**Title: Enhancement of self-sustained muscle activity through external dead space ventilation appears to be associated with hypercapnia**

List of authors: Kei Hatano[a]; Ryouta Matsuura[b], Yoshinori Ohtsuka[c]; Takahiro Yunoki[d]

[a] Graduate School of Education, Hokkaido University, Sapporo, Japan

[b] Graduate School of Education, Joetsu University of Education

[c] Department of Sports and Human Studies, Sapporo International University

[d] Faculty of Education, Hokkaido University, Sapporo, Japan

Corresponding author: Kei Hatano

Address: Graduate School of Education, Hokkaido University, Kita-11, Nishi-7, Kita-ku, Sapporo, 060-0811, Japan

E-mail address: k-hatano@eis.hokudai.ac.jp

**Abstract**

We reported that external dead space ventilation (EDSV) enhanced self-sustained muscle activity (SSMA) of the human soleus muscle, which is an indirect observation of plateau potentials. However, the main factor for EDSV to enhance SSMA remains unclear. The purpose of the present study was to examine the effects of EDSV-induced hypercapnia, hypoxia, and hyperventilation on SSMA. In *Experiment 1* ($n$ = 11; normal breathing [NB], EDSV, hypoxia, and voluntary hyperventilation conditions) and *Experiment 2* ($n$ = 9; NB and normoxic hypercapnia [NH] conditions), SSMA was evoked by electrical train stimulations of the right tibial nerve and measured using surface electromyography under each respiratory condition. In *Experiment* 1, SSMA was significantly higher than that in the NB condition only in the EDSV condition ($P$ < 0.05). In *Experiment 2*, SSMA was higher in the NH condition than in the NB condition ($P$ < 0.05). These results suggest that the EDSV-enhanced SSMA is due to hypercapnia, not hypoxia or increased ventilation.

## 1. Introduction

In the chemical control of breathing, changes in $CO_2$ pressure ($PaCO_2$) and $O_2$ pressure ($PaO_2$) in arterial blood are important factors for modulating breathing through stimulation of central and peripheral chemoreceptors (Nattie and Li, 2012; Whipp and Ward, 1998). In this process, the respiratory center in the medulla receives inputs from each chemoreceptor and generates depolarizing currents called central respiratory drive potentials (CRDPs) in spinal motoneurons (MNs) of the respiratory muscles (Butler, 2007).

It has been reported in decerebrate anesthetized cats that plateau potentials are induced in hindlimb MNs when CRDPs are potentiated by $CO_2$ addition (Kirkwood et al., 2002, 2005). Plateau potentials are self-sustained depolarizations of spinal MNs caused by persistent inward currents (PICs). PICs are a powerful intrinsic property of MNs that are activated via voltage-gated channels in MN dendrites (Heckman et al., 2005; Hounsgaard and Kiehn, 1993) and modulated by the action of monoaminergic drives (i.e., serotonin: 5-HT) onto the MNs (Lee and Heckman, 2000). Serotonergic raphe neurons, which project to the spinal cord (Hultborn et al., 2013; Jacobs and Azmitia, 1992), are a part of the central chemoreceptors (da Silva et al., 2011; Hennessy et al., 2017; Ray et al., 2011) that are activated in response to changes in $PaCO_2$

(Corcoran et al., 2013; Mitchell et al., 2008; Veasey et al., 1995, 1997). Taken together, the phenomenon observed by Kirkwood et al. (2002 and 2005) is considered to suggest that increased $PaCO_2$ can induce not only an increase in breathing but also facilitatory modulation into limb MNs. We (Hatano et al., 2018) have recently shown that external dead space ventilation (EDSV) with hypercapnia and hypoxia enhances self-sustained muscle activity (SSMA) in the soleus muscle, which was measured as an indirect estimation of PIC behavior (Collins et al., 2001; Nozaki et al., 2003), supporting the above possibility.

Self-sustained firing of MNs due to PICs has been considered to provide the baseline tone for fundamental behavior such as maintenance of posture (Heckman et al., 2008; Hounsgaard et al., 1988; Lee & Heckman, 1998), locomotion (Brownstone et al., 1992; Heckman et al., 2009; Jacobs and Fornal 1993), and even production of maximal levels of muscular force (Trajano et al. 2014). Therefore, EDSV has the potential to be an intervention to improve fundamental motor function and would be clinically important to explore in detail the mechanism by which EDSV leads to increased SSMA. Nonetheless, the study by Hatano et al. (2018) leaves open the possibility that CRDPs themselves (Kirkwood et al., 2005) or respiratory afferent activity (Balzamo et al., 1997; Morélot-Panzini et al., 2007), which would be enhanced with increased

ventilation by EDSV, could affect motor neuron excitability of limb muscles. Furthermore, hypoxia has been suggested to cause a form of serotonin-dependent motoneuronal enhancement in limb muscles (Christiansen et al., 2018; Hayes et al., 2014; Trumbower et al., 2012). In addition, hypercapnia may contribute to the enhancement of neural activity by hypoxia (Mateika et al., 2018). As mentioned above, EDSV leads to hypoxia as well as hypercapnia. Therefore, the EDSV-induced increase in SSMA observed in our previous study (Hatano et al., 2018) may have been due to hypoxia rather than hypercapnia or due to a combined effect of both. Thus, the relationship between hypercapnia and SSMA remains to be elucidated.

SSMA is one of the indirect indicators of PICs (Collins et al., 2001; Mesquita et al., 2020; Nozaki et al., 2003; Trajano et al., 2014; Walton et al., 2002). Nozaki et al. (2003) confirmed that SSMA is neither due to the primary motor cortex nor due to reverberating activity within closed neuronal circuits involving MNs, demonstrating that it is due to autonomous motor neuron activity involved in the plateau potential. Therefore, in the present study, our primary purpose was to investigate the possibility that hypoxia and / or increased ventilation equivalent to EDSV may be involved in the enhancement of SSMA in the soleus muscle. If hypoxia and increased ventilation are involved in SSMA, it is predicted that SSMA will be enhanced during hypoxia and / or

hyperventilation. This possibility was examined in *Experiment 1* in this study. Furthermore, if hypercapnia contributes independently to the increase in SSMA, SSMA may be enhanced by hypercapnia without hypoxia. This possibility was examined in *Experiment 2* in this study.

## 2. Methods

### 2.1. Subjects

A total of 11 healthy males (mean ± SD: age, 22 ± 2 years; height, 173.1 ± 5.0 cm; body weight, 68.6 ± 7.9 kg) participated in the present study. All of the subjects received detailed and standardized explanations on the experimental procedure and potential risks involved before they provided informed consent for participation in the study. All 11 subjects participated in *Experiment 1*, and 9 of the 11 subjects also participated in *Experiment 2*. The exclusion criteria for subjects used in order to eliminate factors that might influence neuromuscular parameters were as follows: evidence of neurological disorders such as spinal cord injury and neuritis and strenuous physical activity and alcohol drinking within 24 h before each experiment. The sample size ($n$ = 11) of this study was determined based on a previous study ($n$ = 11, Nozaki et al., 2003) using indicators similar to those in this study. This study was compliant with

the Declaration of Helsinki and was approved by the Human Research Ethics Committee of the Graduate School of Education, Hokkaido University.

