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Focal Microdialysis of CO₂ in the Perifornical-Hypothalamic Area Increases Ventilation during Wakefulness but not NREM sleep

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Abstract

We investigated whether the perifornical-lateral hypothalamic area (PF-LHA), where the orexin neurons reside, is a central chemoreceptor site by microdialysis of artificial cerebrospinal fluid (aCSF) equilibrated with 25% CO₂ into PF-LHA in conscious rats. This treatment is known to produce a focal tissue acidification like that associated with a 6 to 7 mm Hg increase in arterial P_{CO₂}. Such focal acidification in the PF-LHA significantly increased ventilation up to 15% compared with microdialysis of normal aCSF equilibrated with 5% CO₂ only in wakefulness but not in sleep in both the dark (P=0.004) and light (P<0.001) phases of the diurnal cycle. This response was predominantly due to a significant increase in respiratory frequency (11%, P<0.001). There were no significant effects on ventilation in the group with probes misplaced outside the PF-LHA. These results suggest that PF-LHA functions as a central chemoreceptor site in the central nervous system in a vigilant state dependent manner with predominant effects in wakefulness.

Keywords

perifornical-lateral hypothalamus; central chemoreception; orexin

1. Introduction

Central chemoreception, which regulates breathing by detecting changes in CO₂/pH in the interstitial tissue of the brain, has been studied mostly in the brain stem at sites including but not limited to the retrotrapezoid nucleus (RTN) (Guyenet, 2008; Li and Nattie, 2002), the medullary raphe (Nattie and Li, 2001; Taylor et al., 2005; Wang et al., 1998), the locus ceruleus (Biancardi et al., 2008; Noronha-de-Souza et al., 2006), nucleus tractus solitaries (NTS) (Dean et al., 1989; Nattie and Li, 2002) and the caudal ventrolateral medulla (cVLM) (da Silva et al., 2010). The role of higher regions of the central nervous system such as the lateral hypothalamus, which participate in multiple physiological functions, in chemoreception is less well understood.

There are studies that suggest a role for the lateral hypothalamus in the regulation of breathing. Destruction of the lateral hypothalamus in lightly anesthetized cats by high

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frequency currents caused an immediate decrease in rate and/or depth of respiration (Redgate and Gellhorn, 1958). Extracellular recording showed that the neurons located in dorsal and lateral hypothalamic areas increased firing rate in responding to hypercapnic stimulation in lightly-anesthetized rabbits (Cross and Silver, 1963). In the past decade, many studies have shown that orexin/hypocretin neurons, which are exclusively localized in the lateral hypothalamus, are sensitive to CO₂/pH changes both *in vitro* and *in vivo*. A switch from 5% to 10% CO₂ in the artificial cerebrospinal fluid (aCSF) (pH change from 7.02 to 7.25) can induce an increased firing rate of orexin neurons (Williams et al., 2007). A measure of c-Fos activation shows that systemic CO₂ activates orexin neurons in the perifornical region in mice (Sunanaga et al., 2009). In transgenic mice lacking the precursor gene of orexin (OX-KO), prepro-orexin, there is a 50% decrease in the ventilatory CO₂ response in wakefulness but not during sleep (Deng et al., 2007). This hypothesis was further investigated in pharmacological studies with inhibition of both orexin receptors (OX₁R and OX₂R) using the oral administration of a dual orexin receptor antagonist, Almorexant. The CO₂ response was attenuated by 26% only in wakefulness and only during the dark, active, period of the diurnal cycle (Li and Nattie, 2010).

We hypothesize that the neurons in the perifornical lateral hypothalamic area (PF-LHA) including orexin neurons are chemosensitive to CO₂/H⁺ and they participate in the central chemoreception in a vigilance-state dependent manner. To test this hypothesis we focally acidified PF-LHA area with high CO₂ (25%) by using a reverse microdialysis technique and measured the ventilation in wakefulness and NREM sleep during both dark and light phase of the diurnal cycles.

2. Methods

2.1. Ethical approval

The animal experiments and surgical protocols were within the guidelines of the National Institutes of Health for animal use and care and the American Physiological Society's Guiding Principles in the Care and Use of Animals and approved by the Institutional Animal Care and Use Committee at the Dartmouth College Animal Resource Center.

