Activation of Opioid $\mu$-Receptors in Medullary Raphe Depresses Sighs

Zhenxiong Zhang, Ph.D.†, Fadi Xu, M.D. *, Cancan Zhang, B.S.‡, Xiaomin Liang, Ph.D. †

† Postdoctoral Fellow, * Scientist, ‡ Technician, Pathophysiology Program, Lovelace Respiratory
Research Institute, 2425 Ridgecrest Drive, SE, Albuquerque, New Mexico 87108

Running Head: Central chemical control of breathing

*Corresponding Author: F. Xu, Lovelace Respiratory Research Institute, Pathophysiology
Program, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108

Phone: (505) 348-9565
Fax: (505) 348-8567
E-mail: fxu@lrri.org

Funding: This study is supported by the National Heart, Lung, and Blood Institute Grant
HL074183, Bethesda, MD. and by the American Lung Association fellowship RT-83131-N.

The authors thank Jianguo Zhuang, M.D., Ph.D. (Associate research scientist), Pathophysiology
Program, Lovelace Respiratory Research Institute, Albuquerque, New Mexico, for his assistance
of the experiment setup and statistics.

Summary Statement

Hypoxia and hypercapnia elevate the number of sighs in a concentration-dependent
manner and this response is significantly depressed by activation of systemic $\mu$-receptors,
especially those within the caudal medullary raphe region.
Abstract

Sighs, a well known phenomenon in mammals, are substantially augmented by hypoxia and hypercapnia. Because DAMGO, a μ-receptor agonist, injected intravenously and locally in the caudal medullary raphe region (cMRR) decreased the ventilatory response to hypoxia and hypercapnia, we hypothesized that these treatments could inhibit sigh responses to these chemical stimuli. The number and amplitude of sighs were recorded during three levels of isocapnic hypoxia (15%, 10% and 5% O₂ for 1.5 min) or hypercapnia (3%, 7% and 10% CO₂ for 4 min) to test the dependency of sigh responses on the intensity of chemical drive in anesthetized and spontaneously breathing rats. The role of μ-receptors in modulating sigh responses to 10% O₂ or 7% CO₂ was subsequently evaluated by comparing the sighs before and after: (1) intravenous administration of DAMGO (100 ug/kg); (2) microinjection of DAMGO (35 ng/100 nl) into the cMRR; and (3) intravenous administration of DAMGO following CTAP microinjection (100 ng/100 nl), a μ-receptor antagonist, into the cMRR. Hypoxia and hypercapnia increased the number of sighs, but not amplitude, in a concentration-dependent manner, and the responses to hypoxia were significantly greater than those to hypercapnia. Systemic and local injection of DAMGO into the cMRR predominantly decreased the number of sighs, while microinjection into the rostral and middle MRR had no or limited effects. Microinjecting CTAP into the cMRR significantly diminished the systemic DAMGO-induced reduction of the number of sighs in response to hypoxia, but not to hypercapnia. Thus, we conclude that hypoxia and hypercapnia elevate the number of sighs in a concentration-dependent manner in anesthetized rats, and this response is significantly depressed by activating systemic μ-receptors, especially those within the cMRR.
Introduction

Sighs were first noted in man by Haldane in 1919 (22) and have been a subject of interest for nearly a century. Sighs occur in awake, sleeping, and anesthetized states of humans and animals (18, 23, 24, 34, 39, 43, 57) and are characterized by a spontaneously augmented inspiration on the top of the preceding normal tidal volume (7). Several investigators indicated that carotid chemoreceptors and vagal airway mechanoreceptors were critical for the genesis of sighs (3, 4, 20, 46, 55) because bilateral vagotomy or carotid sinus denervation diminished or eliminated sighs in cats (7), rabbits (31), and rats (3, 30). Although the functional significance and possible implications of sighs are not fully understood, they are thought to play a role in maintaining a healthy lung condition by reopening collapsed alveoli (3, 20, 46), causing increased lung compliance and preventing atelectasis (36). Recent studies also suggest that sudden infant death syndrome (SIDS) may be associated with the failure to sigh (25). The number of sighs is greatly augmented by hypoxia and hypercapnia in anesthetized cats (7) and awake dogs (7) and in unanesthetized dogs (3, 20, 55). Interestingly, systemic administration of morphine, a μ-receptor agonist substantially attenuated the ventilatory responses to these chemical challenges (48, 58). It also reduced the number of sighs in patients for postoperative analgesia (14), indicating a modulatory effect of μ-receptors on sighs. However, it has not been explored whether systemic opioids are able to inhibit sigh responses to hypoxia and hypercapnia and, if so, which central sites’ μ-receptors are involved.

There are several lines of evidence implying a possible involvement of MRR μ-receptors in modulating sigh responses to hypercapnia and hypoxia. First, the MRR expresses abundant μ-receptors (12), and our recent studies show that MRR μ-receptors are capable of profoundly
depressing ventilation and the ventilatory responses to hypoxia (60) and hypercapnia (61). Second, neurons within the MRR project extensively to the respiratory-related nuclei, including the pre-Bötzinger Complex (preBötC) (15, 16) and nucleus tractus solitarius (6, 54, 56) that have substantial synaptic inputs from vagal airway mechanoreceptors (32, 33) and carotid chemoreceptors (8, 13, 17). Third, sighs are significantly reduced in SIDS victims (25), in which µ-receptors seem to play an important role (10, 50, 51) and an abnormality in the MRR has been observed (26, 41). To date, direct evidence of MRR µ-receptors’ participation in opioid-induced depression of sighs is lacking.