### 2.2. Measurements and recordings

The subjects breathed through a face mask connected to a hot-wire flow meter and a respiratory gas analyzer (AE-280S, Minato Medical Science, Japan) to measure end-tidal carbon dioxide pressure ($P_{ET}CO_2$), expired ventilation ($\dot{V}_E$) and respiratory flow. $P_{ET}CO_2$ was measured as an index of $PaCO_2$. $P_{ET}CO_2$ and $\dot{V}_E$ were measured throughout the experiment on a breath-by-breath basis under all respiratory conditions in both *Experiment 1* and *Experiment 2*. The flow meter was calibrated using a standard 2-liter syringe. The gas analyzer was calibrated using a precision reference gas ($O_2$, 15.16%; $CO_2$, 5.06%). Arterial oxygen saturation of pulse oximetry ($SpO_2$) was measured throughout the experiments using a pulse oximeter (MLT321, ADInstruments, Australia).

Surface electromyography (EMG) was recorded from the soleus muscle using disposable electrodes (Ag/AgCl; 19 mm × 36 mm, Vitrode F-150S, Nihon-Kohden, Japan). Surface electrodes were placed on the soleus muscle approximately 4 cm distal to the gastrocnemius muscle. The inter-electrode distance was 3 cm. The ground

electrode was placed over the right caput fibula. EMG signals were amplified using an amplifier and filtered between 0.5 Hz and 3 kHz by the amplifier built-in filter (AB-611-J, Nihon-Kohden, Japan).

Respiratory variables, $SpO_2$, and EMG signals were converted into digital signals using an analog-digital converter (PowerLab 16/35, AD Instruments, Australia). The digital signals were stored on a computer at a sampling rate of 4 kHz for offline analysis afterwards (LabChart version 8.1.5, AD Instruments, Australia).

**2.3. Electrical stimulation**

SSMA was induced with reference to previous studies as follows (Collins et al., 2002; Hatano et al., 2018; Nozaki et al., 2003). A constant current stimulator (DS7AH, Digitimer Ltd, UK) was used to deliver percutaneous electrical stimulation (1-ms rectangular pulse) to the tibial nerve of the popliteal fossa of the right leg through stimulation electrodes. The half-ball metal cathode (5 mm in diameter) of the stimulation electrodes was secured to the right leg popliteal fossa, and a silver plate anode (30 mm × 40 mm) was attached to the patella. The electrical stimulation site was determined by adjusting the position of the cathode. Cathode position adjustment was continued until the lowest threshold of the soleus H-reflex was identified while

monitoring EMG of the medial head and lateral head gastrocnemius muscles in the resting state of the subject. The electrical stimulation intensity was unified to the 120% H-reflex threshold during rest breathing under all respiratory conditions. SSMA of the right soleus muscle was induced by three electrical train stimulations (100 Hz, 2-s duration, 2-s intervals, Fig. 1). The subjects were asked to ignore the electrical train stimulation as much as possible.

**2.4. Experimental protocol**

All subjects ($n$ = 11) visited the laboratory before the day of *Experiment 1* for pre-measurements. In pre-measurements, subjects inhaled several concentrations of hypoxic gas (15.8–17.3% $O_2$) using a hypoxic generator (Hypoxico Inc., EVEREST SUMMIT II, USA), and the intensity of hypoxia (15.8% $O_2$) resulting in an $SpO_2$ reduction equivalent to that during EDSV (approximately 90%) was determined. In addition, the volume of low-dose dead space (600–800 mL) to prevent $P_{ET}CO_2$ reduction (i.e., hypocapnia) due to voluntary hyperventilation was determined. Subjects maintained twice the ventilation volume of rest through the mask with 600 ml of external dead space for 1 min with visual feedback of respiratory flow. If $P_{ET}CO_2$ decreased, the volume of the external dead space was increased by 100 ml, and this

voluntary hyperventilation test was repeated with a break of several minutes. Pre-measurements, *Experiment 1* ($n$ = 11), and *Experiment 2* ($n$ = 9) were conducted on different days at intervals of 1 day to 1 week. Therefore, the subjects visited the laboratory 2 or 3 times. Pre-measurements and the two experiments were performed at the about same time of day for each subject. At the time of the two experiments, the room temperature was set to be comfortable (*Experiment 1*: 23.3 ± 1.3 °C, *Experiment 2*: 23.6 ± 1.0 °C) for the subjects. In pre-measurements and each experiment, the subjects lay on a bed in a prone position throughout the measurements or experiment in order to deduce displacement of the cathode position due to slight body movements such as correcting posture. Subjects were allowed to place both arms and face (or chin) on the pillow to ensure a comfortable posture and breathing motion. Prior to start of the experimental protocol in each experiment, the resting respiratory variables of the subjects were measured as baseline values.

*Experiment 1.* The experimental protocol used was based on our previous study (Hatano et al., 2018). A 2-min SSMA-inducing trial was performed three times in each of the four respiratory conditions (normal breathing [NB], external dead space ventilation [EDSV], hypoxia [HX], and voluntary hyperventilation [VH] (12 trials in total). All respiratory conditions were used on the same day. The order of the four

conditions was random among subjects. The interval between trials was 1–10 min: each interval continued until $P_{ET}CO_2$, $SpO_2$ and $\dot{V}_E$ returned to baseline values. In the intervals after the trials in the EDSV and HX conditions, the subjects rested for 5–10 min until respiratory variables returned to baseline values as mentioned above. On the other hand, in the intervals after the trials in the NB and VH conditions, the subjects rested for only 1 min because respiratory variables were normal already or returned immediately. In the NB condition, SSMA was measured while the subjects breathed room air spontaneously through a respiratory mask. In the EDSV condition, an external dead space (1500 mL) was attached to the respiratory mask for 2 min. SSMA was induced 1 min after the start of external dead space. In the HX condition, subjects inhaled hypoxic gas ($O_2$, 15.8%) for 2 min through a respiratory mask with a two-way valve using the hypoxic generator in order to reduce $SpO_2$ equivalent to that during EDSV (approximately 90%). SSMA was induced 1 min after the start of the hypoxic gas inhalation. In the VH condition, subjects were asked to maintain twice the ventilation volume of the baseline with visual feedback of the respiratory flow. SSMA was induced during voluntary hyperventilation. Voluntary hyperventilation continued until the measurement of SSMA in the trial was completed. To avoid hypocapnia in the VH condition, a low-dose external dead space (600–800 mL) was attached to the mask

during voluntary hyperventilation.

*Experiment 2.* To investigate the effect of normoxic hypercapnia (NH) on SSMA, subjects breathed room air (i.e., NB) or a 4% $CO_2$ mixed gas (21% $O_2$, balance $N_2$). SSMA was induced 3 times in each of the NB and NH conditions (6 trials in total). The NB and NH conditions were performed in a random order among the subjects on the same day. The interval between trials was 1–10 min until the respiratory variables returned to the baseline values, as in *Experiment 1*. The NB condition was similar to that in *Experiment 1*. In the NH condition, subjects inhaled 4% $CO_2$ mixed gas for 2 min. SSMA was induced 1 min after start of the 4% $CO_2$ mixed gas inhalation.

For the safety of the subjects, in all respiratory conditions in both experiments, the subjects were asked about their sensation of dyspnea immediately after recording SSMA using the modified 0–10 Borg scale (Borg, 1982) in order to determine whether they could continue the experiment (Gandevia et al., 1993; Nakano et al., 2015).

