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High CO₂/H⁺ dialysis in the caudal ventrolateral medulla (Loeschcke's area) increases ventilation in wakefulness

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Abstract

Central chemoreception, the detection of CO₂/H⁺ within the brain and the resultant effect on ventilation, was initially localized at two areas on the ventrolateral medulla, one rostral (rVLM-Mitchell's) the other caudal (cVLM-Loeschcke's), by surface application of acidic solutions in anesthetized animals. Focal dialysis of a high CO₂/H⁺ artificial cerebrospinal fluid (aCSF) that produced a milder local pH change in unanesthetized rats (like that with a ~6.6 mm Hg increase in arterial P_{CO₂}) delineated putative chemoreceptor regions for the rVLM at the retrotrapezoid nucleus and the rostral medullary raphe that function predominantly in wakefulness and sleep, respectively. Here we ask if chemoreception in the cVLM can be detected by mild focal stimulation and if it functions in a state dependent manner. At responsive sites just beneath Loeschcke's area, ventilation was increased by, on average, 17% (P < 0.01) only in wakefulness. These data support our hypothesis that central chemoreception is a distributed property with some sites functioning in a state-dependent manner.

Keywords

arousal state; control of breathing; central chemoreception

1. Introduction

Central chemoreception, the respiratory response to changes in brain CO₂/H⁺, was originally localized bilaterally at rostral and caudal aspects of the surface of the ventrolateral medulla (VLM) (Mitchell et al., 1963a,b; Schlaefke et al. 1970; Trouth et al., 1973; Loeschcke, 1982). In anesthetized animals, Mitchell et al. (1963a,b) demonstrated that focal application of acidic solutions to a restricted surface area in the rostral aspect of the VLM was followed by an increase in ventilation. A more caudal aspect of the VLM (known as Loeschcke's area) was also reported to be a site for generating respiratory responses after electrical stimulation (Loeschcke, 1982) or pH changes, also under anesthesia (Schlaefke et al., 1970). For a period of time the VLM was believed to be the only or main site for central chemoreception (Mitchell et al., 1963a,b; Loeschcke, 1982) a hypothesis that has narrowed to the rVLM and that remains

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under debate (Richerson et al., 2005; Guyenet et al 2008; Branco et al 2009; Guyenet et al., 2009; Nattie and Li 2009; Corcoran et al., 2009). Our current view is that central chemoreception is a widely distributed function in the brain (Nattie 1999; Nattie 2001; Li & Nattie 2002; Nattie & Li 2006, 2008, 2009).

Recent work *in vitro* and *in vivo* has focused on specific sites and neurons involved in central chemoreception in the rVLM including the retrotrapezoid nucleus; RTN (Li & Nattie, 2002; Guyenet et al., 2008; Nattie & Li, 2009), medullary raphe; MR (Richerson et al., 2005; Corcoran et al., 2009), and locus coeruleus; LC (Biancardi et al., 2008; Hartzler et al., 2008). However, the caudal region of ventrolateral medulla (cVLM or Loeschcke's area) described in the classical literature has received little attention, none in the conscious animal. Data from anesthetized animals with moderately severe acidic stimulation do corroborate the findings reported in the early studies (Mitchell et al. 1963a,b; Schlaefke et al. 1970; Trouth et al., 1973; Loeschcke, 1982) supporting the cVLM as a site for chemoreception. For example; 1) The location of neurons responsive to an acute infusion of CO₂ enriched saline via the vertebral artery includes the cVLM (Arita et al., 1989). 2) Douglas et al. (2001) showed the activation of neurons in this caudal region by mapping the distribution of c-fos expression after acid-base stimulation. 3) Ribas-Salgueiro et al. (2003) demonstrated, in anaesthetized rats the presence of neurons that fired spontaneously (not coupled to the respiratory cycle) and were responsive to acidic stimulus in the cVLM. This region contains 3rd order (propriobulbar) neurons identified by retrograde tracing of retrovirus injected into the diaphragm (Dobbins and Feldman, 1994) and respiratory modulated GABAergic neurons involved in blood pressure regulation (Mandel and Schreihöfer, 2006).

