2</sub>) spinal cord with GABAz/STR (Fig. 5B). Burst duration was significantly longer than that of eupnea and exhibited a decrementing firing pattern (Fig. 5A, B). The decrementing shape of these bursts resembles that observed in C<sub>4</sub> rootlet discharges in the *in vitro* brainstem-spinal cord *en bloc* preparation of the neonatal rat and may thus reflect disinhibition of a latent generator in neonates.<sup>17–20</sup> Regularity of spinal phrenic activity was significantly less than that of eupnea. These synchronous bilateral PhN discharges were completely abolished after an additional C<sub>2</sub>Tx. This may prove an important role for UCRG in the genesis and/or support of spinal central pattern generating mechanisms for respiration.

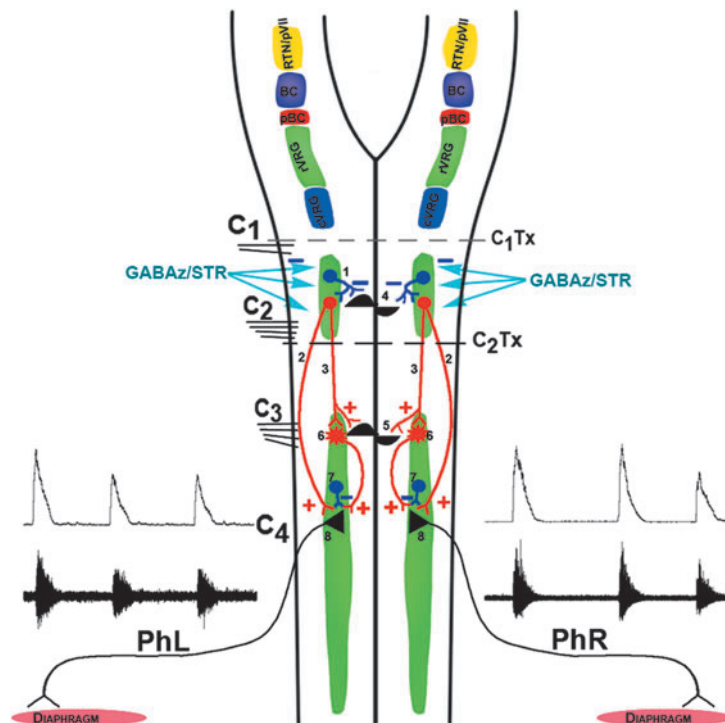


**FIG. 10.** Conceptual models of brainstem and spinal generators underlying phasic activity in phrenic nerve discharge. Color traces indicate excitatory (+, red) and inhibitory (-, blue) synapses. Top: Medullary drive to phrenic nucleus. The retrotrapezoid/parafacial area (RTN/pVII, yellow) is a central chemoreceptor region that is stimulated by hydrogen ions (from the reaction of arterial CO<sub>2</sub> with H<sub>2</sub>O) and provides tonic excitatory support to the pre-Bötzinger complex along with other elements in the medullary respiratory network. The Bötzinger (BC, violet) and pre-Bötzinger (pBC, dark red) complexes interact to generate the respiratory rhythm and control bulbospinal inspiratory neurons in the rostral ventral respiratory group (rVRG, green) and bulbospinal expiratory units in the caudal ventral respiratory group (cVRG, cyan). (1) Gabazine and strychnine (GABAz/STR) treatment of C<sub>1</sub>-C<sub>2</sub> spinal neurons removes inhibition of a generator (4) located among C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons that in turn provide phasic excitation (2) to phrenic motoneurons (PhMNs) directly or via phrenic interneurons. In addition to cessation of phasic excitation, tonic inhibition contributes to burst termination. The data are insufficient to speculate with regard to a source for phasic inhibition. GABAz/STR may disinhibit C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons, which in turn provide tonic excitatory drive to a phrenic generator (5). GABAz/STR-disinhibited excitatory C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons activate PhMNs (8) directly (2) or indirectly (3) via C<sub>3</sub>-C<sub>5</sub> pre-phrenic interneurons (6). PhMNs also receive tonic inhibition from intraphrenic interneurons (7). In this model, the phrenic nucleus is the originator of phasic excitation and possesses tonic inhibitory elements that contribute to burst termination. In either model, the generator may function as a network oscillator or discharge by virtue of the presence of intrinsic bursting units (i.e., pacemaker-like cells). PhL, left phrenic nerve; PhR, right phrenic nerve. Color image is available online at [www.liebertpub.com/neu](http://www.liebertpub.com/neu)

### *Patterns of activity in PhN discharge in response to asphyxia*

The response to asphyxia/anoxia in the bulbospinal intact animal involves a classic four-phase sequence consisting of (1) hyperpnea, (2) primary apnea, (3) gasping, and (4) terminal (or secondary if an autoresuscitation attempt recovers the animal) apnea. Some suggest that gasping (i.e., asphyxia-induced terminal bursting) results from functional reorganization of a common medullary respiratory network that also generates eupnea, while others propose there exist distinct generators mediating eupnea and gasping.<sup>48–57</sup>

According to the current point of view, during severe hypoxia, pre-Bötzinger complex (pre-BötC) neurons (part of the ventral respiratory group circuitry that is responsible for inspiratory rhythm generation), switch their firing mode from network-incorporated elements to pacemaker-like discharge by activation of persistent-sodium channels and appear to be necessary for gasping.<sup>49,50</sup>

In the terminal phase of the response to asphyxia, we observed decrementing bursts reminiscent of classic gasping in a few animals after C<sub>1</sub>- and C<sub>2</sub>Tx, (Fig. 6 and 8). PhN discharges have also been elicited under severe hypoxia in unanesthetized decerebrate C<sub>1</sub>-transected cats, without the aid of supraspinal drive containing the “kernel” of gasping-generating structures (i.e., pre-BötC and surrounding structures).<sup>22</sup> In contrast to that study, in which only one or two bursts occurred in PhN discharge in response to hypoxia, we observed instances of sustained bursting (Fig. 6 and 8).

We believe our results provide evidence for the existence of spinal microcircuits that underlie the generation of spinal gasp-like bursting. Anoxia may thus trigger intrinsic persistent (e.g., sodium) currents and activate spinal pacemaker-like mechanisms located in the UCRG and/or in the vicinity of PhMN pools to generate gasp-like activity. Persistent inward sodium currents have already been described for spinal motoneurons and interneurons and can be used by the spinal respiratory CPG under hypoxic conditions.<sup>49,50,58–62</sup>

It has also been reported that hypoxia also preferentially stimulates nonmyelinated group IV muscle afferents.<sup>63</sup> Activation of muscle afferents during severe hypoxia may serve as an additional factor driving a spinal gasping-like CPG in high cervical Tx animals. Our results suggest the existence and involvement of spinal microcircuits that may underlie the generation of terminal gasps, which may be integrated and function in coordination with gasp-generating elements of the bulbospinal network.

### *Candidate neuroanatomical substrates for generation of phasic activity in spinal respiratory motor outputs*

GABAz/STR treatment of C<sub>1</sub>-C<sub>2</sub> spinal neurons induces phasic activity in PhN discharge post-Tx demonstrating the existence of an intrinsic spinal generator that produces activity, which distributes to phrenic motor outputs. The responsible generator may be located among C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons or the phrenic nucleus proper (Fig. 10). A putative spinal generator located at the C<sub>1</sub>-C<sub>2</sub> group of respiratory-related neurons may be under supraspinal and/or local tonic inhibition; Tx may remove descending supraspinal inhibition of the proposed generator, and treatment of C<sub>1</sub>-C<sub>2</sub> spinal neurons with antagonists of fast inhibitory synaptic transmission may eliminate local tonic inhibition of a latent network or cells with intrinsic bursting properties.

Alternatively, GABAz/STR could act via disinhibition of C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons that function in the capacity of providing tonic excitation to a generator located in the phrenic nucleus (see

below). Last, polysynaptic propriospinal pathways from C<sub>1</sub>-C<sub>2</sub> interneurons to the phrenic nucleus may contribute to the observed activity. Because our goal was to disinhibit C<sub>1</sub>-C<sub>2</sub> spinal neurons via a balanced blockade of both arms of fast inhibitory synaptic transmission, we cannot draw conclusions with respect to whether inhibition of GABA<sub>A</sub>- or glycinergic mechanisms is primarily responsible for the observed activity.

Previous studies have shown that fast inhibitory (i.e., GABA- and glycinergic) mechanisms are essential for shaping of phrenic motor output.<sup>42,64–72</sup> For example, local blockade of GABA synthesis (by application of antglutamate decarboxylase 65/67 siRNA) in the vicinity of the phrenic nucleus leads to increasing tonic and phasic activity in PhN discharge on the side of treatment.<sup>71</sup> It has been shown that tonic inhibition in spinal ventral horn interneurons is mediated by  $\alpha 5$  subunit-containing GABA<sub>A</sub> receptors.<sup>72</sup> Furthermore, it is well accepted that laminae V and VI are the main sources of GABAergic ipsilateral projections to motoneurons with lamina VIII providing contralateral inhibitory and excitatory projections to motoneurons and interneurons.<sup>73,74</sup>

Because spontaneous discharge and asphyxia-induced activity were observed after either C<sub>1</sub>- or C<sub>2</sub>Tx, it is likely that the phrenic nucleus is capable of generating phasic activity independently of the UCRG; it remains to be determined whether this applies to pharmacologically induced discharge as well in the decerebrate adult rat. This is in agreement with the findings of Perségol and Viala,<sup>17</sup> who demonstrated that long-lasting slow bursting may be induced in cervical rootlet discharge by 5-hydroxytryptophan superfusion of the *in vitro* preparation of the neonatal rat.