**2.5. Data processing**

EMG signals were analyzed with a digital band-pass filter of 1.5–1,000 Hz using analysis software. The root mean square (RMS) was calculated from the EMG signals of the right soleus muscle for a 5-s window immediately before (= background

EMG) and for a 30-s window immediately after (= SSMA) electrical train stimulations. The mean value of each respiratory variable was calculated for the same time of SSMA in each trial. The RMSs and respiratory variables in each respiratory condition were averaged for the three trials. For comparisons of SSMA between conditions in each experiment, the RMS of SSMA ($RMS_{SSMA}$) was processed as follow. The difference (ΔSSMA) between $RMS_{SSMA}$ and RMS of background EMG ($RMS_{BG}$) in each trial was calculated and then normalized as a percentage of the mean value of ΔSSMA obtained in all trials (Experiment 1: four conditions * three trials = 12, Experiment 2: two conditions * three trials = 6) for each subject (Danion et al., 2003; Shirakawa et al., 2015). The normalized SSMA was expressed in arbitrary units (AUs).

**2.6. Statistical analysis**

Measured data are presented as means ± standard deviation (SD). Statistical analysis was performed using EZR software of version 1.54, which is a modified version of the R commander (Saitama Medical Center, Jichi Medical University, Japan). Normality of all data was examined using the Shapiro–Wilk test. The variables in *Experiment 1* for which normality was confirmed were examined for the effect of respiratory conditions using one-way repeated measures analysis of variance (ANOVA);

if a main effect was observed, multiple comparisons between the conditions were performed using the Bonferroni *post-hoc* test. If normality was not observed, the effect of respiratory conditions on the variables was examined and compared between conditions using the Friedman test with the Bonferroni-corrected Wilcoxon signed-rank test. In *Experiment 2*, the variables with normality were compared between the respiratory conditions using a paired *t*-test, and the variables without normality were compared using the Wilcoxon signed-rank test. The magnitudes of the changes in variables were expressed as the standardized effect size (Cohen's *d* for a parametric test and *r* for a nonparametric test). The effect size can be classified as small ($0.2 < d < 0.5$, $0.1 < r < 0.3$), medium ($0.5 < d < 0.8$, $0.3 < r < 0.5$), and large ($d > 0.8$, $r > 0.5$) (Cohen, 1988). Pearson's correlation coefficient ($r$) was determined to examine the correlations between the changes in $RMS_{SSMA}$ by each respiratory condition and respiratory variables for which normality was confirmed. Spearman's rank correlation coefficient ($rs$) was determined to examine the correlations between the changes in $RMS_{SSMA}$ and the respiratory variables for which normality was not observed. To analyze the relationship between the changes in $RMS_{SSMA}$ and respiratory variables in each respiratory condition, the percentage of $RMS_{SSMA}$ in each condition to $RMS_{SSMA}$ in NB condition was calculated. Statistical significance was set at $P < 0.05$.

## 3. Results

In *Experiment 1*, ANOVA showed a main effect of respiratory conditions on $P_{ET}CO_2$ ($F_{2, 30} = 41.06$, $P < 0.001$) and $\dot{V}_E$ ($F_{2, 30} = 82.21$, $P < 0.001$). As shown in Table 1, $P_{ET}CO_2$ was significantly higher in the EDSV condition than in the NB ($P < 0.001$, $d = 3.63$), HX ($P < 0.001$, $d = 3.88$) and VH ($P < 0.001$, $d = 2.34$) conditions. There was no significant difference in $P_{ET}CO_2$ between the NB, HX, and VH conditions. $\dot{V}_E$ was significantly higher in the EDSV and VH conditions than in the NB (vs. EDSV: $P < 0.001$, $d = 3.54$; vs. VH: $P < 0.001$, $d = 3.47$) and HX conditions (vs. EDSV: $P < 0.001$, $d = 3.01$; vs. VH: $P < 0.001$, $d = 2.89$). In addition, there were no significant differences in $\dot{V}_E$ between the EDSV and VH conditions and between the NB and HX conditions. The Friedman test showed a main effect of respiratory conditions on $SpO_2$ ($\chi^2_{2, 30} = 22.53$, $P < 0.001$). $SpO_2$ was significantly lower in the HX condition than in the NB ($P = 0.02$, $r = -0.89$) and VH ($P = 0.02$, $r = -0.89$) conditions, and there was no significant difference between the HX and EDSV conditions. Although $SpO_2$ in the EDSV condition was lower than that in both the NB ($P = 0.052$, $r = -0.81$) and VH ($P = 0.02$, $r = -0.89$) conditions, there was a significant difference only between the EDSV and VH conditions. $P_{ET}CO_2$, $\dot{V}_E$ and $SpO_2$ in *Experiment 2* are shown in Table 2. $P_{ET}CO_2$ ($P < 0.001$, $d = 3.39$) and $\dot{V}_E$ ($P < 0.001$, $d = 2.53$) were

significantly higher in the NH condition than in the NB condition. There was no significant difference in $SpO_2$ between the NB and NH conditions.

The normalized SSMA and $RMS_{BG}$ values in *Experiment 1* are shown Fig. 2. ANOVA showed a main effect of respiratory conditions on the normalized SSMA in *Experiment 1* ($F_{2,10} = 5.66$, $P = 0.006$). The normalized SSMA in the EDSV condition (145.6 ± 30.8) was significantly higher than that in the NB condition (69.0 ± 38.8, $P = 0.004$, $d = 2.19$) and that in the HX condition (82.6 ± 30.2, $P < 0.001$, $d = 2.07$) but was not significantly different from that in the VH condition (102.9 ± 56.2). There were no significant differences between normalized SSMA of the NB, HX, and VH conditions. In *Experiment 2*, the normalized SSMA was significantly higher ($P = 0.02$, $d = 1.95$, Fig. 3) in the NH condition (119.8 ± 20.3) than in the NB condition (80.2 ± 20.3). $RMS_{BG}$ was not significantly different between the NB and NH conditions (2.0 ± 1.7 μV vs. 2.2 ± 1.6 μV). In both experiments, there was no significant difference between $RMS_{BG}$ of each respiratory condition (Fig. 2 and Fig. 3). When the changes in $RMS_{SSMA}$ in each condition (percentage to $RMS_{SSMA}$ in the NB condition) were calculated, it showed significant positive correlations with $P_{ET}CO_2$ in the EDSV ($r = 0.72$, $P = 0.01$), HX ($r = 0.61$, $P = 0.04$) and NH condition ($rs = 0.75$, $P = 0.03$, Fig.4). There was no significant correlation between the changes in $RMS_{SSMA}$ and $\dot{V}_E$ or $SpO_2$ in all

conditions.

There was a main effect of respiratory condition on the sensation of dyspnea in *Experiment 1* ($\chi^2_{2, 30}$ = 26.52, $P < 0.001$). The sensation of dyspnea was significantly higher in the EDSV condition (4.4 ± 1.0, moderate – severe) than in the NB condition (0.2 ± 0.3, nothing at all – very slight, $P = 0.02$, $r = -0.89$) and in the HX condition (1.0 ± 1.5, nothing at all – severe, $P = 0.02$, $r = -0.90$) but was not significantly different from that in the VH condition (2.4 ± 2.2, nothing at all – severe). There was a significant difference in the sensation of dyspnea between the NB condition (0.3 ± 0.4, nothing at all – very slight) and the NH condition (2.2 ± 1.5, very slight – severe) in *Experiment 2* ($P = 0.01$, $r = -0.85$). There was no significant correlation between the sensation of dyspnea and changes in $RMS_{SSMA}$ in all conditions.