We hypothesize that the cVLM contains a site for central chemoreception that functions in the unanesthetized rat in an arousal-state dependent manner and is responsive to the mild focal stimulation produced by CO₂ microdialysis. As such the cVLM will join other sites similarly tested whose function varies depending on the arousal state (Nattie 2001; Nattie & Li, 2006, 2008), e.g., the RTN (responsive in wakefulness; Li et al., 1999; Li & Nattie, 2002), the MR (responsive in sleep; Nattie & Li, 2001), and the NTS (responsive in both wakefulness and sleep; Nattie & Li, 2002).

2. Methods

2.1. General

All animal experimentation 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. A total of 21 adult male Sprague–Dawley rats (260–340 g) were used for the experiments in the present study and they were individually placed in the animal facility in a light- and temperature-controlled room with 12 h of light beginning at midnight and 12 h of darkness beginning at noon. Fifteen rats successfully completed the entire study and their data are presented in this paper. All the experiments were carried out during the end of the 12 hr light period between 8.00am–12.00pm. Some of the methods described here were described previously (for more details see Li et al., 1999, Nattie & Li, 2001, 2002; Dias et al., 2008).

2.2. Surgery

Animals were anaesthetized by intramuscular injection of ketamine (100 mg kg⁻¹) and xylazine (15 mg kg⁻¹). The head and a portion of the abdomen were shaved and the skin was sterilized with betadine solution and alcohol. Rats were fixed in a Kopf stereotaxic frame, and a dialysis guide cannula (CMA11, Microdialysis AB, Stockholm, Sweden) was implanted in the caudal

region of VMS. The coordinates for probe placement were 4.4 mm caudal and 1.6 mm lateral from the lambda, and 10.3 mm below the surface of the skull and at a 6° angle (Paxinos & Watson, 1998). The guide cannula was secured with cranioplastic cement. Three EEG electrodes were screwed into the skull, with the frontal electrode 2 mm anterior to bregma and 2 mm lateral to the midline, the parietal electrode 4 mm anterior to lambda and 2 mm lateral to the midline (both in the right side), and the ground electrode between the frontal and parietal electrodes (in the left side). A pair of EMG electrodes was inserted deep into the neck muscle. The skull wound was sutured. A sterile telemetry temperature probe (TA-F20, Data Sciences, St Paul, MN, USA) was placed in the abdominal cavity. The incision was closed, and the animal was allowed to recover for 7 days.

2.3. CO₂ dialysis 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 equilibrated with CO₂. The measured pH of this solution is ~ 6.8 for equilibration with 25% CO₂ and ~7.45 with 5% CO₂. The dialysis pump was run at a speed of 40 µL/min.

2.4. EEG and EMG signals

The signals from the EEG and EMG electrodes were sampled at 150 Hz, filtered at 0.3–50 and 0.1–100 Hz, respectively, and recorded on the computer. Arousal state was determined by analysis of EEG and EMG records. Both wakefulness and NREM sleep states were observed consistently through the experiments but periods of REM sleep were short and were not present in every experiment. Thus ventilation events that occurred during REM or when sleep state was indeterminant were excluded from our analysis.