2.2. General and Diurnal cycle

A total of 28 adult male Sprague–Dawley rats (250–350 g) were used for the experiments and were individually housed in the Animal Resource Center in a light- and temperature-controlled environment with a 12h light and 12h dark cycle. The light cycle was set as 12:00 am lights on and 12:00 pm lights off. Food and water were provided *ad libitum*. Animals were randomly divided into two groups to be tested during either light or dark cycle. The experiments were carried out from 8:00 am ~11:00 am for the light cycle study and from 1:00 pm ~4:00 pm for the dark cycle test.

2.3. Surgery

All the rats were surgically implanted with a microdialysis guide cannula, electroencephalography (EEG) & electromyography (EMG) electrodes, and a telemetric temperature probe. The detailed surgical procedures have been described in our earlier publications (Li et al., 2006; Nattie and Li, 2001). Briefly, sterile surgeries were performed under general anesthesia produced by intramuscular injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). Rats were fixed in a Kopf stereotaxic frame and a microdialysis guide cannula (CMA11, Microdialysis AB, Stockholm, Sweden) was implanted into the PF-LHA. The coordinates for probe placement were 3.3 mm caudal and 1.8 mm lateral from the lambda and 8.5 mm below the surface of the skull (Paxinos and Watson, 1998). Three EEG electrodes were screwed onto the skull and two EMG electrodes were inserted into the

dorsal neck muscles, and all electrode wires were connected to a six-prong plastic pedestal attached to the skull. The guide cannula and the plastic pedestal were secured with cranioplastic cement on the top of the rat's head. A sterile telemetry temperature probe (TA-F20, Data Sciences, St Paul, MN, USA) was implanted into the peritoneal cavity. After the incisions were sutured and cleaned, the animal was allowed to recover for at least 7 days.

2.4. CO₂ Microdialysis Solution

The aCSF was equilibrated with 5 or 25% CO₂. The composition of the aCSF was (in mM) 152 sodium, 3.0 potassium, 2.1 magnesium, 2.2 calcium, 131 chloride and 26 bicarbonate. The calcium was added after the aCSF was acidified by equilibration with CO₂. The measured pH of this solution is ~6.8 for equilibration with 25% CO₂ and ~7.45 with 5% CO₂. The syringe pump for the dialysis was set at a speed of 40 μ l/min for all the experiments.

2.5. Ventilation, Oxygen Consumption and Temperature Measurement

The methods used to measure the ventilation ($\dot{V}_E$), body temperature (T_b) and oxygen consumption ($\dot{V}_{O_2}$) in conscious rats were those commonly used in our lab (Li et al., 2006; Nattie and Li, 2001). In brief, a whole body plethysmograph was used to measure ventilation and the volume of the plethysmograph chamber was 7.6L. The analog output from the pressure transducer was sampled at 150Hz and converted into a digital signal by a computer using the DATAPAC 2K2 system (RUN Technologies, USA). The rate of gas inflow was maintained at 1.4 l min⁻¹ with the plethysmograph at atmospheric pressure. CO₂ and O₂ fractions were sampled from the outflow line at ~100 ml min⁻¹ by the CO₂ and O₂ gas analyser (Applied Electrochemistry). Before each experiment, we calibrated the plethysmograph by obtaining pressure response data from five 0.3 ml air injections made with a 1 ml syringe. Breath-by-breath analysis was performed using the pressure deflections and the respiratory cycle time for each breath.

Tidal volume (V_T) and respiratory frequency (f_R) were calculated per breath to estimate ventilation per breath. V_T was calculated by using the equation published by Bartlett and Tenney in 1970 (Bartlett and Tenney, 1970). $\dot{V}_E$ was calculated as the product of $V_T \cdot f_R$. All ventilatory parameters are expressed as Mean $\pm$ S.E.M. in quiet wakefulness (wakefulness) or NREM sleep. Oxygen consumption was calculated by application of the Fick principle, using the difference in O₂ content between inflow air and outflow air and the flow rate as described previously (Li et al., 2006; Nattie and Li, 2001). Core body temperature was measured continuously using the signal from the telemetric temperature probe inside the abdomen. The temperature of the experimental chamber was measured using a thermometer within the plethysmograph.