## 4. Discussion

The main finding of *Experiment 1* was that SSMA increased in the external dead space ventilation (EDSV) condition compared to that in the normal breathing (NB) condition but did not significantly change in the hypoxia (HX) and voluntary hyperventilation (VH) conditions. $SpO_2$ in the HX condition and $\dot{V}_E$ in the VH condition were equivalent to those in the EDSV condition. In *Experiment 2*, SSMA was

higher in the normoxic hypercapnia (NH) condition than in the NB condition. Therefore, it is likely that hypoxia and increased ventilation were not the main factors increasing SSMA during EDSV, raising the working hypothesis that SSMA is enhanced by hypercapnia.

The EDSV condition resulted in a decrease in $SpO_2$ and increases in $\dot{V}E$ and $P_{ET}CO_2$. These results are similar to those in our previous study (Hatano et al., 2018), in which involuntary hyperventilation was induced by EDSV with hypoxia and hypercapnia, as in the present study. The HX condition reduced $SpO_2$ to the same extent as that in the EDSV condition but did not change $\dot{V}E$ and $P_{ET}CO_2$. On the other hand, the VH condition increased $\dot{V}E$ to the same extent as that in the EDSV condition, while the levels of $SpO_2$ and $P_{ET}CO_2$ were maintained at the same levels as those in the NB condition. These results indicate that the effects of hypoxia and hyperventilation were isolated from the EDSV condition in the HX and VH conditions, respectively. Thus, it can be assumed that the condition setting for examining the effects of hypoxia and increased ventilation during the EDSV condition as independent factors on SSMA was established.

SSMA is an indirect measure of PICs (Collins et al., 2001; Mesquita et al., 2021; Nozaki et al., 2003; Trajano et al., 2014). While there are several methods for

deriving SSMA, in the present study, we referenced the methods of Collins et al. (2002) and Nozaki et al. (2003). Reflex responses, which are similar to those in previous studies (Hatano et al., 2018; Nozaki et al., 2003), were elicited during the train stimulation. Therefore, we probably induced neuromuscular activity as a self-sustained muscle activity that is likely to be associated with PICs.

In the present study, to investigate whether hypoxia during EDSV independently affects SSMA, SSMA was induced during similar levels of hypoxia in the HX ($SpO_2$ = 91.0 ± 2.8%) and EDSV ($SpO_2$ = 91.7 ± 3.6%) conditions. As a result, in contrast to the EDSV condition, the HX condition did not alter SSMA (Fig. 2). This result indicates that hypoxia may have not independently enhanced SSMA during EDSV. Regarding the effects of hypoxia, it has been reported that ankle strength in humans with incomplete spinal cord injuries (Trumbower et al., 2012) and corticospinal transmission of a finger muscle (Christiansen et al., 2018) in able-bodied subjects increased after 30-min acute intermittent hypoxia exposure ($FiO_2$ = 9%, $O_2$ saturation = ~81%). It has been considered that the effect of hypoxia on these neural activities may involve the activation of carotid chemoafferents that stimulate episodic 5-HT release by acute hypoxia exposure (Fuller et al., 2000; MacFarlane and Mitchell, 2009), enhancing synaptic input and motor output in the respiratory and somatic motor nuclei (Hayes et

al., 2014). In the HX condition, intensity ($FiO_2$ = 15.8%, $SpO_2$ = 91.0 ± 2.8%) and duration (2 min) of hypoxic exposure were set to be comparable to those in the EDSV condition. Consequently, the hypoxic exposure in the present study was milder and shorter than that in previous studies in which hypoxia-induced facilitation was observed in somatic motor function. Therefore, although the possibility that hypoxia also enhances SSMA cannot be completely excluded, mild hypoxia (2 min, $SpO_2$: ~90%), such as the HX condition in the present study, is not expected to induce SSMA enhancement.

The normalized SSMA in *Experiment 1* was significantly higher than that in the NB condition only in the EDSV condition. In *Experiment 2*, normalized SSMA was significantly increased by inhalation of 4% $CO_2$ mixed gas, which causes hypercapnia without hypoxia. These results support the results of our previous study (Hatano et al., 2018) suggesting that SSMA is enhanced by hypercapnia. Although the mechanism underlying the hypercapnia-induced increase in SSMA is still unknown, it has been postulated that hypercapnia may induce serotonin-dependent enhancement of PICs into spinal motoneurons of the soleus muscle by stimulating serotonergic raphe neurons (Corcoran et al., 2013; Mitchell et al., 2008; Veasey et al., 1995, 1997), which are part of the central chemoreceptors that respond directly to changes in $PaCO_2$ (da Silva et al.,

2011; Hennessy et al., 2017; Ray et al., 2011). In the present study, a significant positive correlation between increases in SSMA and $P_{ET}CO_2$ was found not only in the EDSV condition but also in the HX condition (Fig. 4). This reinforces the above-stated assumption and also suggests that it is important for SSMA to have a certain level of $P_{ET}CO_2$ even in the HX condition.

Resistive breathing has been reported to increase the tonic vibratory reflex (Balzamo et al., 1997) and H-reflex (Morélot-Panzini et al., 2007) in lower limb muscles. This indicates that afferent input associated with respiratory muscle activity may increase the excitability of spinal motoneurons. In other words, it is possible that the increase in SSMA during EDSV is related to an increase in the afferent input associated with increased ventilation. In the present study, there was no significant difference between SSMA in the VH and NB conditions, whereas the EDSV condition augmented SSMA (Fig. 2). $\dot{V}_E$ in the VH condition was higher than that in the NB condition, and $\dot{V}_E$ in the VH condition was the same as that in the EDSV condition (Table 1). In addition, there was no significant correlation between increased SSMA and $\dot{V}_E$ in each condition (Fig. 4). These results suggest that increased respiratory afferent activity and central respiratory drive during EDSV are not the cause of enhanced SSMA.

It has been reported that corticospinal excitability of the vastus lateralis muscle correlates with effort sense of breathing (Shirakawa et al., 2015), and that corticospinal excitability of the biceps brachii muscle increases with respiratory discomfort (Gandevia et al., 1998). Those previous studies suggest that respiratory sensations and arousal may be involved in changes in neuromuscular activity in the extremities. In this study, although we did not measure effort sense of breathing, there was no significant correlation between dyspnea and changes in SSMA. Therefore, changes in respiratory sensations and arousal associated with EDSV are not likely to be involved in SSMA.

The plateau potentials or PICs on which SSMA is based are believed to play an important role in posture (Heckman et al., 2008; Hounsgaard et al., 1988; Lee & Heckman, 1998) and locomotion (Heckman et al., 2009; Brownstone et al., 1992; Jacobs and Fornal, 1992). In addition, a recent study suggested that PICs are also involved in maximal force production of the plantar flexors (Trajano et al., 2014). Therefore, it can be assumed from the present study that EDSV or NH has the potential to be an intervention to improve fundamental motor function. However, it has been reported that exposure to 7% $CO_2$ mixed gas for 10–15 minutes reduces the H-reflex amplitude of the soleus muscle (Beekley et al., 2004, 2014; Maloney and Beekley, 2011). Furthermore, excessive serotonin release can reduce motor neuron activity via

inhibitory 5-HT receptors (5-$HT_{1A}$ receptors) (Perrier and Cotel, 2015). Therefore, more severe $CO_2$ concentrations or prolonged exposure may result in suppression of SSMA caused by electrical train stimulation to afferent nerves. The effects of intensity and duration of hypercapnia on SSMA need to be further studied before SSMA can be generalized as an intervention to improve motor function. Therefore, we concluded that the increase in SSMA by EDSV is due to hypercapnia, not due to hypoxia or increased ventilation. The results suggest that acute stimulation of the central $CO_2$ chemoreceptor by EDSV may be associated with an enhanced SSMA, although the mechanism and clinical significance is not established.