Our results are in partial agreement with a previous study where application of L-glutamate (1 M) pledgets to the dorsal surface of C<sub>1</sub>-C<sub>2</sub> spinal segments in decerebrate rats increased phasic activity in PhN discharge and tonic activity in intercostal neurograms, suggesting distributed tonic control of upper cervical propriospinal neurons over spinal respiratory motor outputs.<sup>39</sup> This group of cells was likely disinhibited by our local treatment of C<sub>1</sub>-C<sub>2</sub> spinal segments and could be either directly or indirectly (i.e., tonic excitation of phrenic generator) involved in the formation of phasic activity in PhN discharge post-Tx (Fig. 5). In contrast to the aforementioned study, we eschewed the use of anesthetic induction using parenteral agents (i.e., pentobarbital), any residual of which may have unpredictable suppressive effects on respiratory-related neural output. Moreover, our use of a 3 h recovery period after spinal Tx before drug application may have provided adequate time for resolution of spinal shock and the appearance of phasic activity.

Alternatively, disinhibition of C<sub>1</sub>-C<sub>2</sub> spinal neurons may drive the tonic excitation of a burst/rhythm generator located in the phrenic nucleus (Fig. 5). In a previous study, we performed a systematic analysis of phrenic motoneuron discharge patterns in bulbospinal-intact decerebrate rats, which revealed distinct subpopulations of units segregating by maximal firing frequency and, within a frequency class, qualitatively exhibiting highly analogous time-frequency profiles.<sup>75</sup>

While these firing dynamics may reflect the activity of bulbospinal inputs, they likely also represent a segregation of phrenic motoneurons by intrinsic biophysical membrane properties (i.e., action potential duration, time and space constants, and active conductances).<sup>76–81</sup> These motoneuronal classes, in conjunction with interneuronal elements may interact to generate phasic activity after the elimination of overriding excitatory supraspinal drive and/or descending tonic inhibition by spinal transection in conjunction with disinhibition of C<sub>1</sub>-C<sub>2</sub> pre-phrenic interneurons with GABAz/STR.

Upper cervical inspiratory neurons sending fibers toward motor neuron pools in phrenic and intercostal nuclei were originally identified in the cat and later demonstrated in rats.<sup>82–84</sup> Intracellularly traced C<sub>1</sub>–C<sub>2</sub> units send collaterals near the phrenic nucleus and continue into upper thoracic levels where they project to intercostal motor nuclei.<sup>82,83</sup> In the unanesthetized decerebrate adult rat, cross-correlation analysis revealed monosynaptic connections between C<sub>1</sub>–C<sub>2</sub> inspiratory units with PhMNs caudally.<sup>82</sup> Coupling for either generator may occur at C<sub>1</sub>–C<sub>2</sub> or phrenic nucleus levels, perhaps through commissural interneurons located mainly in Rexed's layer VIII, the inhibitory and excitatory variety of which is responsible for left-right synchronization in respiratory<sup>85–90</sup> and alternation in locomotor rhythmic outputs.<sup>91–94</sup>

## Conclusion

We report on different patterns of spontaneous activity occurring in PhN discharge after complete high cervical spinal cord Tx, including tonic and phasic activity, slow oscillations, and long tonic discharges. We also demonstrate asphyxia- and GABA<sub>A</sub>/STR-induced bursting in phrenic discharge after high cervical spinalization. Our findings suggest that UCRG may provide important support for spinal cord mechanisms generating phasic activity in spinal respiratory motor outputs. The details of the microcircuit structure providing rhythmic activity of the spinal CPG for breathing remain to be determined, as well as to what extent such activity is functionally unique to respiratory versus other spinal motor outputs and their capacity to generate alveolar ventilation capable of sustaining life.

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## Author Disclosure Statement

No competing financial interests exist.

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**Control/Tracking Number:** 2017-S-14454-SfN

**Activity:** Scientific Abstract

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**Epidural stimulation and pharmacological blockade of fast inhibition improve respiratory pacing following complete spinal cord injury**

**AUTHOR BLOCK:** \*V. MARCHENKO, T. BEZDUDNAYA, M. A. LANE;  
Dept Neurobiology/Anatomy, Drexel Univ. Col. of Med., Philadelphia, PA

**Abstract:**

The use of epidural stimulation (ES) in patients with spinal cord injury (SCI) has gained increased media attention in the past decade with significant success demonstrated in human studies, including improvement of lower limb motor function in paraplegic patients. DiMarco, Kowalski and colleagues were first to investigate ES applied to T1-T2 ventral surfaces to restore respiratory function following high cervical spinal cord transection. In the current study we combine ES (at C3-C5 segments corresponding to area of phrenic motor pool) and pharmacological strategies (blocking of GABA<sub>A</sub> and Glycine inhibitory receptors) for more specific activation of spinal interneurons and motoneurons involved in the shaping of respiratory motor output following C1 transection (C1Tx). All experiments were performed in decerebrate, unanesthetized adult Sprague-Dawley rats, 5-6 h post C1Tx. ES was applied to ventrolateral surface of C3-C5 cervical segments bilaterally via teflon-coated silver (0.01" bare and 0.013" coated, AM-Systems) stimulating electrodes (0.2 ms biphasic stimulation, 100-200 Hz during 0.3 s, one train per sec). Prior to pacing procedure, the minimal thresholds (Tr) of current ( $87.4 \pm 10.3$  for C3,  $73.6 \pm 8.2$  for C4 and  $84.7 \pm 8.2$  mA for C5 segments) affecting tracheal flow and end-tidal CO<sub>2</sub> level were detected. ES applied to the C4 level ( $\sim 5$  Tr,  $350.7 \pm 41.2$  mA) produced non-fatigue contraction of chest and diaphragm muscles with stable tracheal flow ( $2.3 \pm 0.21$  ml of tidal volume) and end-tidal CO<sub>2</sub> ( $4.5 \pm 0.3$  %) during 1 h. In contrast, ES applied to C3 or C5 segments required much higher current ( $\sim 7$  Tr,  $633.8 \pm 84.5$  mA) with development of muscle fatigue in 5 out of 8 rats. Ten minutes after intrathecal administration of 30  $\mu$ l (25 mMol) of GABAzine and strychnine (blockers of GABA<sub>A</sub> and glycine inhibitory receptors, respectively) the minimal thresholds were significantly decreased for all segments ( $62 \pm 7.9$  for C3,  $41.1 \pm 6.4$  for C4 and  $67.9 \pm 7.3$  mA for C5).

The tidal volume was increased by 43% ( $3.3 \pm 0.27$  ml) during C4 pacing and fatigue of respiratory muscles was observed in 2 out of 8 rats during ES of C3 or C4 segments. Based on our results, we conclude that spinal respiratory circuits are tonically inhibited after C1Tx and their pharmacological modulation has the potential to improve ES in patients with SCI.

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**Presentation Preference (Complete):** Poster Only

**Linking Group (Complete):** None selected

**Theme and Topic (Complete):** E.09.c. Spinal cord injury: Training, rehabilitation, and repair ;  
E.09.d. Neurophysiology

**Nanosymposium Information (Complete):**

**Keyword (Complete):** SPINAL CORD INJURY ; STIMULATION ; SPINAL CORD

**Support (Complete):**

**Support:** Yes

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**Control/Tracking Number:** 2018-S-9685-SfN

**Activity:** Scientific Abstract

**Current Date/Time:** 6/3/2018 12:32:33 PM

**The role of fast inhibition in facilitation of phrenic nerve and diaphragm activity during epidural stimulation following complete cervical spinal cord injury in rats**

**AUTHOR BLOCK:** \*V. MARCHENKO, T. BEZDUDNAYA, M. A. LANE;

Dept Neurobiology/Anatomy, Drexel Univ. Col. of Med., Philadelphia, PA

**Abstract:**

Spinal cord injury (SCI) at mid- to high-cervical spinal levels often results in life-threatening respiratory complications and requires long-term mechanical ventilator assistance. Thus, restoring diaphragm activity and regaining voluntary control of breathing are the primary clinical goals for patients with respiratory dysfunction following cervical SCI. Epidural stimulation (EDS) is a promising strategy that has been explored extensively for non-respiratory functions, and to a limited extent within the respiratory system. The goal of the present study was to test the efficacy for EDS applied to the center of phrenic nucleus location (C4 cervical segment, C4-EDS) in combination with intrathecal GABA<sub>A</sub> and glycine inhibitory receptors antagonists (GABAzine and strychnine) administration on paced breathing following complete C1 cervical transection (C1Tx). To avoid the suppressive effect of anesthesia all experiments were performed in decerebrate, unanesthetized, non-paralyzed or paralyzed animals. High-frequency C4-EDS (100-400 Hz) (240-350  $\mu$ A, 0.2 ms of biphasic pulse duration, stimulation during 0.3 s, one train per sec) was able to maintain breathing with stable diaphragm EMG (DiEMG), normal end-tidal CO<sub>2</sub> level and raise blood pressure. In addition, 100-300 Hz of C4-EDS showed time- and -frequency dependent changes (short-term facilitation) of evoked phrenic nerve (PN) and DiEMG responses during of each stimulus train that may serve as a target mechanism for pacing of phrenic motor circuits. C4-EDS applied with frequencies 350-400 Hz causes depression of PN and DiEMG responses. Ten minutes after intrathecal application of GABAzine and strychnine, (GABAz+STR, 25  $\mu$ Mol - 30  $\mu$ l) over C3-C5 segments and C4-EDS (200-300Hz), respiratory flow was increased by  $26 \pm 7.3\%$  ( $p < 0.05$ ), DiEMG - by  $19 \pm 5.6\%$ , and PN responses by  $354 \pm 42.9\%$ . Based on these results, we conclude that respiratory circuits at the level of phrenic nucleus are tonically inhibited after C1Tx and their pharmacological modulation has the potential to enhance efficacy of EDS in people with SCI.