2.6. Determination of vigilance state

The EEG and EMG signals from the electrodes implanted on the skull and in the neck muscles were sampled at 150Hz, filtered at 0.3–70 and 0.1–100 Hz, respectively, and were acquired continuously using the DATAPAC system. The vigilance states were determined by analysis of EEG and EMG records as previously described (Li et al., 2006; Nattie and Li, 2001). In brief, a fast Fourier transform was performed on the EEG and EMG signal at 4.0-s-long epochs. We categorized the vigilance states as wakefulness, NREM and REM sleep. Both wakefulness and NREM sleep states were observed consistently through the experiments but periods of REM sleep were short and sometimes were absent during the experiment period. Thus ventilation events that occurred during REM or when sleep state was indeterminant were excluded from our analysis.

2.7. Experimental protocol

At least 7 days after surgery, the rat was weighed and then a microdialysis probe was gently inserted into the guide cannula and EEG and EMG electrode cable was connected. The animals were placed into the plethysmograph chamber and allowed 1 h to acclimate. All experiments were performed at room temperature (24–25 °C) with the rat breathing room air. During acclimation, we began reverse-microdialysis with 5% CO₂ - equilibrated aCSF without recording any data. After acclimatization, baseline measurements were recorded for 30 min with dialysis of 5% CO₂. The microdialysis solution was then switched to 25% CO₂-equilibrated aCSF and measurements were continued for another 30min followed by second period of dialysis with 5% CO₂ with data recorded for another 30 min. All animals were submitted to the same protocol and received only one microdialysis probe insertion and 1-day of experimentation. The animals first were randomly divided into two groups based on the diurnal cycle that the experiments were conducted, as dark (N=14) and light (N=14) groups, and then within the dark and light group the animals were further divided into two sub-groups based on the placement of the microdialysis probe, as within-PF-LHA (N=8 & 9 respectively) and outside-PF-LHA groups (N=6 & 5 respectively) (Fig1B & C).

2.8. Anatomy analysis

Upon conclusion of the experiment, the rats were euthanized with an overdose of sodium pentobarbitone injected intraperitoneally (>75mg/kg). The whole brain was removed, frozen, and sectioned at 50 µm thickness with a Reichert–Jung cryostat. The sections were then stained with cresyl violet to identify the locations of the microdialysis probe tips.

2.9. Statistics

The data are presented as mean ± standard error of the mean (S.E.M.). We analyzed data measured during wakefulness and NREM sleep separately with a one-way repeated measures (RM) ANOVA to compare V_T , f_R , $\dot{V}_E$, $\dot{V}_{O_2}$, and T_b changes within each of three groups; 1) control (5% CO₂ dialysis), 2) acidification (25% CO₂ dialysis), and 3) control post focal acidosis (5% CO₂ dialysis). Bonferroni post hoc test was applied when appropriate.

3. Results

3.1. Microdialysis probe placement

A representative example of the anatomic location of a microdialysis probe placement in the PF-LHA is shown in Fig. 1A (4x magnification). A series of schematic cross-sections of the hypothalamus in a caudal to rostral organization demonstrating the placement of the microdialysis probe tips (black filled symbols) are shown in Fig 1B and 1C. Based on the diurnal cycle and anatomic locations of the probe placement the rats were divided into four groups, 1) the probe tips were within the PF-LHA and rats studied in the dark phase (Fig 1B left, N=8); 2) the probe tips were within the PF-LHA and rats studied in the light phase (Fig 1B right, N=9); 3) the probe tips were misplaced outside PF-LHA and rats studied in the dark phase (Fig 1C left, N=6); the probe tips were outside PF-LHA and rats studied in the light phase (Fig 1B right, N=5). The anatomical location of the microdialysis probes of 17 rats in group 1 and 2 are within the PF-LHA, which are comparable with the location of the orexin neurons in the LHA demonstrated by orexin-immunoreactive perikarya staining in the adult rat brain (Nambu et al., 1999).

Minor tissue damage and gliosis were observed surrounding the location of the probe tip, but never extended more than a 0.4-mm radius from the tip center. This damage did not appear to alter ventilatory responses induced by focal acidification in control rats.