**Data availability**

The data associated with the paper are not publicly available but are available from the corresponding author upon reasonable request.

**Acknowledgments**

This study was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, and Culture (JSPS KAKENHI Grant Number 17K01612).

## Authors' Contribution

KH, MR, and TY conceived and designed the study. KH, OY, and TY conducted the experiments. KH and TY analyzed the data. KH performed the statistical analyses. KH and TY wrote the manuscript. All authors read and approved the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

## References

Balzamo, E., Vuillon-Cacciuttolo, G., Burnet, H., Jammes, Y., 1997. Influence of respiratory afferents upon the proprioceptive reflex of skeletal muscles in healthy humans. Neurosci. Lett. 236, 127–130. https://doi.org/10.1016/S0304-3940(97)00782-9.

Beekley, M.D., Cullom, D.L., Brechue, W.F., 2004. Hypercapnic impairment of neuromuscular function is related to afferent depression. Eur. J. Appl. Physiol. 91, 105–110. https://doi.org/10.1007/s00421-003-0936-x.

Beekley, M.D., 2014. Carbon dioxide alters the Hoffmann reflex independent of hydrogen ions. Int. J. Neurosci. 124, 518–523. https://doi.org/10.3109/00207454.2013.855760.

Borg, G., 1982. Psychophysical bases of perceived exertion. Med. Sci. Sports Exerc. 14, 377–381.

Brownstone, R.M., Jordan, L.M., Kriellaars, D.J., Noga, B.R., Shefchyk, S.J., 1992. On the regulation of repetitive firing in lumbar motoneurones during fictive locomotion in the cat. Exp. Brain Res. 90, 441–455. https://doi.org/10.1007/BF00230927.

Butler, J.E., 2007. Drive to the human respiratory muscles. Respir Physiol Neurobiol. 159, 115–126. https://doi.org/10.1016/j.resp.2007.06.006.

Christiansen, L., Urbin, M.A., Mitchell, G.S., Perez, M.A., 2018. Acute intermittent hypoxia enhances corticospinal synaptic plasticity in humans. Elife. 7, e34304. https://doi.org/10.7554/eLife.34304.

Cohen, J., 1988. Statistical power analysis for the behavioral sciences, 2nd ed. Lawrence Erlbaum Associates, Hillsdale, New York.

Collins, D.F., Burke, D., Gandevia, S.C., 2001. Large involuntary forces consistent with plateau-like behavior of human motoneurons. J. Neurosci. 21, 4059–4065. https://doi.org/10.1523/jneurosci.21-11-04059.2001

Collins, D.F., Burke, D., Gandevia, S.C., 2002. Sustained contractions produced by plateau-like behaviour in human motoneurones. J. Physiol. 538, 289–301. https://doi.org/10.1113/jphysiol.2001.012825

Corcoran, A.E., Richerson, B.G., Harris, M.B., 2013. Serotonergic mechanisms are necessary for central respiratory chemoresponsiveness in situ. Respir. Physiol. Neurobiol. 186, 214–220. https://doi.org/10.1016/j.resp.2013.02.015.

da Silva, G.S.F., Giusti, H., Benedetti, M., Dias, M.B., Gargaglioni, L.H., Branco, L.G.S., Glass, M.L., 2011. Serotonergic neurons in the nucleus raphe obscurus contribute to interaction between central and peripheral ventilatory responses to hypercapnia. Pflugers Arch. Eur. J. Physiol. 462, 407–418. https://doi.org/10.1007/s00424-011-0990-x.

Danion, F., Latash, M.L., Li, S., 2003. Finger interactions studied with transcranial magnetic stimulation during multi-finger force production tasks. Clin Neurophysiol. 114, 1445–1455. https://doi.org/10.1016/S1388-2457(03)00105-6.

Fuller, D.D., Bach, K.B., Baker, T.L., Kinkead, R., Mitchell. G.S., 2000. Long term facilitation of phrenic motor output. Respir. Physiol. 121, 135–146. https://doi.org/10.1016/S0034-5687(00)00124-9.

Gandevia, S.C., Killian, K., McKenzie, D.K., Crawford, M., Allen, G.M., Gorman, R.B., Hales, J.P., 1993. Respiratory sensations, cardiovascular control, kinaesthesia and transcranial stimulation during paralysis in humans. J Physiol. 470, 85–107. https://doi.org/10.1113/jphysiol.1993.sp019849.

Gandevia, S.C., Butler, J.E., Taylor, J.L., Crawford, M.R., 1998. Absence of viscerosomatic inhibition with injections of lobeline designed to activate human pulmonary C fibres. J. Physiol. 511, 289–300. https://doi.org/10.1111/j.1469-7793.1998.289bi.x.

Hatano, K., Shirakawa, K., Usuda, N., Matsuura, R., Ohtsuka, Y., Yunoki, T., 2018. Effect of hypercapnia on self-sustained muscle activity. Respir. Physiol. Neurobiol. 250, 28–30. https://doi.org/10.1016/j.resp.2018.02.002.

Hayes, H.B., Jayaraman, A., Herrmann, M., Mitchell, G.S., Rymer, W.Z., Trumbower, R. D., 2014. Daily intermittent hypoxia enhances walking after chronic spinal cord injury: a randomized trial. Neurology. 82, 104–113. https://doi.org/10.1212/01.WNL.0000437416.34298.43.

Heckman, C.J., Gorassini, M.A., Bennett, D.J., 2005. Persistent inward currents in motoneuron dendrites: Implications for motor output. Muscle and Nerve 31, 135–156. https://doi.org/10.1002/mus.20261.

Heckman, C.J., Hyngstrom, A.S., Johnson, M.D., 2008. Active properties of motoneurone dendrites: Diffuse descending neuromodulation, focused local inhibition. J. Physiol. 586, 1225–1231. https://doi.org/10.1113/jphysiol.2007.145078

Heckman, C.J., Mottram, C., Quinlan, K., Theiss, R., Schuster, J., 2009. Motoneuron excitability: The importance of neuromodulatory inputs. Clin. Neurophysiol. 120, 2040–2054. https://doi.org/10.1016/j.clinph.2009.08.009.

Hennessy, M.L., Corcoran, A.E., Brust, R.D., Chang, Y.J., Nattie, E.E., Dymecki, S.M., 2017. Activity of Tachykinin1-expressing Pet1 raphe neurons modulates the respiratory chemoreflex. J. Neurosci. 37, 1807–1819. https://doi.org/10.1523/JNEUROSCI.2316-16.2016.

Hounsgaard, J., Hultborn, H., Jespersen, B., Kiehn, O., 1988. Bistability of alpha-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. J Physiol. 405, 345–367. https://doi.org/10.1113/jphysiol.1988.sp017336.

Hounsgaard, J., Kiehn, O., 1993. Calcium spikes and calcium plateaux evoked by differential polarization in dendrites of turtle motoneurones in vitro. J Physiol. 468, 245–259. https://doi.org/10.1113/jphysiol.1993.sp019769.

Hultborn H., Zhang M., Meehan C.F., 2013. Control and role of plateau potential properties in the spinal cord. Curr Pharm Des. 19, 4357–4370. https://doi.org/10.2174/1381612811319240004.

Jacobs B.L, Azmitia E.C., 1992. Structure and function of the brain serotonin system. Physiol Rev. 72, 165–229. https://doi.org/10.1152/physrev.1992.72.1.165.

Jacobs, B.L., Fornal, C.A., 1993. 5-HT and motor control: a hypothesis. Trends Neurosci. 16, 346–352. https://doi.org/10.1016/0166-2236(93)90090-9.