2.5. Ventilation measurement

Ventilation was obtained by whole body plethysmography and the chamber used in these experiments is as previously described (Li et al., 1999, Nattie & Li, 2001, 2002; Dias et al., 2008). The animal chamber operates at atmospheric pressure, with the inflow and outflow of inspired gases balanced to prevent hyper- or hypobaric conditions in the box. The inflow gas was humidified, and the flow rate was controlled by a flowmeter (model 7491T; Matheson) and the chamber temperature was measured by a thermometer inside the chamber. The outflow port was connected to the in-house vacuum system via a flowmeter. A high-resistance “bleed” of the outflow line provided 100 mL/min of outflow gas to the O₂ and CO₂ analyzers (Applied Electrochemistry). The flow rate through the plethysmograph was maintained at or above 1.4 L/min to prevent CO₂ rebreathing. The plethysmograph was calibrated with 0.3-ml injections. The analog output of the pressure transducer was converted to a digital signal and directly sampled at 150 Hz by a computer using the DataPac 2000 system. Tidal volume (V_T) and breathing frequency (f) were calculated per breath to estimate ventilation ($\dot{V}_E$) per breath. Using EEG and EMG criteria, $\dot{V}_E$ was calculated in each animal and during each state condition (wakefulness or NREM sleep). V_T was calculated using the formula published by Bartlett and Tenney (1970).

Two analyses were performed on these ventilatory variables. First, we examined the time course of the response to CO₂ dialysis. For this, whenever possible, $\dot{V}_E$ was calculated each five minutes during wakefulness and sleep periods throughout the experimental protocol. Second, we averaged the $\dot{V}_E$ values during the dialysis protocol (5% CO₂ and 25% CO₂) according to arousal state (wakefulness and sleep). Because the animals did not have the sleep-wake cycles at the same times, it was difficult to express mean group values for the first time

course analysis. So we present in the results section examples of the time course analysis for individual animals and the analysis averaged by arousal state for each group.

2.6. Oxygen consumption and body temperature

Oxygen consumption ($\dot{V}_{O_2}$) was calculated by the Fick principle using the difference in O_2 content between inspired and expired gas and the flow rate through the plethysmograph and normalized to $mL \cdot 100g \text{ body wt}^{-1} \cdot h^{-1}$. The inflow O_2 content was measured at the beginning of each experiment, and the outflow content of O_2 was read from the O_2 and CO_2 sensors constantly during the experiment. Rat body temperature (T_b) was measured by using the analog output via telemetry from the temperature probe in the peritoneal cavity.

2.7. Anatomy analysis

At the end of the experiment, the rats were killed with an overdose of sodium pentobarbitone injected intraperitoneally ($>75 \text{ mg kg}^{-1}$), the brainstems were frozen and then sectioned at $50 \mu m$ thickness with a Reichert–Jung cryostat. The sections were stained with cresyl violet. The distance between interaural line and the probe tip (rostral-caudal level) was calculated by measuring the distance between the center of the probe tip and the ending of the inferior olive (medial nucleus; IOM) in the caudal region of medulla (-5.30 mm relative to interaural line; Paxinos & Watson, 1998).

2.8. Experimental protocol

Seven days after surgery, the rats were weighed and then gently held while the dummy cannula was removed and the dialysis probe was inserted into the guide tube and EEG and EMG electrode cable connected. By 7 days the rats are eating well, gaining weight and have normal body temperatures and activity. The animals were placed into the plethysmograph chamber and allowed 30–40 min to acclimate. All experiments were performed at room temperature ($23\text{--}25^\circ\text{C}$) with the rat breathing room air. Dialysis with 5% CO_2 -equilibrated aCSF began when the animals were placed into the chamber. They were dialyzed with 5% CO_2 during a period without any measurement (acclimatization period), and then baseline measurements were made over 30 min. The dialysis solution was then switched to 25% CO_2 -equilibrated aCSF and measurements were made over 35 min. After that, the dialysis solution was changed again to 5% CO_2 and measurements were made during 20 min. All animals were submitted to this same protocol and received only one dialysis probe insertion and 1 day of experimentation.