3.2 Typical experiments

The representative experiments with time course analysis of before, during and after high 25% CO₂ microdialysis are illustrated in Fig. 2, which shows that the increase of $\dot{V}_E$ during focal acidification depended on the animal's arousal state. Fig. 2A shows EEG and EMG recording throughout the experimental protocol for this individual animal. As expected, the animal cycled between wakefulness and sleep periods before, during and after focal acidification. Fig. 2B shows $\dot{V}_E$ changes during the time course throughout the experimental periods in both dark (Fig. 2B, upper panel) and light (Fig. 2B, lower panel) phases in two individual animals. Comparing to the control 5% CO₂ dialysis, 25% CO₂ dialysis into the PF-LHA significantly increased $\dot{V}_E$ only in wakefulness but not in NREM sleep. The similar response was observed during both dark and light cycles.

3.3. Ventilatory effects: focal acidification of PF-LHA

Fig. 3 summarizes the effects of focal acidification of PF-LHA on $\dot{V}_E$, f_R and V_T in wakefulness and NREM sleep during both dark and light phases.

In wakefulness during the dark cycle, $\dot{V}_E$ was 70 ± 3 (ml min⁻¹ 100g⁻¹) during first 5% CO₂ dialysis period and increased to 80 ± 2 (ml min⁻¹ 100g⁻¹) during higher 25% CO₂ dialysis, which was a 15% increase ($P = 0.004$). $\dot{V}_E$ then returned to 71 ± 3 (ml min⁻¹ 100g⁻¹) after switching back to 5% CO₂ dialysis. The significantly increased $\dot{V}_E$ by the focal acidification of PF-LHA was primarily contributed by respiratory frequency. During dark cycle, f_R increased from 71 ± 2 (min⁻¹) during the first period of 5% CO₂ microdialysis to 79 ± 3 (min⁻¹) during 25% CO₂ microdialysis, an 11% increase ($P < 0.001$). During the second period of 5% CO₂ dialysis, f_R fell back to 70 ± 3 (min⁻¹). V_T was not significantly affected by any period of CO₂ microdialysis. In wakefulness during the dark cycle, V_T was 1.00 ± 0.04 , 1.04 ± 0.05 , and 1.03 ± 0.05 (ml 100g⁻¹) during first 5% CO₂, 25% CO₂ and second 5% CO₂ dialysis period, respectively. There is no significant difference on $\dot{V}_E$, f_R and V_T between the first and second periods of 5% CO₂ dialysis ($P = 0.05$). The similar ventilatory effects were observed in wakefulness during the light phase (Fig. 2B and 3).

Distinct from the responses observed during wakefulness, focal acidification did not induce any significant changes on $\dot{V}_E$, f_R or V_T during NREM sleep in either dark or light phase.

3.4. Ventilatory effects: focal acidification outside of PF-LHA

Fig. 4 shows the changes of $\dot{V}_E$, f_R and V_T in the groups that microdialysis probe tips were mis-located outside the PF-LHA during 5% and 25% CO₂ dialysis in wakefulness and sleep during either dark or light phase. Focal acidification outside the PF-LHA with 25% CO₂ compared to 5% CO₂ did not significantly change $\dot{V}_E$, f_R , V_T , body temperature and metabolic rate in wakefulness or NREM sleep during either light or dark phases.

3.5. Body temperature and metabolic rate

There were no significant effects on body temperature and oxygen consumption by microdialysis of either 5% or 25% CO₂ in wakefulness and sleep during dark and light phases (data not shown).

4. Discussion

The key finding of this study is that focal acidification of PF-LHA by CO₂ significantly increased ventilation predominantly by increasing respiratory frequency only in wakefulness but not in NREM sleep during both light and dark phases of the diurnal cycle. Our data suggest that the PF-LHA may play a role in the central chemoreception depending on the animal's arousal state.

4.1. Focal acidification by microdialysis of CO₂/H⁺

Using the reverse microdialysis with a high CO₂/H⁺ aCSF to produce a focal acidosis in a specific brain site for studying the central chemoreception in wakefulness and sleep in conscious animals has been well established (Biancardi et al., 2008; da Silva et al., 2010; Li and Nattie, 2002; Martino et al., 2006; Nattie and Li, 2001, 2002). The approach does not distinguish the specificity of the stimulation at cellular level, e.g., CO₂ vs H⁺, but it does address the functional difference in the central chemoreception at various brain regions in different arousal state and diurnal cycle.