Kirkwood, P.A., Lawton, M., Ford, T.W., 2002. Plateau potentials in hindlimb motoneurones of female cats under anaesthesia. Exp. Brain Res. 146, 399–403. https://doi.org/10.1007/s00221-002-1163-0.

Kirkwood, P., Denton, M.E., Wienecke, J., Nielsen, J., Hultborn, H., 2005. Physiological roles for persistent inward currents in motoneurones: insights from the central respiratory drive. Biocybern. Biomed. Eng. 25, 31–38.

Lee, R.H., Heckman, C.J., 1998. Bistability in spinal motoneurons in vivo: Systematic variations in rhythmic firing patterns. J. Neurophysiol. 80, 572–582. https://doi.org/10.1152/jn.1998.80.2.572.

Lee, R.H., Heckman, C.J., 2000. Adjustable amplification of synaptic input in the dendrites of spinal motoneurons in vivo. J Neurosci. 20, 6734–6740. https://doi.org/10.1523/JNEUROSCI.20-17-06734.2000.

MacFarlane, P.M., Mitchell, G.S., 2009. Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism. J. Physiol. 587, 5469–5481. https://doi.org/10.1113/jphysiol.2009.176982.

Maloney, B.J., Beekley, M.D., 2011. Effect of breathing 7% carbon dioxide on the human soleus Hoffmann-reflex recruitment curve. Clin. Neurophysiol. 122, 373–377. https://doi.org/10.1016/j.clinph.2010.07.007.

Mateika, J.H., Panza, G., Alex, R., El-Chami, M., 2018. The impact of intermittent or sustained carbon dioxide on intermittent hypoxia initiated respiratory plasticity. What is the effect of these combined stimuli on apnea severity? Respir. Physiol. Neurobiol. 256, 58–66. https://doi.org/10.1016/j.resp.2017.10.008.

Mesquita, R.N.O., Taylor, J.L., Kirk, B., Blazevich, A.J., 2021. Involuntary sustained firing of plantar flexor motor neurones: effect of electrical stimulation parameters during tendon vibration. Eur. J. Appl. Physiol. 121, 881–891. https://doi.org/10.1007/s00421-020-04563-7

Mitchell, G.S., Turner, D.L., Henderson, D.R., Foley, K.T., 2008. Spinal serotonin receptor activation modulates the exercise ventilatory response with increased dead space in goats. Respir. Physiol. Neurobiol. 161, 230–238. https://doi.org/10.1016/j.resp.2008.02.007.

Morélot-Panzini, C., Demoule, A., Straus, C., Zelter, M., Derenne, J.P., Willer, J.C., Similowski, T., 2007. Dyspnea as a noxious sensation: inspiratory threshold loading may trigger diffuse noxious inhibitory controls in humans. J. Neurophysiol. 97, 1396–1404. https://doi.org/10.1152/jn.00116.2006.

Nakano, T., Iwazaki, M., Sasao, G., Nagai, A., Ebihara, A., Iwamoto, T., Kuwahira, I., 2015. Hypobaric hypoxia is not a direct dyspnogenic factor in healthy individuals at rest. Respir. Physiol. Neurobiol. 218, 28–31. https://doi.org/10.1016/j.resp.2015.07.009

Nattie, E., Li, A., 2012. Central chemoreceptors: locations and functions. Compr Physiol. 2, 221–254. https://doi.org/10.1002/cphy.c100083.

Nozaki, D., Kawashima, N., Aramaki, Y., Akai, M., Nakazawa, K., Nakajima, Y., Yano, H., 2003. Sustained muscle contractions maintained by autonomous neuronal activity within the human spinal cord. J. Neurophysiol. 90, 2090–2097. https://doi.org/10.1152/jn.00200.2003.

Perrier, J.F., Cotel, F., 2015. Serotonergic modulation of spinal motor control. Cur. Opin. Neurobiol. 33, 1–7. https://doi.org/10.1016/j.conb.2014.12.008.

Ray, R.S., Corcoran, A.E., Brust, R.D., Kim, J.C., Richerson, G.B., Nattie, E., Dymecki, S.M., 2011. Serotonergic Neuron Inhibition. Science. 983, 637–642. https://doi.org/10.1126/science.1205295.

Shirakawa, K., Yunoki, T., Afroundeh, R., Lian, C.S., Matsuura, R., Ohtsuka, Y., Yano, T., 2015. Voluntary breathing increases corticospinal excitability of lower limb muscle during isometric contraction. Respir Physiol Neurobiol. 217, 40–45. https://doi.org/10.1016/j.resp.2015.07.003.

Trajano, G.S., Seitz, L.B., Nosaka, K., Blazevich, A.J., 2014. Can passive stretch inhibit motoneuron facilitation in the human plantar flexors? J. Appl. Physiol. 117, 1486–1492. https://doi.org/10.1152/japplphysiol.00809.2014.

Trumbower, R.D., Jayaraman, A., Mitchell, G.S., Rymer, W.Z., 2012. Exposure to acute intermittent hypoxia augments somatic motor function in humans with

incomplete spinal cord injury. Neurorehabil. Neural Repair 26, 163–172. https://doi.org/10.1177/1545968311412055.

Veasey, S.C., Fornal, C.A., Metzler, C.W., Jacobs, B.L., 1995. Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. J. Neurosci. 15, 5346–5359. https://doi.org/10.1523/JNEUROSCI.15-07-05346.1995.

Veasey, S.C., Fornal, C.A., Metzler, C.W., Jacobs, B.L., 1997. Single-unit responses of serotonergic dorsal raphe neurons to specific motor challenges in freely moving cats. Neurosci. 79, 161–169. https://doi.org/10.1016/S0306-4522(96)00673-2.

Walton, C., Kalmar, J.M., Cafarelli, E., 2002. Effect of caffeine on self-sustained firing in human motor units. J. Physiol. 545, 671–679. https://doi.org/10.1113/jphysiol.2002.025064.

Whipp, B.J., Ward, S.A., 1998. Determinants and control of breathing during muscular exercise. Br. J. Sports Med. 32, 199–211. http://dx.doi.org/10.1136/bjsm.32.3.199.

**Figure legends**

**Fig. 1.** Schematic representation of the experimental protocol. The trial to induce self-sustained muscle activity (SSMA) was repeated 12 times in *Experiment 1* and 6 times in *Experiment 2*. The interval between trials was 1–10 min. In each trial, SSMA was induced by electrical train stimulations 1 min after the start of the trial. Normal breathing (NB), external dead space ventilation (EDSV), hypoxia (HX), and normoxic hypercapnia (NH) started 60 s before the stimulation, and voluntary hyperventilation (VH) started 30 s before the stimulation. Four conditions (NB, EDSV, HX and VH) were performed in *Experiment 1*, and two conditions (NB and NH) were performed in *Experiment 2*.

**Fig. 2.** A: Typical examples of EMG signal of the soleus muscle and respiratory flow in one subject in *Experiment 1*. SSMA was induced by repetitive electrical train stimulations (*vertical black bars*) under 4 respiratory conditions. B: Comparisons of root mean squares (RMS) of background EMG (*left panel*) and normalized SSMA values (*right panel*) in the normal breathing (NB), external dead space ventilation

(EDSV), hypoxia (HX), and voluntary hyperventilation (VH) conditions in *Experiment 1*. Data are expressed as means ± SD for $n = 11$. * = significant difference ($P < 0.05$).