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**Presentation Preference (Complete):** Poster Only

**Linking Group (Complete):** None selected

**Theme and Topic (Complete):** C.11.c. Spinal cord injury: Therapeutic strategies: In vivo ; E.07.c. Neuromodulation

**Nanosymposium Information (Complete):**

**Mapped Theme :** Spinal Cord Injury and Recovery

**Dynamic Poster :** False

**Great Science :** False

**Suspicious :** False

**Sensitive :** False

**Keyword (Complete):** SPINAL CORD INJURY ; STIMULATION ; RESPIRATION

**Support (Complete):**

**Support:** Yes

**Grant/Other Support :** The United States Department of Defense (USAMRAH #SC140038)

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**Mapped Theme One :** Spinal Cord Injury and Recovery

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**Control/Tracking Number:** 2016-S-13226-SfN

**Activity:** Scientific Abstract

**Current Date/Time:** 5/5/2016 2:15:39 PM

**Distribution of respiratory-related neurons in C3-C5 cervical segments and their responses to blockade of GABA<sub>A</sub> and Glycine receptors in decerebrate rats**

**AUTHOR BLOCK:** T. G. BEZDUDNAYA, M. A. LANE, \*V. MARCHENKO;  
Dept Neurobiology/Anatomy, Drexel Univ. Col. of Med., Philadelphia, PA

**Abstract:**

It is well known that respiratory interneurons play an important role in formation of respiratory motor output for intercostal nerves. However, the detailed distribution and role of respiratory interneurons at the level of phrenic nucleus is still underinvestigated. In 29 experiments using decerebrate, vagotomized and paralyzed (vecuronium Br) rats with bilateral pneumothorax 122 respiratory-related neurons were extracellular recorded from C3-C5 cervical segments corresponded to location of phrenic motoneurons. Motor-(n=35) and interneurons (n=87) were identified by the presence or absence of antidromic response to phrenic nerve stimulation. These units were categorized as follows: Inspiratory (Insp) (n=35) (27 units were identified as motoneurons), Full expiratory (Exp) interneurons (n=17), Pre-inspiratory or 'expiratory type 2' (E2) interneurons (n=15), Inspiratory interneurons with tonic background activity (Insp+BG) (n=27), Inspiratory-expiratory phase-spanning interneurons (Insp-Exp) (n=9), Late-inspiratory (late-Insp) (n=13) (8 units were identified as motoneurons), Exp-Insp (wide phase-spanning) (n=6). Topographical analyses of the phrenic motoneurons reveal two distinct group of inspiratory motoneurons located at C4-cervical level ventro-medial and ventrolateral, accordingly. This bimodal distribution of phrenic motoneurons was confirmed by retrograde labeling using different tracers (Fast blue, Texas Red, Cholera Toxin subunit B, Cascade blue). Also, it was found, that Insp+BG interneurons located mostly dorso-medial to motoneuron pools. However, some of them were found in vicinity of the phrenic motoneurons population. GABAzine (high selective antagonist of postsynaptic GABA<sub>A</sub> receptors) and Strychnine (STR, antagonist of glycine postsynaptic receptors) were applied to 61 cells with microiontophoresis (+20 nA, 2 min) using multibarrel (Carbostar-3) electrode with follow labeling by Neurobiotin 2% , +10 nA, 30 min). In response to application of GABAzine, high-frequency Insp+BG cells did not show significant changes in firing rate while STR elicited increasing of background activity. In response to STR or GABAzine, middle- and low-frequency (late-Insp, e.g.) units

showed an altered firing pattern to become early-inspiratory with increasing of tonic background activity. These data evidence that spinal respiratory neurons represent high-heterogeneity network with firing patterns described for ponto-medullary level. The functional significant of fast inhibitory mechanisms in respiratory pattern formation is discussed.

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**Presentation Preference (Complete):** Poster Only

**Nanosymposium Information (Complete):**

**Theme and Topic (Complete):** E.07.a. Cellular properties: Interneurons and motor neurons ; E.08.b. Respiratory regulation: Respiratory rhythm and pattern generation

**Linking Group (Complete):** None selected

**Keyword (Complete):** SPINAL CORD ; MOTONEURON ; INTERNEURON

**Support (Complete):**

**Support:** Yes

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## RESEARCH ARTICLE

# Paced breathing and phrenic nerve responses evoked by epidural stimulation following complete high cervical spinal cord injury in rats

Tatiana Bezdudnaya, Michael A. Lane, and Vitaliy Marchenko

Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, Pennsylvania

Submitted 2 October 2017; accepted in final form 8 May 2018

**Bezdudnaya T, Lane MA, Marchenko V.** Paced breathing and phrenic nerve responses evoked by epidural stimulation following complete high cervical spinal cord injury in rats. *J Appl Physiol* 125: 687–696, 2018. First published May 17, 2018; doi:10.1152/japplphysiol.00895.2017.—Spinal cord injury (SCI) at the level of cervical segments often results in life-threatening respiratory complications and requires long-term mechanical ventilator assistance. Thus restoring diaphragm activity and regaining voluntary control of breathing are the primary clinical goals for patients with respiratory dysfunction following cervical SCI. Epidural stimulation (EDS) is a promising strategy that has been explored extensively for nonrespiratory functions and to a limited extent within the respiratory system. The goal of the present study is to assess the potential for EDS at the location of the phrenic nucleus ( $C_3$ – $C_5$ ) innervating the diaphragm: the main inspiratory muscle following complete  $C_1$  cervical transection. To avoid the suppressive effect of anesthesia, all experiments were performed in decerebrate,  $C_1$  cervical transection, unanesthetized, nonparalyzed ( $n = 13$ ) and paralyzed ( $n = 7$ ) animals. Our results show that  $C_4$  segment was the most responsive to EDS and required the lowest threshold of current intensity, affecting tracheal pressure and phrenic nerve responses. High-frequency (200–300 Hz) EDS applied over  $C_4$  segment ( $C_4$ -EDS) was able to maintain breathing with normal end-tidal  $CO_2$  level and raise blood pressure. In addition, 100–300 Hz of  $C_4$ -EDS showed time- and frequency-dependent changes (short-term facilitation) of evoked phrenic nerve responses that may serve as a target mechanism for pacing of phrenic motor circuits. The present work provides the first report of successful EDS at the level of phrenic nucleus in a complete SCI animal model and offers insight into the potential therapeutic application in patients with high cervical SCI.

**NEW & NOTEWORTHY** The present work offers the first demonstration of successful life-supporting breathing paced by epidural stimulation (EDS) at the level of the phrenic nucleus, following a complete spinal cord injury in unanesthetized, decerebrate rats. Moreover, our experiments showed time- and frequency-dependent changes of evoked phrenic nerve activity during EDS that may serve as a target mechanism for pacing spinal phrenic motor networks.

breathing; complete spinal cord injury; epidural stimulation; phrenic nerve; rats

## INTRODUCTION

Around 13.3% of tetraplegic patients (~2,300 new cases yearly in USA) with high cervical ( $C_1$ – $C_4$ ) spinal cord injury (SCI) require long-term ventilation support (50a). Mechanical

ventilation, while important and necessary for life support, contributes to disuse atrophy of the diaphragm, affects venous return, increases the risk of respiratory infections, and decreases life expectancy. Thus weaning from ventilator dependency is the primary clinical goal for these patients. Electrical stimulation of phrenic nerve and diaphragm are among those strategies being increasingly used in clinic (10, 24, 30, 40, 51, 61). Epidural stimulation (EDS) is an alternative neural interfacing strategy that has been explored extensively for nonrespiratory functions (52, 64), and to a lesser extent within the respiratory system (6, 7, 9, 12, 25). DiMarco et al. (6, 7, 9) showed that  $Th_2$ – $Th_3$  low frequency (15–40 Hz) EDS in dogs was effective for life-supporting breathing, even after bilateral phrenicotomy. However, the first clinical trial with EDS for quadriplegics with bilaterally compromised phrenic nerves yielded little success (8). More successful EDS pacing of intercostal muscles, combined with diaphragm pacing, was achieved in patients with at least one phrenic nerve function (11). In preclinical studies using anesthetized dogs (including those  $C_2$ -transected), DiMarco and Kowalski (12) described effective high-frequency EDS (HF-EDS) (100–300 Hz) applied at the  $Th_2$ – $Th_3$  level. The same efficiency of HF-EDS was demonstrated in urethane-anesthetized  $C_1$ -transected ( $C1Tx$ ) rats (14). Interestingly, diaphragm contraction has also been confirmed during activation of intercostal muscles (14, 34). The authors attribute this to interconnections between prephrenic and thoracic interneurons, which have also been documented anatomically (35).

An important consideration is that the majority of previous studies with EDS have been pursued in anesthetized animals (6, 7, 9, 12, 13). However, it is well known that anesthesia suppresses respiratory function (20, 67) and activity of spinal neurons (3, 65) that may affect experimental data and interpretation of results. Therefore, in the present work, we used decerebrate preparation that allowed for experiments in unanesthetized animals. We hypothesized that the use of EDS to pace respiratory muscles in unanesthetized animals would require less current intensity. Furthermore, we predicted that it would help to identify EDS parameters capable of more specific diaphragm pacing, avoiding side effects such as off-target (locomotor) muscle contractions. Using unanesthetized animals can reveal effects of EDS that might not be seen under anesthesia. While upper-thoracic EDS has been the focus of previous studies, stimulation of cervical segments that correspond to location of the phrenic motor nucleus ( $C_3$ – $C_5/C_6$ ) innervating the diaphragm has not been investigated in a model of complete SCI. As the diaphragm is the primary inspiratory muscle, contributing ~88% of inspiratory effort during rest and

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~75% during forced breathing (1, 48, 68), the present work explores whether midcervical EDS represents an effective therapeutic target. The primary goal of the present study is to establish an effective C<sub>3</sub>–C<sub>5</sub> EDS strategy in the absence of anesthesia, capable of producing life-supporting, paced breathing in decerebrate rats following complete C<sub>1</sub> spinal transection.