There are two groups, and rats were placed into each group based on their ventilatory response to focal acidification (25% CO_2 dialysis). Based on previous studies using the same protocol and stimulus intensity (Li et al., 1999; Nattie & Li, 2001; Li & Nattie, 2002), we considered responsive animals those that had an increase in averaged ventilation ($> 10\%$ of the baseline value in either arousal state) during 30 min of 25% CO_2 dialysis test (responsive group, $n=8$). Animals that did not increase ventilation (by this defined criteria) were grouped in the non-responsive group ($n=7$). We decided to group the animals using their response to CO_2 dialysis as criteria rather than the anatomical localization of dialysis probes, because the anatomical boundaries in this caudal region proposed to be involved in central chemoreception are not well established. In the study by Ribas-Salgueiro et al., (2003), in which they described the presence of neurons sensitive to H^+ stimulus in the caudal region, they found low H^+ -sensitive neurons loosely distributed in the caudal region studied, while high H^+ -sensitive neurons were located close to the ventral surface of the medulla. The description of anatomy and the methods that they used to measure rostral-caudal level were imprecise. Hence, we grouped the animals in the present study as responsive or non-responsive and showed the anatomy figures with the probe placement for each animal.

2.9. Statistics

We applied to the awake and the sleep data sets separately a repeated measures (RM) ANOVA to determine if $\dot{V}_E$, V_T , f changed during the CO₂ dialysis comparing the control (5% CO₂ dialysis), acidification (25% CO₂ dialysis) and return after acidosis (5% CO₂ dialysis). Post hoc comparisons were performed using Dunnett's test. In addition we applied a two-way RM ANOVA to the awake and sleep data sets together to determine if there was an interaction between state and CO₂ dialysis. Post hoc comparisons were made using a modified Bonferroni's test.

3. Results

3.1. Anatomy

A stained cross section of the medulla of one responsive animal with the location of the microdialysis probe tip is shown in figure 1A. Figure 1B shows a series of schematic cross sections of the medulla in a rostral to caudal organization demonstrating the location of the dialysis probe tip for each rat included in this study. Different symbols are used to show and compare the location of responsive with non-responsive animals. For responsive animals (black filled symbols, Fig. 1B), the mean distance from interaural line for eight probe tips was -4.66 ± 0.09 mm (mean $\pm$ S.E.) with a range of -4.20 to -4.96 mm. In the responsive group, six of 8 animals had the probe close to the ventrolateral surface of medulla, while two animals had the probes located more dorsally. Figure 1B also schematically shows (highlighted areas in the left side) an approximate distribution of H⁺-sensitive neurons described by Ribas-Salgueiro et al. (2003). In the responsive group, five of 8 animals had the probe within the H⁺- sensitive neurons containing-region while the other 3 were just adjacent to this area.

For the non-responsive animals (grays filled symbol, Fig. 1B) the mean distance from interaural line for seven probe tips was -5.05 ± 0.10 mm (mean $\pm$ S.E.) with a range of -4.60 to -5.35 mm. Of these 7 probe locations, one was within the area described by Ribas-Salgueiro et al., (2003) while three were caudal and three were dorsal.

3.2. Focal acidification: Individual example

The time course analysis of $\dot{V}_E$ before, during, and after the high CO₂ dialysis test is presented for an individual rat in Fig. 2, which shows that the increase of $\dot{V}_E$ during focal acidification depends on the animal's arousal state. Fig. 2A shows EEG and EMG recording throughout the experimental protocol for this animal. As expected, the animal cycled between wakefulness and sleep periods before, during and after focal acidification. Fig. 2A also shows that the time course increase of $\dot{V}_E$ is awake-sleep dependent. $\dot{V}_E$ increased during focal acidification in wakefulness periods but not in sleep periods. Interestingly, in the beginning of the dialysis of high CO₂ $\dot{V}_E$ did not increase if the animal was asleep, but did increase when the animal was awake. In this animal during focal acidification in wakefulness, the peak response of $\dot{V}_E$ was ~47% higher than the control value right before high CO₂ dialysis.