**Fig. 3.** A: Typical examples of EMG signal of the soleus muscle and respiratory flow in one subject in *Experiment 2*. SSMA was induced by repetitive electrical train stimulations (*vertical black bars*) under 2 respiratory conditions. B: Comparisons of root mean squares (RMS) of background EMG (*left panel*) and normalized SSMA values (*right panel*) in the normal breathing (NB) and normoxic hypercapnia (NH) conditions in *Experiment 2*. Data are expressed as means ± SD for $n = 9$. * = significant difference ($P < 0.05$).

**Fig. 4.** Relationships between changes in self-sustained muscle activity (SSMA) and respiratory variables ($P_{ET}CO_2$ [*upper panels*], $\dot{V}_E$ [*middle panels*] and $SpO_2$ [*lower panels*]) in the external dead space (EDSV), hypoxia (HX), voluntary hyperventilation and normoxic hypercapnia (NH) conditions. Data presented are each subject's mean value calculated in each condition. The change in SSMA was expressed as percentage of root mean square (RMS) of SSMA ($RMS_{SSMA}$) in each condition to $RMS_{SSMA}$ in the normal breathing (NB) condition was calculated (%NB).

Fig. 1

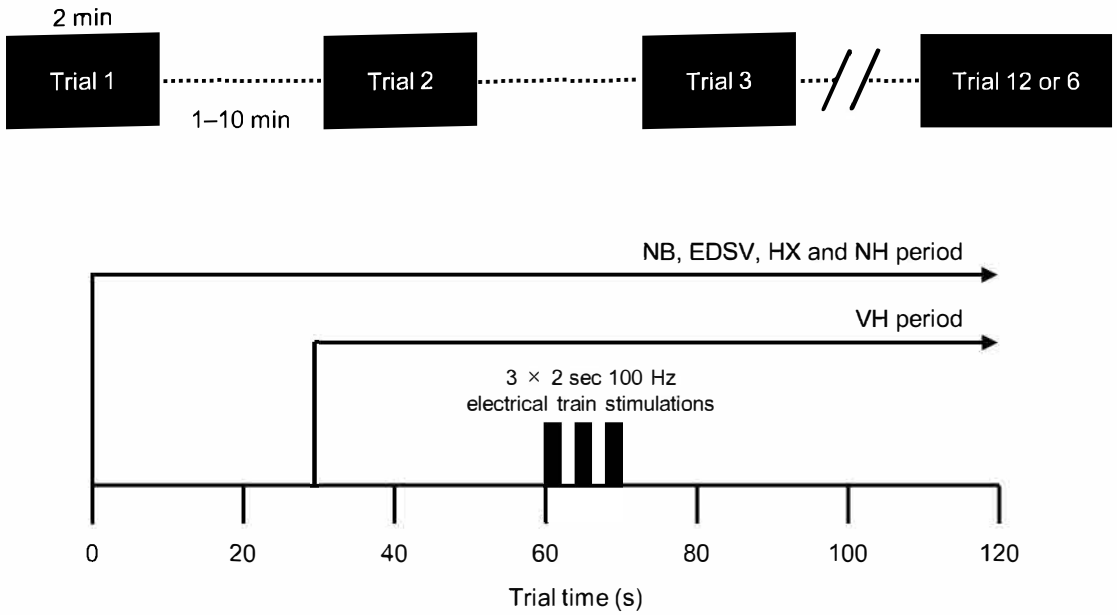
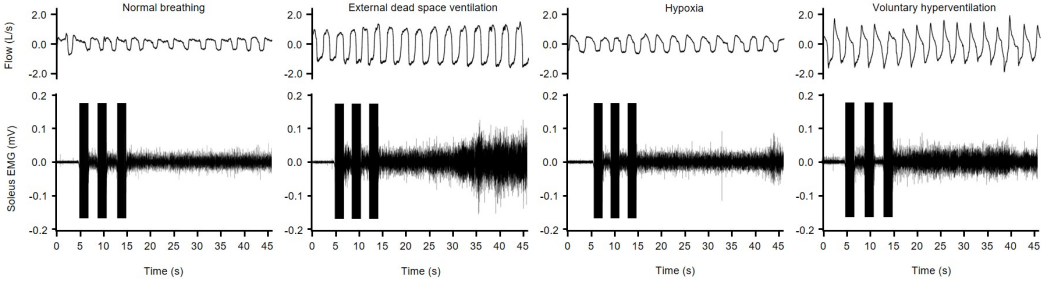
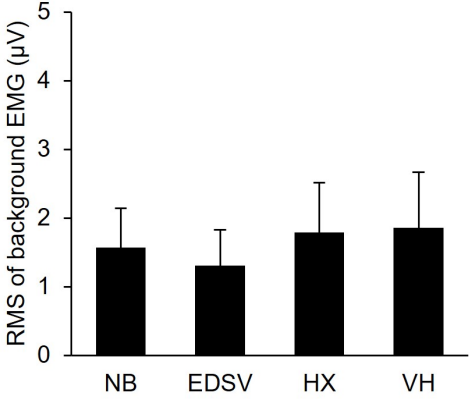

2 min
Trial 1
1–10 min
Trial 2
Trial 3
Trial 12 or 6
NB, EDSV, HX and NH period
VH period
3 × 2 sec 100 Hz
electrical train stimulations
0
20
40
60
80
100
120
Trial time (s)

Fig. 2

A


Normal breathing
External dead space ventilation
Hypoxia
Voluntary hyperventilation
Flow (L/s)
Soleus EMG (mV)
Time (s)

B


RMS of background EMG (μV)
NB
EDSV
HX
VH

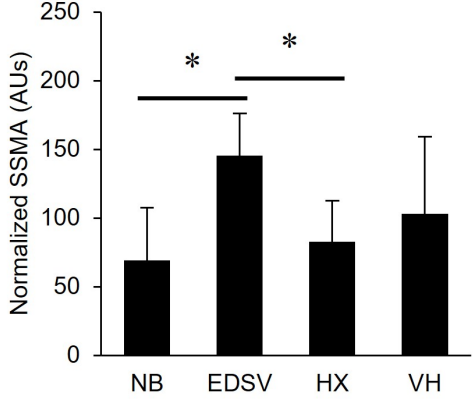
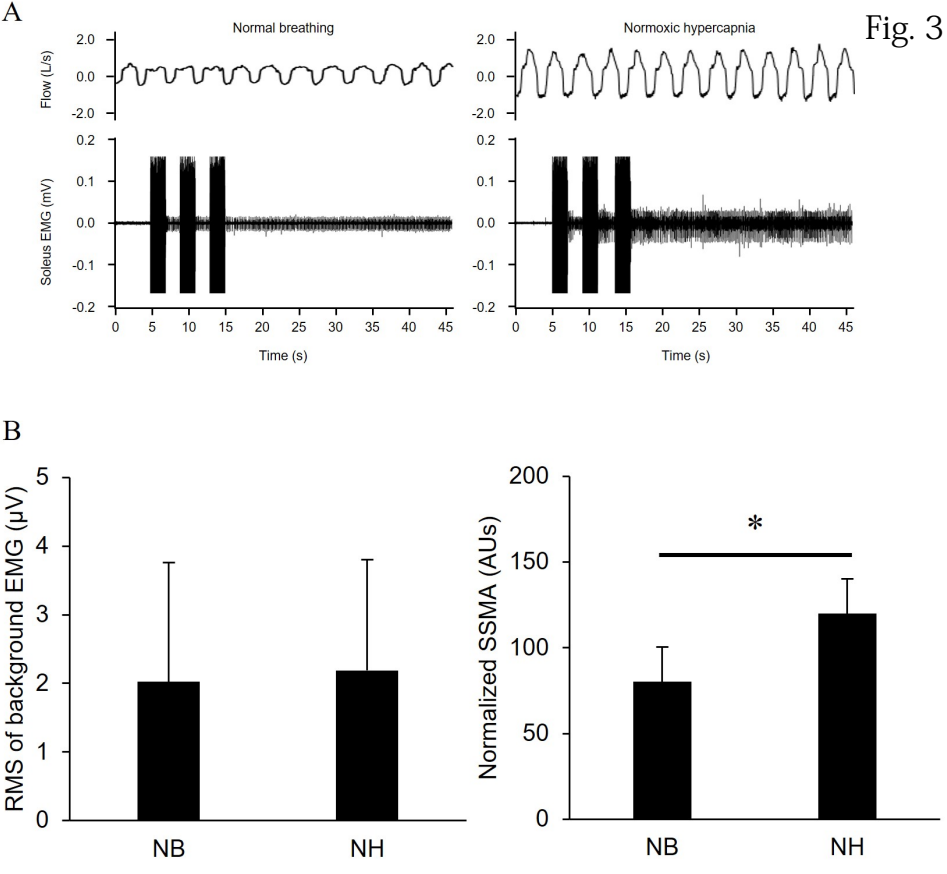