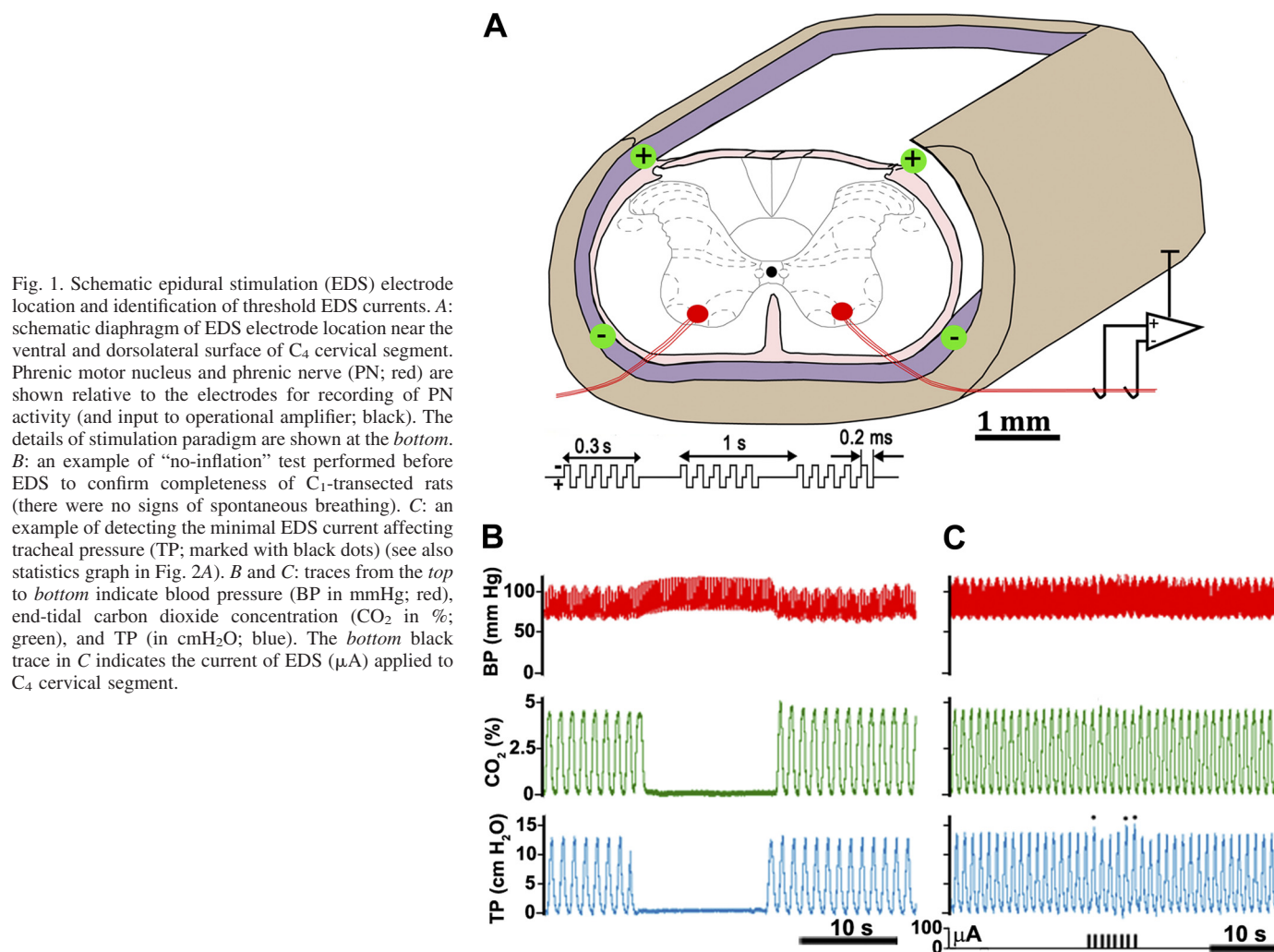
## METHODS

Experiments were performed on 20 decerebrate, unanesthetized male Sprague-Dawley rats (330–370 g) with a C1Tx. Two groups of animals were studied under terminal electrophysiological conditions: nonparalyzed ( $n = 13$ ) and pharmacologically paralyzed ( $n = 7$ ). All surgical and animal care procedures were approved by the Drexel University Institutional Animal Care and Use Committee and adhered to American Association for Accreditation of Laboratory Animal Care/National Institutes of Health guidelines.

**Surgical procedure.** Spontaneously breathing animals were anesthetized in an induction chamber and then via nose mask (2.5% isoflurane vaporized in 100% of O<sub>2</sub>; MDS Matrix). Following tracheotomy, animals were artificially ventilated with the same gas mixture (45–65 cycles/min, 2.5- to 3.0-ml tidal volume; Columbus Apparatus). The femoral artery and vein were cannulated for measurement of arterial pressure and infusion of drugs/fluids, respectively. Rectal temperature was maintained at  $37.0 \pm 0.1^\circ\text{C}$  via a servo-controlled

heating blanket coupled to a rectal thermometer (TCAT-2DF, Physitemp). In seven animals (paralyzed group), phrenic nerves were dissected free from the surrounding tissue and transected immediately superior to the subclavian vessels. The internal carotid arteries were ligated to prevent bleeding after decerebration. To attenuate cerebral and spinal edema following decerebration and C1Tx, dexamethasone (1 mg/kg) was injected subcutaneously (4).

**Decerebration.** Animals were placed prone in a stereotaxic device, and the vertebral column was raised by the Th<sub>2</sub> vertebral spinous process to align the spinal cord and medulla. Arterial and tracheal cannulas were connected to pressure transducers (CDXII, Argon Medical) for monitoring arterial blood pressure (BP) and tracheal pressure (TP), respectively, using conventional amplifiers (Gould Statham). End-tidal CO<sub>2</sub> was maintained between 5.0 and 5.2% (Capstar CWE) by adjusting ventilation rate (54–60 cycles/min). Biparietal craniectomies were made using a surgical drill (Foredom Electric), the dura was opened using iridectomy scissors, and the superior sagittal sinus was ligated proximally and distally. The neuraxis was transected at the rostral border of the superior colliculus using a blunt-edged microspatula. Brain tissue rostral to the transection was removed by suction, and residual bleeding was prevented by packing the anterior and middle cranial fossae with small pieces of gelfoam (USP, Pharmacia) soaked in cold thrombin solution (50 U/ml USP, dissolved in 1 ml physiological saline). After decerebration, anesthesia was slowly withdrawn until rats started to breathe spontaneously (1.5–2 h). During recovery time (2 h), dextrose (1 ml of 5%



wt/vol in Locke-Ringer solution) was injected intravenously (every 15–30 min) to stabilize BP.

**Laminectomy.** Following recovery after decerebration, C<sub>3</sub>–C<sub>5</sub> laminectomy was performed under artificial ventilation and isoflurane (2%) anesthesia. Dorsal neck muscles were removed, and C<sub>3</sub>–C<sub>5</sub> vertebral arches were removed with rongeurs. The previously ventrally dissected and transected phrenic nerves were retrieved dorsally in seven animals (paralyzed group).

**High cervical transection.** Immediately following laminectomy, atlanto-occipital membrane and dura were opened, and isoflurane (4%) was added to the ventilated gas mixture to minimize spinal shock from subsequent spinal cord lesioning (33, 55). When mean arterial pressure was reduced to 70–80 mmHg, a complete spinal cord transection was performed at the upper border of C<sub>1</sub> rootlets using a sharp microscalpel (cat no. 10073–14, FST). Anesthesia was withdrawn ~10 min after injury, and animals recovered over the next 4 h. During recovery (4 h) and recording (3 h), dextrose (5% wt/vol in Locke-Ringer solution) was continuously infused (5 ml·kg<sup>-1</sup>·h<sup>-1</sup>; syringe pump, NE-300, New Era Pump Scientific) via the vein catheter to stabilize mean BP (70–80 mmHg).

**Epidural stimulation.** Silver Teflon-coated wires (0.008-in. bare and 0.011-in. coated, AM-Systems) with ~1-mm uninsulated atraumatic (rounded) tips were used for bipolar EDS. Electrodes were gently inserted ventrolateral (toward ventral roots, cathode) and dorsolateral (2 mm from the midline, anode) at the both sides of the spinal cord (Fig. 1A). Stimulus parameters were comparable to those previously reported for epidural pacing of intercostal muscles (14). EDS was produced using two Iso-Flex isolated units (AMPI) connected to PG400-NEURODATA stimulator (Cygnus Technology). The minimal current (threshold) of EDS affecting TP was identified in non-paralyzed rats using a single biphasic pulse of different lengths (0.1, 0.2, and 0.5 ms) applied to C<sub>3</sub>–C<sub>5</sub> segments. In series with HF-EDS stimulation, biphasic current with intensity of 3 thresholds (230–250  $\mu$ A) and 0.2 ms of single pulse length was applied to C<sub>4</sub> segment once per second (1 Hz) during 0.33 s (train duration) with different frequencies (100, 200, and 300 Hz) (stimulation paradigm outlined in Fig. 1A).

**Recording.** In the first experimental group of animals (non-paralyzed rats,  $n = 13$ ), EDS was used to detect the following: 1) the minimal current (threshold) applied by a single pulse (0.1, 0.2, and 0.5 ms) to the C<sub>3</sub>–C<sub>5</sub> level affecting TP ( $\geq 15\%$  changes above control); 2) the most effective EDS-pacing paradigm maintaining stable breathing for at least 30 min. Artificial ventilation was turned off during HF-EDS pacing, and the tracheal tube was connected to open respiratory circuits with a small steady flow of 100% O<sub>2</sub> (10 ml/min). In the open-circuit mode, the tracheal tube was attached to the flow head (cat. no. MLT1L, ADInstruments) connected to the differential pressure transducer (Gould) (2). Respiratory flow (ml/sample rate) with bias correction was measured during EDS-paced breathing. The values of tidal volume were obtained by integration of respiratory flow during pacing (inspiratory) time (0.33 s) (2). The respiratory flow was calibrated with different frequencies of artificial ventilation and tidal volume before and after the experiment. All recorded parameters and stimulus marks were digitized at a 10,000 sample rate (PowerLab, ADInstruments), and stored using Chart 5 software (ADInstruments).