3.3. Focal acidification: mean values for responsive vs. non-responsive animals

Fig. 3 shows the $\dot{V}_E$, f and V_T responses of the responsive group during control and high CO₂ dialysis tests in different states. Baseline values were not different in the two groups (Figs. 3 and 4). In wakefulness, dialysis of high CO₂ increased $\dot{V}_E$ significantly compared to control (first period of control dialysis) ($p = 0.003$, one-way RM ANOVA; $p < 0.001$ Dunnett's *post hoc* comparison). $\dot{V}_E$ increased due to a significant increase of V_T ($p = 0.049$; one-way RM ANOVA; $p < 0.01$ Dunnett's *post hoc* comparison) and f ($p = 0.04$; one-way RM ANOVA;

$p < 0.01$ Dunnett's *post hoc* comparison). Comparing mean values before and during focal acidification, $\dot{V}_E$ increased 17%. During sleep, focal acidification (dialysis of high CO_2) had no effect on $\dot{V}_E$, f and V_T . Two-way RM ANOVA revealed a significant interactive effect between arousal state and high CO_2 dialysis on $\dot{V}_E$ ($p < 0.001$, modified Bonferroni's *post hoc* comparison).

Fig. 4 shows the $\dot{V}_E$, f and V_T responses of the non-responsive group during control and high CO_2 dialysis tests in different arousal states. Focal dialysis of high CO_2/H^+ aCSF did not affect $\dot{V}_E$, f and V_T in this group in wakefulness or sleep. Baseline values of $\dot{V}_E$, f and V_T did not differ between the responsive and non-responsive groups.

3.4. $\dot{V}_{O_2}$ and body temperature

Because of the characteristics of $\dot{V}_{O_2}$ measurement (relatively slow due to the time it takes for wash-out of the plethysmograph), it was not possible to calculate and express it according to animal arousal state. However, oxygen consumption did not vary significantly between groups during CO_2 dialysis tests. The mean values for responsive group during control, high CO_2 and control dialysis respectively are: 1.05 ± 0.06 , 0.99 ± 0.05 and $1.04 \pm 0.07 \text{ mL} \cdot 100\text{g} \cdot \text{h}^{-1}$ (mean $\pm$ S.E). For these same times in the non-responsive group the values are: 1.03 ± 0.08 , 1.02 ± 0.06 and $1.02 \pm 0.06 \text{ mL} \cdot 100\text{g} \cdot \text{h}^{-1}$ (mean $\pm$ S.E). Rat body temperature (T_b) also did not change significantly between groups during CO_2 dialysis tests (data not shown). Moreover, the mean initial and final T_b did not differ throughout the experimental protocol.

4. Discussion

4.1. Focal dialysis of CO_2/H^+ ; rationale

We apply reverse microdialysis with a high CO_2/H^+ aCSF to produce a focal change in CO_2 and H^+ in a specific brain region in unanesthetized rats. This approach allows application of a change of CO_2 and H^+ that are of a mild to moderate degree (see below) and to a focal region. The responses of the rat can be examined in sleep and wakefulness; the dialysis per se does not appear to affect sleep wake cycling. This approach does not allow distinction of the stimulus specificity, i.e., whether it is CO_2 or H^+ , nor does it yield mechanistic information at the cellular level. It does yield important systems level information about the function of different brain region in central chemoreception in different arousal states. Our hypothesis was that the caudal area would respond during wakefulness, much like the more rostral RTN region (Li et al., 1999).

4.2. Focal dialysis of CO_2/H^+ ; degree of acidification

In the present study we applied the high CO_2/H^+ microdialysis in a caudal region of medulla just dorsal to Loeschcke's area of the cVLM surface, as described in the classical literature. In the rostral aspect of the medulla, previous studies have used high CO_2/H^+ dialysis in single chemoreceptor sites to evaluate ventilatory responses to this stimulus in different arousal states. With dialysis in the rat RTN of an aCSF solution equilibrated with 25% CO_2 , Li & Nattie (2002) showed that the average focal pH change of 0.069 pH units measured within 200 μm of the dialysis probe was equivalent to that induced by a 6.6 mm Hg increase in arterial P_{CO_2} . Dialysis with aCSF equilibrated with 5 % CO_2 produced no change in brain pH. In marked contrast, if performed under anesthesia the focal dialysis at the RTN with aCSF equilibrated with 25% CO_2 produced a focal pH change like that observed with a 35 mm Hg increase in estimated arterial P_{CO_2} , which was limited to within 600 μm of the 2 dialysis probe (Li et al., 1999). Thus, our application of this technique in the conscious, unanesthetized rat allows examination of focal central chemoreceptor sites using a stimulus applied directly within