Normalized SSMA (AUs)
*
*
NB
EDSV
HX
VH

Fig. 3


A
Normal breathing
Normoxic hypercapnia
Flow (L/s)
Soleus EMG (mV)
Time (s)
B
RMS of background EMG (μV)
Normalized SSMA (AUs)
*
NB
NH

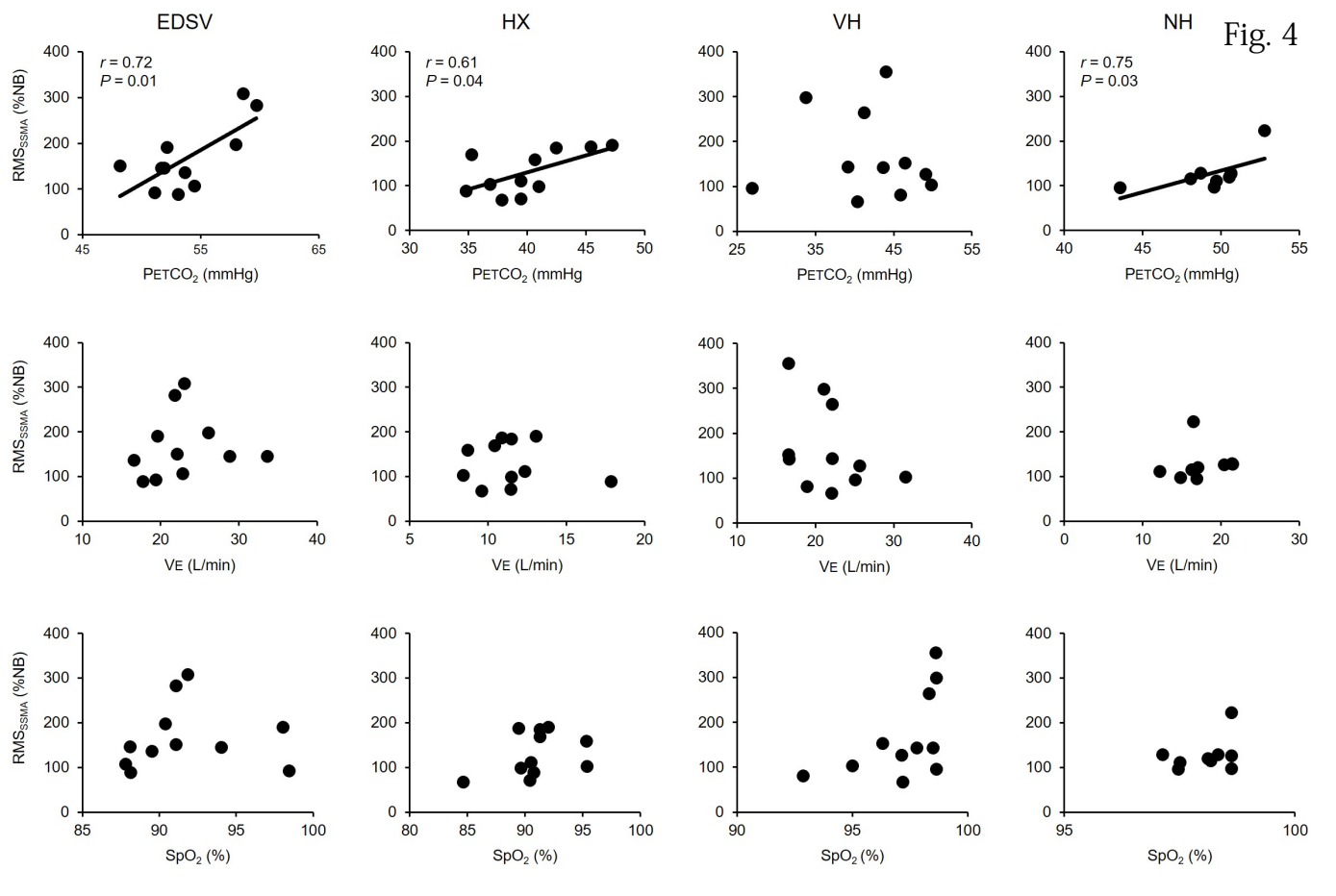

EDSV
HX
VH
NH
Fig. 4
r = 0.72
P = 0.01
r = 0.61
P = 0.04
r = 0.75
P = 0.03
RMSSSMA (%NB)
PETCO2 (mmHg)
VE (L/min)
SpO2 (%)

Table 1. Respiratory variables and $SpO_2$ under the normal breathing (NB), external dead space ventilation (EDSV), hypoxia (HX) and voluntary hyperventilation (VH) conditions in *Experiment 1*.

| | NB | EDSV | HX | VH |
|---|---|---|---|---|
| $P_{ET}CO_2$ (mmHg) | 38.8 ± 4.8 | 53.9 ± 3.4$^{*\dagger\#}$ | 40.0 ± 3.7 | 41.8 ± 6.4 |
| $\dot{V}_E$ (l/min) | 10.0 ± 1.9 | 22.9 ± 4.8$^{*\dagger}$ | 11.4 ± 2.4 | 21.7 ± 4.4$^{*\dagger}$ |
| $SpO_2$ (%) | 97.5 ± 0.8 | 91.7 ± 3.6$^{\#}$ | 91.0 ± 2.8$^{*\#}$ | 97.2 ± 1.8 |

Values are expressed as means ± SD. $^{*}$ = significant difference ($P < 0.05$) vs. the NB condition. $^{\dagger}$ = significant difference ($P < 0.05$) vs. the HX condition. $^{\#}$ = significant difference ($P < 0.05$) vs. the VH condition.

Table 2. Respiratory variables and $SpO_2$ under the normal breathing (NB) and normoxic hypercapnia (NH) conditions in *Experiment 2*.

| | NB | NH |
|---|---|---|
| $P_{ET}CO_2$ (mmHg) | 41.4 ± 2.4 | 49.3 ± 2.4* |
| $\dot{V}_E$ (l/min) | 11.6 ± 1.4 | 17.4 ± 2.9* |
| $SpO_2$ (%) | 97.7 ± 0.7 | 98.1 ± 0.5 |

Values are expressed as means ± SD. * = significant difference ($P < 0.05$) vs. the NB condition.