The second group of animals (paralyzed,  $n = 7$ ), was used to detect the changes in phrenic nerve activity in response to different frequencies of EDS. The recording of phrenic nerve activity was chosen based on its linear correlation with a lung tidal volume (18, 19, 66). To avoid any movement artifact during phrenic nerve recording, animals were paralyzed by an intravenous bolus injection (2 mg/kg) of vecuronium bromide (Abbott Laboratories), dissolved (0.4 mg/ml) in lactated Ringer solution, followed by continuous infusion (4 mg·kg<sup>-1</sup>·h<sup>-1</sup>). The central ends of the phrenic nerves were placed on bipolar silver electrodes and immersed in a mineral oil pool. Efferent phrenic nerve activity was recorded, amplified ( $\times 10,000$ ), filtered (10–5,000 Hz; Neurolog, Digitimer), digitized (PowerLab, ADInstruments), and

stored onto the hard disk of a personal computer using Chart 5 software (ADInstruments).

**Data analysis.** Recorded data were analyzed with Spike2 (version 6, Cambridge Electronic Design), MATLAB (version R2017b, The MathWorks), and custom-written scripts. Using Spike2 software, stimulus-triggered averaging of phrenic nerve responses was calculated for the first and second half of HF-EDS. The changes in amplitude of evoked phrenic nerve activity in response to single stimulus (identification of threshold) and HF-EDS were identified as peak maximums relative to prestimulus background (“zero”) level. The estimation of statistics for mean BP, TP, respiratory flow, and tidal volume was performed on 1-min records taken at the middle of pacing time in MATLAB. Data distribution was evaluated using Lilliefors test for normality in MATLAB. Accordingly, significant difference between means was evaluated by applying either parametric (one-way ANOVA) or nonparametric (Kruskal-Wallis) repeated-measures analysis of variance. Post hoc Tukey-Kramer test was applied with multiple comparison to calculate the correct significant

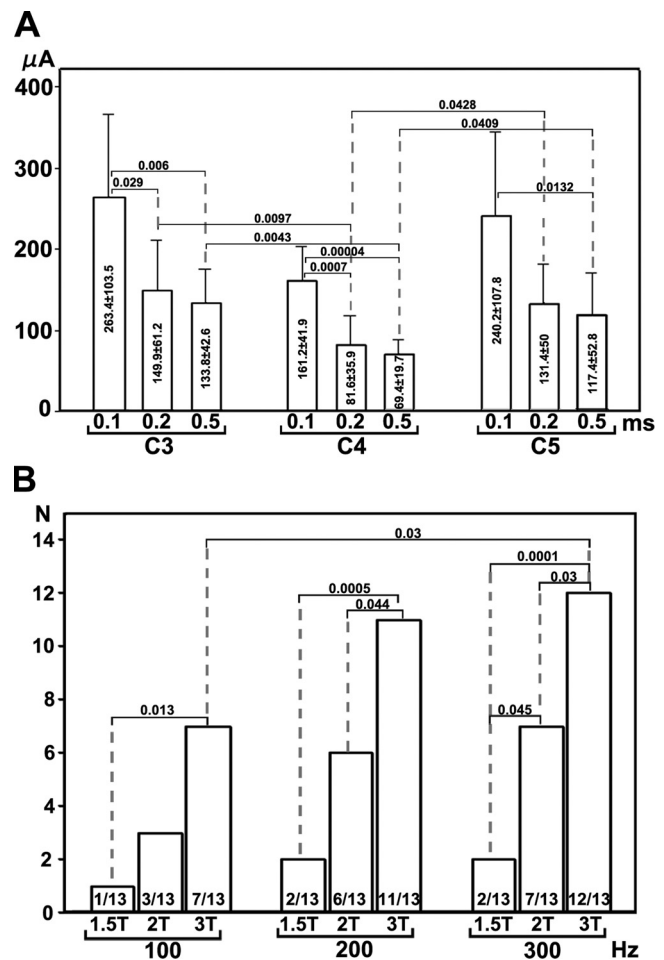


Fig. 2. Statistical graphs of thresholds (A) and successful vs. unsuccessful (B) epidural stimulation (EDS)-paced breathing in C<sub>1</sub>-transected rats. A: average minimal thresholds of EDS current affecting tracheal pressure (see Fig. 1C), applied with different pulse lengths (0.1, 0.2, and 0.5 ms) to C<sub>3</sub>–C<sub>5</sub> segments (C<sub>3</sub>, C<sub>4</sub>, and C<sub>5</sub>), are shown with relative significant differences (horizontal lines) and corresponding *P* values. The mean ( $\pm$ SD) threshold values are shown inside the bars. B: the differences between proportions of successful and unsuccessful C<sub>4</sub>-EDS trials are indicated with corresponding *P* values. The proportions (ratio between successful EDS and all EDS trials) are shown inside the bars. Current intensity of EDS in thresholds applied with different frequencies (100, 200, and 300 Hz) are as follows: 1.5 $\times$  threshold (1.5T), 2 $\times$  threshold (2T), and 3 $\times$  threshold (3T).

difference between all data groups: e.g., between thresholds of single EDS pulses (0.1, 0.2, and 0.5 ms) affecting TP ( $\geq 15\%$  changes above control) within each cervical segment and between segments for each pulse length (Fig. 2A). Post hoc Dunnett's test was applied during multiple comparisons between data groups and the control group: e.g., between changes of the first N1, second N2, and sum of N1 and N2 (N1 + N2 = total phrenic nerve output) phrenic nerve components relative to their control values during HF-EDS (100, 200, and 300 Hz) (see Figs. 5B and 6). The peak values of the N1, N2, and N1 + N2 components observed in response to the first pulse of each frequency train were selected as the control amplitudes (blue dashed line for N1; red dashed line for N2; black dashed line for N1 + N2; see Fig. 5B). Furthermore, the changes in amplitudes of N1, N2, and N1 + N2 components were normalized (in %) by the first N1 component (was chosen as the base control level, 100%) (Table 1 and see Fig. 5B). All significant differences for N1, N2, and N1 + N2 stimulus-triggered averaged peak amplitude were calculated during the first and second half of the frequency trains (see Fig. 5B and Table 1). The "N-1"  $\chi^2$  test for the comparison of two proportions was used for estimation of significance difference between successful (nonfatigue) vs. unsuccessful (fatigue) EDS pacings (MedCalc, version 17.9.7). All values are given as means  $\pm$  SD. Differences were considered significant at the 95% confidence limit.

**Verification of C1Tx completeness.** At the end of each experiment, animals were transcardially perfused with 200 ml of normal saline (20°C, pH 7.4) with heparin (100 U/ml), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The spinal cord and brain stem were removed and postfixed in the same fixative for 48 h at 4°C. Verification of a complete C<sub>1</sub> transection was determined by post hoc analysis using a stereo microscope (magnification  $\times 10$ , Smart Optic operational microscope). Careful removing of dura mater from fixed tissue caused complete separation of the brain stem from the spinal cord (only blood clots were observed between them).

## RESULTS

**Defining the EDS-thresholds in non-paralyzed animals.** In the present study, all animals received a complete C1Tx and recovered for 4 h before undergoing electrophysiological recordings. Epidural electrodes were placed as described in METHODS (Fig. 1A). Before all experimental manipulations, a functional "no-inflation" test was applied in nonparalyzed rats, and before vecuronium infusion in those paralyzed, to confirm that the C1Tx was complete (Fig. 1B). In nonparalyzed rats ( $n = 13$ ), we then identified minimal current of EDS (threshold) affecting TP using a single biphasic pulse of different lengths (0.1, 0.2, and 0.5 ms) applied to C<sub>3</sub>–C<sub>5</sub> segments. Figure 1C shows an example of a record during stimulation,

where changes in TP to a single pulse are indicated (black dots). The average threshold values and differences between them are presented in the graph in Fig. 2A. While no significant difference was detected between threshold currents applied to C<sub>3</sub> and C<sub>5</sub> segments, stimulation of C<sub>4</sub> level resulted in significantly lower thresholds at all pulse lengths at 0.2 and 0.5 ms. Thresholds at 0.2- and 0.5-ms pulse width did not show significant differences, whereas pulse duration of 0.1 ms resulted in significantly higher thresholds for all segments (Fig. 2A). Furthermore, 0.2-ms pulse widths produced much smaller artifacts than 0.5-ms pulses and decreases unnecessary disturbing of nerve tissue by electrical current, and is thus perhaps more optimal for EDS. The results of this experimental group also indicate that C<sub>4</sub> EDS yields more optimal effects than stimulation at C<sub>3</sub> or C<sub>5</sub>.

**Optimal parameters for EDS-paced breathing.** In the same set of animals, trains of EDS were applied to C<sub>4</sub> segment (C<sub>4</sub>-EDS) once per second (1 Hz) during 0.33 s, with an optimal stimulus length (0.2 ms) and different frequencies (100, 200, and 300 Hz) (stimulation paradigm outlined in Fig. 1A) that were in the physiological range of phrenic nerve frequency bands MFO ( $\sim 110$ – $150$  Hz, middle-frequency oscillations), HFO ( $\sim 155$ – $220$  Hz, high-frequency oscillations), and UHFO ( $\sim 225$ – $285$  Hz, upper HFO) in rats (43–45). The train duration (0.33 s) was chosen based on average inspiratory time in adult rats (4a). EDS with frequencies of 200–300 Hz successfully paced stable breathing at  $3 \times$  threshold ( $3 \times T$ ) (220–240  $\mu$ A) for more than 30 min in 11–12 of 13 trials (see graph in Fig. 2B and an example of record in Fig. 3). The tidal volume maintained with EDS train frequency 200 Hz ( $1.34 \pm 0.1$  ml) and 300 Hz ( $1.37 \pm 0.12$  ml) showed significant difference vs 100 Hz ( $1.22 \pm 0.07$  ml) ( $P = 0.03$  [100 vs 200 Hz] and  $P = 0.0016$  [100 vs 300 Hz]). A current intensity of  $1.5 \times T$  ( $\sim 100$ – $120$   $\mu$ A) failed to support paced breathing, with a progressive decrease in tidal volume and unstable end-tidal CO<sub>2</sub> in almost all trials (11 of 13) (see graph in Fig. 2B and an example of record in Fig. 4). This nonstable paced fatigue breathing developed within 3–5 min of EDS. The present work used a 30-min period (6 times longer) to ensure that EDS produced stable breathing. While stimulation at  $3 \times T$  of optimal current intensity did not produce any visible limb muscle contractions, more than  $5 \times T$  ( $>400$   $\mu$ A) resulted in contraction of upper and lower limb muscles and were, therefore, avoided.