the brain at an intensity that is approximately equivalent to a 6.6 mm Hg increase in arterial P_{CO_2} , arguably a physiological level of stress. In contrast, when 2 breathing 7% CO_2 the arterial P_{CO_2} increases by 15 mm Hg (Li & Nattie, 2002).

Others have applied the technique of focal dialysis of aCSF equilibrated with high CO_2 in unanesthetized goats. In a series of studies the Forster lab has shown: 1) increases in ventilation with stimulation of the caudal medullary raphe in wakefulness during the daytime (Hodges et al., 2004a); 2) greater responses with simultaneous stimulation of multiple as compared to a single medullary raphe site in wakefulness (Hodges et al., 2004b); 3) both stimulation and inhibition by CO_2/H^+ in the fastigial nucleus of the cerebellum (Martino et al., 2006); and 4) stimulation of breathing frequency by CO_2/H^+ in the pre-Bötzinger complex (Krause et al., 2009). In general these studies required the use of higher concentration of CO_2 in the dialysate as well as a larger dialysis probe. However, the measured changes in tissue pH adjacent to the probes was of a similar magnitude, 0.06 pH units (Hodges et al., 2004a) to 0.18 pH unit (Hodges et al., 2004a, b).

4.3. Focal dialysis of CO_2/H^+ ; homogeneity of tissue responses

We did not measure focal tissue pH in this study as we did in our initial study (Li & Nattie, 2002). Measurements of tissue pH in conscious goats before and during an increase in inspired CO_2 have described a similar degree of tissue pH regulation in the RTN, facial nucleus, gigantocellularis reticularis nuclei, and caudal and rostral medullary raphe (Hodges et al., 2004b). In contrast, tissue pH regulation is absent in the pre-Bötzinger complex of conscious goats (Krause et al., 2009) and estimates of tissue pH in anesthetized animals indicates the presence of some heterogeneity (Arita et al., 1989). Our focus in this study, the cVLM, lies within the ventral medullary regions that appear to be relatively homogenous in respect to local tissue pH regulation at least as studied in conscious animals.

4.4. Focal dialysis of CO_2/H^+ ; responses at different sites

To date in the rat, three sites (RTN, medullary raphe and NTS) have been assessed with regard to their awake-sleep dependent response to focal dialysis of high CO_2/H^+ . In the RTN $\dot{V}_E$ was increased by about 24% due to increases in V_T (Li & Nattie, 1999). This response occurred in wakefulness; no response was observed if the animal was asleep. In the medullary raphe, high CO_2/H^+ dialysis induced a ~20% increase of $\dot{V}_E$ mediated by respiratory frequency only during sleep (Nattie & Li, 2001). And in the caudal aspect of the NTS, focal high CO_2/H^+ dialysis increased $\dot{V}_E$ by 20-30% in both wakefulness and sleep (Nattie & Li, 2002).

In the present study, we used the same stimulus intensity as in these previous studies (25% CO_2 dialysis) to address the question of whether or not focal high CO_2/H^+ dialysis in the caudal ventrolateral region of the medulla would increase $\dot{V}_E$ and if so, verify the state dependence of this response. Our results in conscious, freely moving, unanaesthetized animals showed a significant increase in $\dot{V}_E$ (~17% on average) only in wakefulness. Although the response seems to be smaller than in the RTN, it had a similar awake-sleep pattern (Li et al., 1999). Further, the peak response was often greater as shown in Fig. 2. While in the RTN $\dot{V}_E$ increased mainly due to V_T , the observed increase in $\dot{V}_E$ in our results was mediated by both V_T and f . These results fit well into the idea of multiple sites for central chemoreception (Nattie, 2001; Nattie & Li, 2005; Nattie & Li, 2008; 2009) and arousal state dependence was postulated as one hypothesis to explain the presence of widespread sites for sensing CO_2 ; different sites may vary in their role dependent on the state of arousal (Nattie, 2001; Nattie & Li, 2005; Nattie & Li, 2008; 2009).