We have demonstrated in our previous study that aCSF solution equilibrated with 25% CO₂ only provides a mild to moderate focal acidification in the region (Li and Nattie, 2002). Focal acidification of the RTN area by 25% CO₂, the same stimulating intensity used in this study, induced an average focal pH change of 0.069 pH units measured within 200 μm of the microdialysis probe, which was equivalent to that induced by a 6.6 mm Hg increase in arterial P_{CO₂} (Li and Nattie, 2002). Here we showed that this modest acidification in the area of PF-LHA significantly stimulated breathing mainly by increasing respiratory frequency in wakefulness while the same acidification in the area outside PF-LHA showed no effects on breathing in either arousal state (Fig. 1C & 4).

It is also important to note that in this study the enhanced respiratory response to the focal acidification by dialysis of CO₂ was only noted when the probe was placed within the area of PF-LHA; no such respiratory response to the same focal acidification was observed when the probe was placed outside PF-LHA (see figures 1, 3 and 4). These data indicate that: 1) not all neurons or areas of the brain are chemosensitive to CO₂/H⁺ and respond to such focal acidosis generated by microdialysis of high CO₂; 2) microdialysis of CO₂ can produce a specific stimulation in specific areas that can excite breathing.

4.2. Lateral hypothalamus and central chemoreception

The LHA has been found to regulate body homeostasis, blood pressure, heart rate, food intake, and energy balance and stress response. Orexin (or hypocretin) neurons are specifically localized within the hypothalamus (LHA) including the PF-LHA, dorsomedial hypothalamic nucleus (DMH), and posterior hypothalamic area (Nambu et al., 1999). The orexin receptors (OX₁R and OX₂R) and orexin axonal projections are widely distributed in the central nervous system including many sites that are important in cardiorespiratory regulation (Nambu et al., 1999; Trivedi et al., 1998). Recent studies have found that the orexin neurons may participate in central chemoreception (Deng et al., 2007; Song et al., 2012; Sunanaga et al., 2009; Williams et al., 2007). The *c-Fos* expression in orexin neurons were significantly increased in the perifornical region and the dorsomedial hypothalamus after systemic hypercapnic stimulation in mouse (Sunanaga et al., 2009). In mouse brain slices, the spontaneous firing rate of identified orexin neurons is profoundly affected by physiological changes of H⁺ and CO₂. The firing rate of the orexin neurons increases as pH decreases with ~100% rate increase per 0.1 pH unit (Williams et al., 2007). Song et al. (2012) showed that the acid-sensing ion channel 1 (ASIC1) is co-localized with orexin-A in the LHA, and focal injection of acidified aCSF into the LH significantly increased phrenic nerve activity in anesthetized rats (Song et al., 2012). It is possible that the neurons in the PF-LHA area sense CO₂/H⁺ and participate in the central chemoreception through the ASIC1 located on the orexin neurons in the region.

We acknowledge that other types of neurons are also located in the PF-LHA area, e.g. melanin concentrating hormone (MCH) neurons, and that they may also contribute to the response observed in this study.

4.3. Multiple central chemoreceptor sites and sleep-wake states

Previous studies by our group and others have shown that there are multiple central chemoreceptor sites in the brain, for example, the locus ceruleus, medullary raphe, caudal ventrolateral medulla, the RTN and caudal NTS (Coates et al., 1993; da Silva et al., 2010; Haxhiu et al., 1996; Hodges and Richerson, 2010; Li and Nattie, 2002; Nattie and Li, 2002). Many of these central chemoreceptor sites function in an arousal state dependent manner, e.g. focal microdialysis of 25% CO₂: 1) in the RTN, increased ventilation (mostly V_T) by 24% only in wakefulness (Li and Nattie, 2002); 2) in the caudal medullary raphe, increased ventilation (mostly f_R) by 20% only in NREM sleep (Nattie and Li, 2001); 3) in the cVLM increased ventilation (both V_T and f_R) by 17% only in wakefulness (da Silva et al., 2010); 4) in the caudal NTS significantly increased ventilation by 16% in sleep and 28% in wakefulness (Nattie and Li, 2002). Here we show that focal dialysis of 25% CO₂ into the PF-LHA increased ventilation (mostly f_R) by 15% only in wakefulness. The data supports the hypothesis that among multiple central chemoreceptor sites in the brain some function in an arousal state dependent manner. The multiple central chemoreceptor sites may work together to control the breathing and maintain the homeostasis and physiological functions in different arousal states.