Table 1. Changes of phrenic nerve components in response to different frequencies of C<sub>4</sub>-EDS

| Stimulation                     | Amplitude               |                                  |                         |                                  |                              |                                    |
|---------------------------------|-------------------------|----------------------------------|-------------------------|----------------------------------|------------------------------|------------------------------------|
|                                 | N1, mV $\times 10^{-2}$ | N1, %                            | N2, mV $\times 10^{-2}$ | N2, %                            | N1 + N2, mV $\times 10^{-2}$ | N1 + N2, %                         |
| Control ( $3 \times$ threshold) | $1.53 \pm 0.17$         | $100 \pm 10.999$                 | $0.15 \pm 0.02$         | $10.14 \pm 1.01$                 | $1.68 \pm 0.17$              | $110.1 \pm 10.42$                  |
| 100-1/1                         | $1.49 \pm 0.16$         | $97.7 \pm 10.78$                 | $0.17 \pm 0.02$         | $10.87 \pm 1.14$                 | $1.66 \pm 0.13$              | $108.56 \pm 10.59$                 |
| 100-2/2                         | $1.35 \pm 0.14$         | $88.42 \pm 9.02$                 | $0.33 \pm 0.04$         | $21.51 \pm 2.09^* (P < 0.00001)$ | $1.68 \pm 0.15$              | $109.93 \pm 10.19$                 |
| 200-1/1                         | $1.29 \pm 0.13$         | $84.43 \pm 8.18^* (P = 0.01179)$ | $0.46 \pm 0.05$         | $29.91 \pm 3.01^* (P < 0.00001)$ | $1.75 \pm 0.16$              | $114.33 \pm 8.75$                  |
| 200-2/2                         | $1.17 \pm 0.14$         | $76.61 \pm 8.99^* (P = 0.00618)$ | $0.61 \pm 0.06$         | $39.74 \pm 3.99^* (P < 0.00001)$ | $1.78 \pm 0.18$              | $116.35 \pm 9.08$                  |
| 300-1/1                         | $1.28 \pm 0.12$         | $83.53 \pm 7.88^* (P = 0.00015)$ | $0.74 \pm 0.07$         | $48.12 \pm 4.77^* (P < 0.00001)$ | $2.02 \pm 0.23$              | $131.64 \pm 7.46^* (P < 0.00011)$  |
| 300-2/2                         | $1.12 \pm 0.13$         | $73.36 \pm 8.42^* (P = 0.00002)$ | $0.89 \pm 0.09$         | $58.49 \pm 5.98^* (P < 0.00001)$ | $2.01 \pm 0.21$              | $131.85 \pm 10.62^* (P < 0.00078)$ |

Values are means  $\pm$  SD. The changes of stimulus-triggered phrenic nerve averaged components (N1, N2, and N1 + N2) in response to  $3 \times$  threshold C<sub>4</sub>-epidural stimulation (EDS) at different frequencies (stimulation) are presented in amplitude (mV, mV  $\times 10^{-2}$ ) and normalized (%) units. Control ( $3 \times$  threshold) (100%) corresponds to the amplitude of N1 component in the first response of phrenic nerve during the first (1/1) and second (2/2) half of train stimulation (100-1/1 to 300-2/2) for 100, 200, and 300 Hz. All comparisons were made between control values within corresponding component (N1, N2, and N1 + N2) in %. \*Significant differences between control and phrenic components means are marked, with attached  $P$  values given.

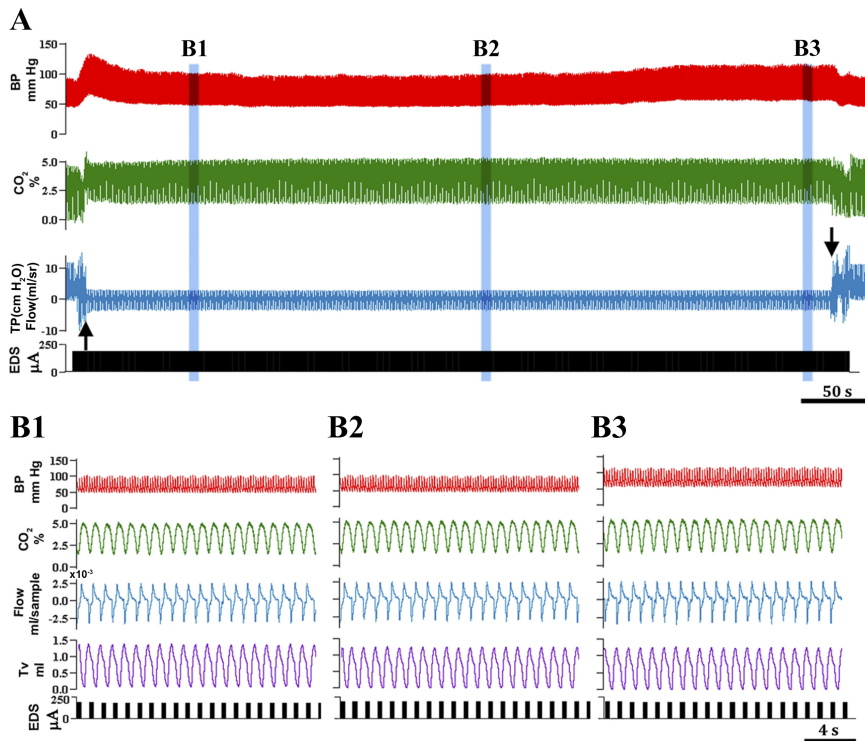


Fig. 3. A: an example of the stable breathing during high-frequency (300 Hz) C<sub>4</sub>-epidural stimulation (EDS) pacing with  $3\times$  thresholds of current intensity. Black arrows indicate turning assisted ventilation off or on (up and down arrows, respectively). B1–B3: highlighted segments demonstrate stable changes of paced breathing in expanded time scale during EDS with respiratory flow (cyan) and tidal volume (Tv; purple). Labels and legends for three top traces in A and B1–B3 are as follows (from top to bottom): blood pressure (BP in mmHg; red), end-tidal carbon dioxide concentration (CO<sub>2</sub> in %; green), tracheal pressure (TP in cmH<sub>2</sub>O; blue) was recorded before and after C<sub>4</sub>-EDS, respiratory flow in milliliters per sample rate (Flow, ml/sample rate; blue) was recorded during C<sub>4</sub>-EDS. The bottom black trace on in A and B1–B3 indicates the current of EDS ( $\mu$ A) applied to C<sub>4</sub> cervical segment.

*Changes of phrenic nerve responses during C<sub>4</sub>-EDS in paralyzed animals.* In the second experimental group (phrenic nerve recording), we used paralyzed animals ( $n = 7$ ) to eliminate moving artifacts associated with mechanical ventilation. Phrenic nerve activity was recorded in response to a

single biphasic pulse (0.2-ms duration) with different current intensities at the C<sub>4</sub> segment to identify the threshold and optimal stimulus paradigm (Fig. 5A). The minimum current intensity ( $1 \times T$ ) elicited phrenic response was  $77.96 \pm 11.59 \mu$ A (Fig. 5A,  $1 \times T$ ), which approximated the threshold current

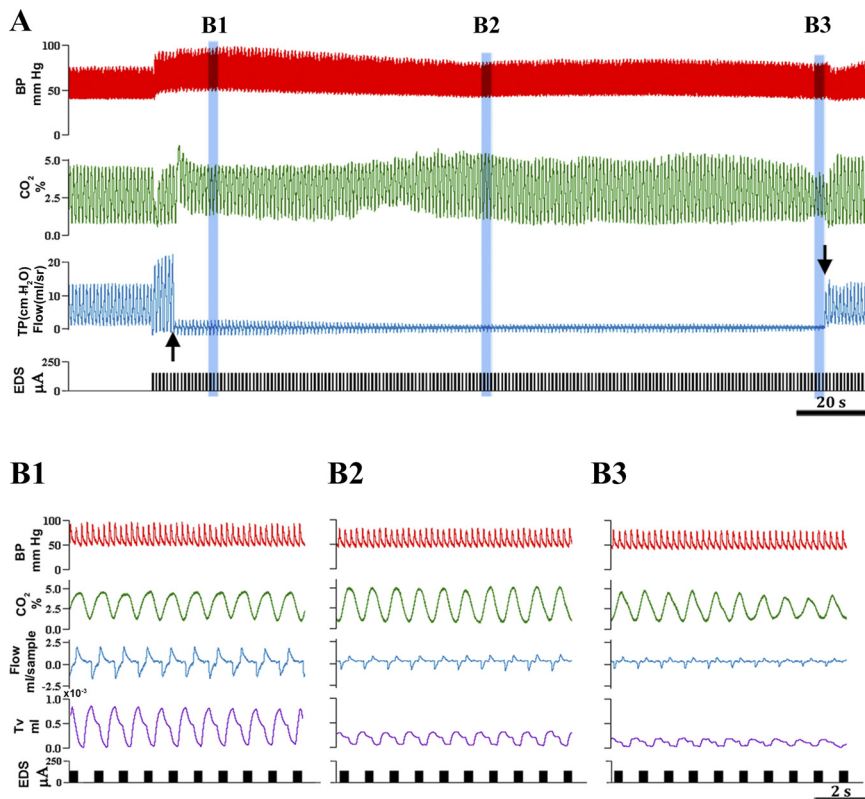


Fig. 4. A: an example of ineffective breathing during high-frequency (300 Hz) C<sub>4</sub>-epidural stimulation (EDS) pacing with  $1.5\times$  threshold current intensity. A series of EDS pulses (0.2 ms, 300 Hz,  $1.5\times$  threshold, train duration 0.33 s, 1 Hz) was applied at C<sub>4</sub> level. Black arrows indicate turning assisted ventilation off or on (up and down arrows, respectively). B1–B3: highlighted segments demonstrate unstable (declined) paced breathing in expanded time scale during EDS with respiratory flow (cyan) and tidal volume (Tv; purple). Labels and legends for three top traces in A and B1–B3 are as follows (from top to bottom): blood pressure (BP in mmHg; red), end-tidal carbon dioxide concentration (CO<sub>2</sub> in %; green), tracheal pressure (TP in cmH<sub>2</sub>O; blue) was recorded before and after C<sub>4</sub>-EDS, respiratory flow in milliliters per sample rate (Flow, ml/sample rate, blue) was recorded during C<sub>4</sub>-EDS. The bottom black trace in A and B1–B3 indicates the current of EDS ( $\mu$ A) applied to C<sub>4</sub> cervical segment. Note decrease in respiratory flow and Tv in the middle part and the end of EDS pacing.