We found a smaller increase in $\dot{V}_E$ during focal high CO_2/H^+ dialysis in the present study compared to previous ones, however it does not necessarily imply less importance for this region in central chemoreception. In fact the overall high system sensitivity for respiratory responses to systemic hypercapnia may be due to several sites acting together, hence the more central chemoreceptor sites that participate the less the sensitivity that needs to be attributed to each site (Li & Nattie, 2002). Also, the increased $\dot{V}_E$ induced by the focal high CO_2/H^+ dialysis may lower arterial P_{CO_2} and inhibit other chemoreceptor sites (Li & Nattie, 2002). Thus the observed response to focal acidification is likely to be an underestimate of the true contribution of that site.

4.5. Central chemoreception sites: rostral vs. caudal

Recent studies on central chemoreception have focused on structures located more rostrally in the brain stem. The RTN region has been extensively studied and is believed to correspond to the VLM surface area described earlier in the literature as area M (Mitchell et al. 1963a,b; Loeschcke, 1982). The RTN has anatomical connections with the respiratory network (Cream et al. 2002; Rosin et al., 2006) and contains putative chemosensitive neurons of a specific phenotype (Phox2b and VGlut2 expressing) (Guyenet et al., 2008). The medullary raphe and LC have also been studied in some depth and neuronal phenotypes involved in chemoreception have been described (serotonergic neurons in raphe and catecholaminergic neurons in the LC) (Richerson et al., 2005; Biancardi et al., 2008; Hartzler et al., 2008; Corcoran et al., 2009).

In the caudal medulla, neurons of the nucleus raphe obscurus (ROb) (presumably serotonergic) respond to a hypercapnic stimulus in the conscious cat *in vivo* (Veasey et al. 1995). More recently, inhibition (Li et al., 2006) or stimulation (by focal high CO_2/H^+ dialysis) (Dias et al. 2008) of the ROb enhanced the effects of inhibition or stimulation, respectively, of the RTN indicating a functional interaction between the ROb and RTN in central chemoreception.

However, few recent studies have explored the cVLM region, none in the conscious animal. In anesthetized animals, Ribas-Salgueiro et al., (2003) reported the presence of H^+ -sensitive neurons in the cVLM. Microiontophoretic injection of H^+ stimulated neurons in an area including the caudal region underlying Loeschcke's area a region that was subsequently shown to contain projections to respiratory-related regions in the brain stem (Ribas-Salgueiro et al., 2005). Our results support the role of the cVLM in the central chemoreception and add valuable information indicating its functional significance in response to small changes in CO_2/H^+ in the unanesthetized state and the arousal state dependence of its involvement on the process of CO_2 sensing at these stimulus intensities.

4.6. Focal acidification and $\dot{V}_E$ response: grouping animals by response

In the present study, we grouped the animals by the criterion of their response to CO_2 rather than by the anatomical localization of dialysis probes as in previous studies because the anatomical boundaries in this caudal region believed to be involved in central chemoreception are not well established. Loeschcke's surface area is classically described to be around the hypoglossal rootlets. More recently, in the Ribas-Salgueiro's study cited above, the presence of neurons sensitive to H^+ stimulus were in a region beneath Loeschcke's area. Low H^+ -sensitive neurons were loosely distributed in the caudal ventrolateral region studied, while high H^+ -sensitive neurons were located close to the ventral surface of the medulla (see Fig. 1). Despite the lack of clear anatomical boundaries, one could reasonably ask how our data would look if we had grouped the animals according to an anatomical classification rather than a functional one. In order to address to this question, we considered the H^+ -sensitive region described by Ribas-Salgueiro's study (highlighted area in Fig 1B) and placed animals into two

groups according to probe placement: inside or outside this H^+ -sensitive region. In the animals that had the probe tip inside this region, $\dot{V}_E$ increased ~12% on average ($P < 0.01$) during high CO_2 dialysis compared to control dialysis. And in the animals that had the probe outside this region $\dot{V}_E$ increased by ~3%.