Orexin neurons discharge maximally during active wakefulness and the firing rate gradually slows down during quiet wakefulness in the absence of movement and ceases during sleep (Lee et al., 2005). The level of orexin in the CSF has a diurnal-rhythm, with a peak level at the end of the dark, predominantly wake-active period, and low levels toward the end of the light, predominantly sleep period (Desarnaud et al., 2004). Studies have shown that orexin participates in the control of breathing in an arousal state dependent manner. For examples, OX-KO mice, lacking orexins, have a significantly attenuated CO₂ response during wakefulness but not during sleep (Nakamura et al., 2007). Antagonism of both orexin receptors by Almorexant attenuates the CO₂ response by 26% only in wakefulness during the dark period of the diurnal cycle (Li and Nattie, 2010). In this study we have demonstrated that the focal acidification of the PF-LHA only stimulates breathing when the animals are awake. All of these data suggest that the LHA and orexin system participate in central chemoreception in a vigilance-state-dependent manner.

In summary, focal acidification of the PF-LHA by CO₂/H⁺ significantly increased breathing (predominantly respiratory frequency) only in wakefulness but not in sleep. Our study suggests that the higher regions of the central nervous system, such as the lateral hypothalamus, which participate in multiple physiological functions, may also play a role in the central chemoreception.

Acknowledgments

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Highlights

- We focal acidified Perifornical-Lateral Hypothalamic Area (PF-LHA) by CO₂/H⁺ in conscious rat.
- Focal acidification of PF-LHA significantly increased ventilation mainly due to breathing frequency.
- The enhanced ventilatory response was only observed in wakefulness not in sleep.
- Focal acidification of outside the PF-LHA had no effect on respiration.

Figure 1.

Anatomical locations of the tip of the microdialysis probe. (A) a photomicrograph of a stained coronal section of the lateral hypothalamus of one representative rat with the tip of microdialysis probe near the fornix indicated in the white rectangle. Scale bar represents 0.5 mm. The schematized anatomical cross sections show in (B) the probe placement within the PF-LHA in the dark (left, N=8) and light (right, N=9) phase groups; and (C) the probe placement outside PF-LHA in the dark (left, N=6) and light (right, N=5) phase groups. The dark filled ovals represent the locations of the microdialysis probe tips. The numbers on the left refer to millimeters caudal to Bregma. Abbreviations: mt, mammillothalamic tract; f, fornix; 3v, third ventricle. The drawings are modified from the atlas of Paxinos & Watson (Paxinos and Watson, 1998).

Figure 2.

Illustrative example of one single experiment in one animal with the microdialysis probe located in the PF-LHA. (A) EEG and EMG recordings with breathing tracing before, during and after focal acidification. Scale bar represents 6 min. The open rectangles refer to 6 s. (B) time course of changes in $\dot{V}_E$ before, during and after 25% CO_2 microdialysis in the perifornical hypothalamic area in dark (upper panel) and light (lower panel) phase in two individual rats. Dark circles refer to $\dot{V}_E$ during quiet wakefulness and open circles NREM sleep. The grey rectangles represent the period of microdialysis of 25% CO_2 .

Figure 3.

$\dot{V}_E$, f_R and V_T (Mean $\pm$ S.E.M.) during CO₂ microdialysis in the groups with the tips located within the PF-LHA in both dark (N = 8, left panel) and light (N=9, right panel) phases. $\dot{V}_E$ and f_R increased significantly during high CO₂/H⁺ (25% CO₂) dialysis compare to normal 5% CO₂ in wakefulness (!) but not in NREM sleep (!) during dark and light phase of the diurnal cycle. * P < 0.01, ** P < 0.001, one-way RM ANOVA; Bonferroni post hoc test.

Figure 4.

$\dot{V}_E$, f_R and V_T (Mean $\pm$ S.E.M.) during CO₂ microdialysis in the animals with the tips located outside the PF-LHA in both dark (N = 6, left panel) and light (N=4, right panel) phases. $\dot{V}_E$, f_R and V_T were not significantly changed during high CO₂/H⁺ (25% CO₂) dialysis compare to normal 5% CO₂ in both wakefulness (!) and NREM sleep (!) during dark and light phase of the diurnal cycle (P > 0.05, one-way RM ANOVA).