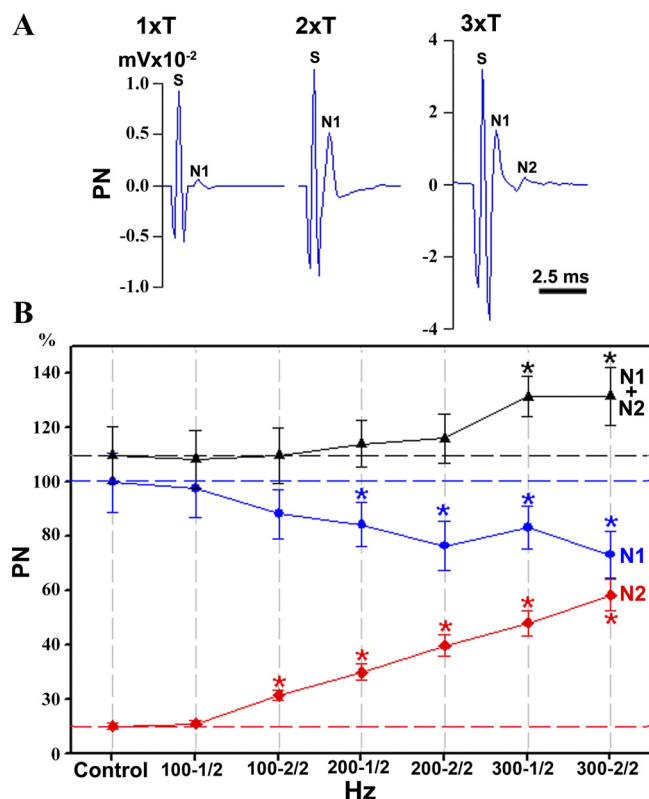


Fig. 5. The responses of phrenic nerve to single pulse and train of epidural stimulation (EDS) applied to C<sub>4</sub> segment with different frequencies. A: phrenic nerve (PN) responses (in mV,  $mV \times 10^{-2}$ ) to single pulse (0.2 ms) of EDS with different current intensity (1–3× thresholds, 1 × T to 3 × T). S, N1, and N2 are abbreviations used for marking the stimulus artifact (S) and the first (N1) and second (N2) phrenic nerve components, respectively. Phrenic nerve response to 2 × T to 3 × T of current intensity has a high-amplitude stimulus artifact that partially overlaps the onset of N1 component. This overlap is absent at 1 × T of current intensity. B: the graph of phrenic nerve components (% for numbers, see Table 1) changes in response to 3 × T of EDS-train stimulation at different frequencies (blue circles, N1; red diamonds, N2; black triangles, N1 + N2 components). x-Axis (100–1/2 to 300–2/2) shows stimulus-triggered averaging of phrenic nerve components during the first (1/1) and second (2/2) half of EDS-train stimulation for 100, 200, and 300 Hz. Control (100%) corresponds to the amplitude of N1 component in the first response of phrenic nerve during stimulations (blue dashed line). Relative controls for N2 and sum of components (N1 + N2) are shown in red and black dashed lines, respectively. \*Significant differences of component amplitude means were estimated relative to their controls (see Table 1 for *P* values).

( $81.6 \pm 35.9 \mu A$ ) affecting TP in nonparalyzed animals (Figs. 1C and 2A). Stimulus intensity at 3 × T elicited two distinct components in phrenic nerve recordings: 1) early, high-amplitude N1, with latency  $1.08 \pm 0.02$  ms; and 2) delayed, lower amplitude N2 ( $10.14 \pm 1.01\%$  of N1 amplitude; Control (3 × T) in Table 1), with latency of  $2.54 \pm 0.05$  ms (Fig. 5A, 3 × T). EDS trains (100–300 Hz) were applied during 0.33 s with a biphasic pulse (0.2 ms) every 1 s (as described for nonparalyzed animals). Our results show that N1 and N2 components changed in a reciprocal way (Fig. 5B) in time- and frequency-dependent mode. While N1 was depressed by increasing frequencies of stimulation, the N2 component demonstrated a significant increase in amplitude, starting from the second half of the 100-Hz train (Fig. 5B and Table 1). Moreover, a significant decrease in the N1 component was observed at the 200- ( $P = 0.012$ ) and 300-Hz ( $P = 0.00015$ ) trains. The

N2 component increased by 5.77 times: from  $10.14 \pm 1.01$  (N2 control) to  $58.49 \pm 5.98\%$  during the second half of the 300-Hz train (Fig. 5B and Table 1;  $P < 0.00001$ ). It was also observed that total phrenic nerve output (N1 + N2) significantly increased ( $P = 0.00078$ ; Fig. 5B and Table 1) during a 300-Hz train. An example of phrenic nerve responses to 100- to 300-Hz C<sub>4</sub>-EDS is shown in Fig. 6. Stimulus-triggered averaged phrenic responses from this experiment are presented in Fig. 6, bottom. Stimulation of phrenic nerve with single pulse at 1-Hz frequency just after the end of each train resulted in no detectable changes of phrenic nerve components compared with the control. The latencies of N1 and N2 components were not significantly different along the EDS.

**Blood pressure and heart rate changes during C<sub>4</sub>-EDS.** An important clinically relevant finding from the present work was that C<sub>4</sub>-EDS at 300 Hz showed a significant increase in mean BP from  $67.84 \pm 7.31$  mmHg before to  $82.57 \pm 9.04$  mmHg ( $P = 0.028$ ,  $n = 7$ ) with nonsignificant changes (by  $8.41 \pm 5.33\%$ ) in heart rate. This moderate and stable increase in BP could be considered beneficial for the experimental animal's condition, because low BP (by  $26.7 \pm 8.2\%$ ,  $P = 0.002$ ) is always observed posttransection.

## DISCUSSION

**General findings.** The present work demonstrates the following: 1) that the C<sub>4</sub>-cervical segment is the most excitable spinal area of those assessed, as it has the lowest thresholds affecting TP in response to a single pulse of EDS (graph in Fig. 2A); 2) that C<sub>4</sub>-EDS applied with 200- to 300-Hz frequency and 3 × T current intensity resulted in a stable paced breathing (Fig. 3); 3) time- and frequency-dependent reciprocal changes of phrenic nerve components (N1 and N2) in response to EDS with different frequencies (Fig. 5B); 4) significant increase of total phrenic output (N1 + N2) at 300 Hz of EDS (Fig. 5B); and 5) significant increase and stabilization of BP during C<sub>4</sub>-EDS that may play an important role in improving blood tissue perfusion and metabolism postinjury.

**Optimal parameters for EDS.** Identification of threshold currents was a necessary step for standardization/estimation of the optimal stimulation paradigm for EDS. Previous anatomical studies have revealed that C<sub>4</sub> cervical segment represents the center of the phrenic motor nucleus in rats, containing ~62–72% of all phrenic motoneurons (26, 27). This may explain the minimal threshold value for EDS of C<sub>4</sub> segment. Our results show that stimulation at the C<sub>4</sub> segment, with trains at 200 and 300 Hz, 0.2 ms of pulse duration, and 3 × T of current intensity elicited stable breathing with tidal volume equal to, or higher than, that seen in naive animals (1.1–1.4 ml) (31). The same pulse duration (0.2 ms) was used for EDS-evoked stepping activity in paraplegic cats (39). A similar pulse width (0.2–0.215 ms) is traditionally used to provide adequate EDS current intensity in SCI patients (29, 70).