4.7. Summary

We show in this study for the first time that focal dialysis of a high CO_2/H^+ aCSF with a presumed moderate stimulus intensity in the caudal region overlapping Loeschcke's area increases ventilation in conscious animals and this response occurs in an arousal state dependent manner (during wakefulness but not sleep). Our results indicate that this region may play a contributory role in the ventilatory response to systemic hypercapnia depending on the animal's arousal state. The identification of neuron(s) phenotype(s) and anatomical connections underlying the responses observed here will provide valuable information on how this region is related to and integrated in central chemoreception.

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Fig 1. Anatomical locations of the tip of the dialysis probes

A - photomicrograph of a stained coronal section of the medulla of one responsive rat shows the location of the tip of the dialysis probe (arrow). B - on the right side of each cross section we show the anatomical location of dialysis probe tips for all animals. The black filled symbols represent the probe placement in the animals that increased ventilation during the increased focal CO₂ dialysis test (responsive group; n=8) and the gray filled symbols represent the location of dialysis probe tips in the non-responsive animals (n=7). In the left side of the cross sections, the highlighted areas show schematic distributions of H⁺-sensitive neurons reported by Ribas-Salgueiro et al. (2003). The areas were drawn only in the closest cross sections reported by the authors. Note that five responsive animals had the dialysis probe in the region

containing H⁺-sensitive neurons. The numbers below the cross sections refer to millimeters caudal to the interaural line. Abbreviations: 4V, fourth ventricle; Sol, nucleus of the solitary tract; Amb, nucleus ambiguous; ROb, raphe obscurus nucleus; 12, hypoglossal nucleus; AP, area postrema; py, pyramidal tract; pyx, pyramidal decussation; IOM, inferior olive (medial nucleus). The drawings are modified from the atlas of Paxinos & Watson (1998).

Fig 2. Illustrative example of a single experiment in a responsive animal

The period of focal acidification is depicted by the gray rectangle with control periods shown before and after. The EEG and EMG recordings shown in panel A indicate the presence of wakefulness and sleep periods before, during and after focal acidosis. The $\dot{V}_E$ data in the bottom of panel A shows that the increase of ventilation during high CO₂ dialysis is present only in wakefulness. Note that at the onset of high CO₂ dialysis when the rat was asleep (open symbols) $\dot{V}_E$ did not increase but quickly did so when the animal woke up (filled symbols). In panel B, we show actual recordings of the plethysmograph pressure signal (upper trace), the EEG (middle trace) and the EMG (lower trace) taken from the averaged data points depicted by the lines and arrows.

Fig 3. Ventilation, f and V_T during CO₂ dialysis tests in the responsive group (N=8)

$\dot{V}_E$ increased significantly during high CO₂/H⁺ dialysis in wakefulness but not in sleep. Mean $\pm$ S.E.M. values are shown. *Values that are significantly different comparing control dialysis to high CO₂ dialysis during wakefulness ($P < 0.01$ repeated measures ANOVA; $P < 0.01$; post hoc comparison, Dunnett's test). There was no difference between control dialysis before and after focal high CO₂/H⁺ dialysis.

Fig 4. Ventilation, f and V_T during CO₂ dialysis tests in the non-responsive group (N=7)

$\dot{V}_E$ did not increase during high CO₂/H⁺ dialysis neither in wakefulness nor in sleep. Mean $\pm$ S.E.M. values are shown.