**Changes of phrenic nerve responses during C<sub>4</sub>-EDS in paralyzed animals.** Phrenic nerve responses to EDS demonstrated time- and frequency-dependent reciprocal changes between N1 and N2 components: while N1 was suppressed with higher frequencies (200 and 300 Hz), N2 showed a significant increase in amplitude at the second half of the 100-Hz trains (Figs. 5 and 6). In a study by the Edgerton laboratory (38) performed on low-transected (Th<sub>6</sub>–L<sub>4</sub>) chronic rats, three com-

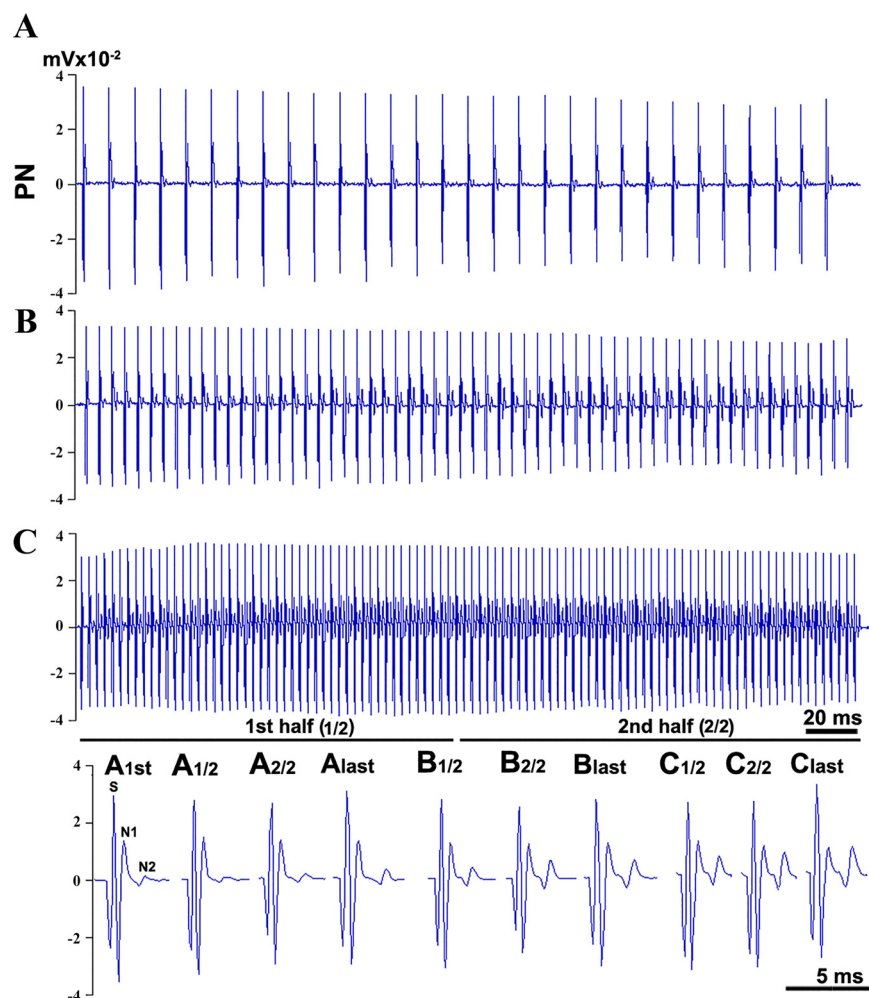


Fig. 6. An example of phrenic nerve component changes in response to different frequencies of C<sub>4</sub>-epidural stimulation (EDS). A–C: phrenic nerve (PN) responses (in mV,  $\text{mV} \times 10^{-2}$ ) to C<sub>4</sub>-EDS at different frequencies (A, 100 Hz; B, 200 Hz; and C: 300 Hz) with  $3 \times$  threshold current intensity (0.33-s train length, 0.2-ms pulse duration). Bottom: A<sub>1/2</sub> to C<sub>1/2</sub> and A<sub>2/2</sub> to C<sub>2/2</sub> are stimulus-triggered averaging of phrenic nerve responses during first and second half period of stimulation at different frequencies (each half is marked with solid line below C). A<sub>1st</sub> to C<sub>1st</sub> and A<sub>last</sub> to C<sub>last</sub> are the first and last phrenic nerve responses at the beginning and the end of each frequency train. S, N1, and N2 are abbreviations used for marking the stimulus artifact (S) and the first (N1) and second (N2) phrenic nerve components, respectively.

ponents (direct, monosynaptic, and polysynaptic) of tibial muscle EMG were detected in response to EDS applied to the first sacral segment. Consistent with this, the N1 component with short latency ( $1.08 \pm 0.02$  ms) may reflect direct activation of phrenic motoneurons, whereas N2 with longer latency ( $2.54 \pm 0.05$  ms) can be related to synaptic origin.

The short-latency N1 component can be evoked by direct stimulation of cell bodies, dendrites extending dorsolateral (22, 49), and antidromic/orthodromic activation of proximal segment of axons. In anesthetized C1Tx rats, it has been shown that high-frequency (100 Hz) antidromic stimulation resulted in hyperpolarization of phrenic motoneurons rather than sustained activation (28). This postspike slow hyperpolarization is partially dependent on  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  outward current ( $\text{K}_{\text{Ca}}^{+}$ ) and plays an important role in limiting neuronal firing frequency (5, 46, 47). Therefore, we hypothesize that the observed depression of the N1-component can be explained by activation of  $\text{K}_{\text{Ca}}^{+}$  current in a time- and frequency-dependent mode.

The synaptic origin of the N2 component can be explained by activation of prephrenic interneurons and descending pathway from the upper cervical (C<sub>1</sub>–C<sub>2</sub>) respiratory group (15, 16, 35–37, 41, 50). The drastic increase of the N2 component (by almost 6-fold) at the end of 300 Hz could reflect a short-term

facilitation, as no changes were detected in phrenic nerve activity in response to low-frequency (1 Hz) stimulation immediately after the 300-Hz trains. In the present work, it is likely that high-frequency stimulation enhances presynaptic release of a neuromodulator (5, 73). This N2 short-term facilitation is also time dependent and was significantly greater in the second half of stimulus trains. The postsynaptic mechanisms of short-term facilitation should also be considered (59). HF-EDS may lead to sustained membrane depolarization and may unblock *N*-methyl-D-aspartate (NMDA) channels by voltage-dependent removal of  $\text{Mg}^{2+}$  (the main blocker of NMDA receptors). Thus there is no need to enhance presynaptic neuromodulator release. The same (NMDA-dependent) mechanism has been proposed in developing short-term potentiation observed around 1 min after focal high-frequency stimulation (100 Hz) of dorsolateral funiculus at the C<sub>1</sub>–C<sub>2</sub> level in anesthetized C1Tx rats (28). It was also reported that EDS (300 Hz) applied during 60 s to C<sub>4</sub> and Th<sub>2</sub> segments elicited short-term potentiation ( $\sim 3$  min) of phrenic nerve activity in anesthetized C<sub>2</sub>-hemisectioned rats, ipsilateral to injury (25). In addition, the severed bulbospinal pathways that may remain functional during first 4–6 h posttransection might also be excited by electrical EDS current. For example, it has been shown that spinal sensory fibers of dorsal columns showed no

abnormality rostral to lesion (L<sub>2</sub>) 24 h postinjury in cats (42). However, in a modeling study that used results from rat lumbar spinal cord EDS experiments, it was shown that a pulse width of 0.2 ms requires the highest current intensity for excitation of spinal pathways (71). Therefore, 0.2-ms pulse duration used in the present work might not be sufficient for excitation of spinal pathways.

The combined (total) phrenic nerve output (N1 + N2) was also measured, to assess whether it was a more accurate estimation of EDS efficacy. The total phrenic nerve output showed a significant increase at 300-Hz frequency compared with 100 and 200 Hz. Higher frequencies were not tested, as their suppressive effect on EDS-paced breathing has been recently reported in dog and rats (13, 14).

**Blood pressure changes during C<sub>4</sub>-EDS.** Our results show a moderate, but significant, increase in BP during EDS. This BP increase may represent an important mechanism for improving tissue perfusion following complete high cervical injury in the present experimental conditions. It is well-known that patients with complete cervical trauma have much lower BP interposed with episodes of cardiovascular dysreflexia, compared with individuals with incomplete injury or paraplegia (21, 69, 72). Cardiovascular dysfunction can lead to hypotension and bradycardia associated with high risk of deep venous thrombosis and coronary heart disease (17, 56, 58). Clinical assessment of EDS for the treatment of pain has been reported to nonsignificantly increase BP during thoracic Th<sub>1</sub>–Th<sub>2</sub> stimulation and decrease BP during Th<sub>5</sub>–Th<sub>6</sub> stimulation (62). A significant increase in cerebral blood flow in response to EDS of the cervical segment in rats was also reported (60). The brief EDS (300 Hz, 60 s) applied to the C<sub>4</sub> segment in anesthetized C<sub>2</sub>-hemisected rats elicited a significant increase of BP at 2 and 12 wk postinjury (25). The increase in BP during C<sub>4</sub>-EDS in the present work can be explained by excitation of descending presympathetic pathways originating from the medullo-cervical pressure zone located between the spinal-medullary junction and C<sub>3</sub> segment (63). Stimulation of these pathways may provide controlled rhythmic activation of sympathetic preganglionic efferent neurons located in thoracic segments and prevent orthostatic hypotension and life-threatening increase of BP during episodes of autonomic dysreflexia in tetraplegic patients (57).

**Conclusion and future perspectives.** The present work highlights the advantage of EDS at the level of the phrenic motor nucleus and short-term changes of phrenic nerve components during high-frequency (100–300 Hz) stimulation. Detailed mechanisms of these phenomena remain unknown, however, and require further investigation to improve the therapeutic potential for EDS pacing of respiratory muscles in patients with complete SCI. It would also be beneficial to assess whether the application of neurotransmitter receptors agonist/antagonists (e.g., by intrathecal delivery) could enhance the efficacy of EDS. For instance, our published data revealed that, in unanesthetized C1Tx rats, spinal circuits are capable of generating synchronous phrenic nerve discharges after disinhibition of GABA<sub>A</sub> and glycine receptors (23). In combination, these methods may lead to developing more effective new approaches to maintain life-supporting ventilation and gas exchange in high-tetraplegic patients. Another consideration is that activation of interneurons can be responsible for time- and frequency-dependent increase of the late phrenic nerve com-

ponent found in the present work, although this requires further conformation with extra/intracellular records from phrenic motoneurons and prephrenic interneurons. In addition, one of the ways to improve parameters of EDS is to use a ramp-shaped paradigm of pacing with different frequencies and current intensity that may resemble the natural phrenic nerve and diaphragm activity (43–45). The effectiveness of incrementing ramplike direct spinal cord (Th<sub>1</sub>–Th<sub>3</sub>) stimulation for pacing of intercostal muscles in spinal dogs has been previously described (53, 54).

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

T.B. and V.M. performed experiments; T.B., M.A.L., and V.M. analyzed data; T.B. and V.M. prepared figures; T.B., M.A.L., and V.M. drafted manuscript; T.B., M.A.L., and V.M. edited and revised manuscript; M.A.L. and V.M. conceived and designed research; M.A.L. and V.M. interpreted results of experiments; V.M. approved final version of manuscript.

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