To address these issues, we tested in anesthetized and spontaneously breathing rats whether: (1) hypercapnia and hypoxia augmented the number and amplitude of sighs in a concentration-dependent manner; (2) systemic or local injection of DAMGO [(d-Ala2, N-Me-Phe4, Gly-ol)-Enkephalin], a selective µ-receptors agonist, into the MRR altered sighs during hypoxia and hypercapnia; and (3) systemic DAMGO-induced attenuation of sigh responses to hypoxia and hypercapnia was diminished by a pre-microinjection of CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2), a µ-receptor antagonist, into the MRR.
Materials and Methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Lovelace Respiratory Research Institute, Albuquerque, NM, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, USA. The experiments were performed in tracheotomized and spontaneously breathing Sprague-Dawley adult male rats (350 – 450 g).

General Animal Preparation

Rats (n = 46) were initially anesthetized with urethane (1200 mg/kg, intraperitoneally), and supplemental urethane was administered, if needed, to reach an adequate level of anesthesia at which rats exhibited neither an eye-blink nor limb-withdrawal reflex throughout the experiment. The animal preparation was similar to that we previously reported (61). Briefly, the right femoral vein and artery were cannulated, the former for drug administration and the latter for monitoring mean arterial blood pressure (BP) and heart rate (HR). The trachea below the larynx was exposed through a midline incision, tracheotomized by blunt dissection, and cannulated. A pneumotachograph was connected to the tracheal cannula to record airflow and a set-up that allowed the rat to be exposed to different chemical challenges. The pneumotachograph had a linear flow-pressure relationship in the range of 2 – 20 ml/s, a flow resistance of 0.046 cm H2O ml/s, and a dead space of 0.2 ml. During isocapnic hypoxia, CO2 was added to maintain the end tidal carbon dioxide pressure (PETCO2) within 2 mmHg deviated from the baseline value (53). PETCO2 was measured via a carbon dioxide analyzer (MicroCapStar end-tidal carbon dioxide analyzer, Model 15-10000; CWE, Inc. Ardmore, PA) connected to a side-port of the tracheal
cannula. Animals were placed into a rigid metal frame with their heads fixed and centered in a stereotaxic apparatus (Model 1404, Kopf, Tujunga, CA). A hole (~10 mm diameter) was drilled at the midline of the skull in some rats for microinjecting DMAGO or CTAP into the MRR. The animals’ core temperature was monitored with a rectal probe and maintained at 36.5 – 37.5°C by a heat pad and radiant heat lamp.

**Hypercapnic and Hypoxic Exposure**

Hyperoxia (30% O₂ balanced with nitrogen) was applied to serve as control at which a sigh was rarely observed. To test the hypoxic concentration-dependency of sighs, the animal was exposed to 15%, 10%, and 5% O₂ (balanced with varied percentages of nitrogen) for 1.5 min as brief hypoxia is believed to act mainly on the carotid body (5). With respect to hypercapnia, 3%, 7%, and 10% CO₂ (balanced with 30% O₂ and varied percentages of nitrogen) for 4 min, respectively, were applied. This exposure was chosen because it would primarily stimulate central chemoreceptors, and less than 4 min of hypercapnic exposure failed to sufficiently evoke sighs, especially under lower CO₂ concentration in our pilot study. A 3 min-interval was allowed for recovery between two chemical challenges. Subsequently, in order to clarify the effect of intravenously or locally injected DAMGO on sighs during hypoxia or hypercapnia, the middle, rather than higher levels of chemical stimulations (10% O₂ followed by 7% CO₂) were chosen to avoid severe impact of these stimulations.

**Systemic Administration or Microinjection of DAMGO**

To evaluate the role of systemic µ-receptors in modulating sigh responses to hypoxia and hypercapnia, rats were exposed to these stimulations before and after intravenous injection of
DAMGO (100 µg/kg). This dose has been demonstrated in our previous studies to substantially depress the ventilatory response to hypoxia (60) and hypercapnia (61). For microinjection, a 0.5µl microneedle with the tip OD 0.25 mm (Hamilton, Reno, NV) prefilled with DAMGO (Sigma-Aldrich, St. Louis, MO) was inserted into the selected MRR region. DAMGO (0.35 µg/µl) was made in a solution of 0.9% saline containing 1% Chicago Sky Blue (Sigma Chemical, St. Louis, MO). According to the rat stereotaxic atlas of Paxinos and Watson (1998) and earlier studies (61), the MRR, extending from 9 – 12 mm caudal to the bregma, was divided into three subregions: rostral, middle, and caudal MRR (rMRR, mMRR, and cMRR), located at 9.0, 10.5, and 12.0 mm caudal to the bregma, respectively. The rMRR contained the magnus nucleus (RMg), the mMRR contained the RMg and its neighboring pallidus nucleus (RPa), and the cMRR contained the obscurus nucleus (ROb) and RPa. The central sites for the mMRR and rMRR were localized 9 mm ventral to the cerebellar surface, and each site received a 100-nl microinjection. Because the two nuclei in the cMRR were located separately and distantly, two injections (100 nl each) were given. The first injection was given when the needle was placed into the site 8.3 mm ventral to the cerebellar surface, corresponding to the center of the ROb. After this injection, the needle was advanced 1 mm deeper, corresponding to the RPa, and was followed by the second injection. This relative large volume of microinjection (100 nl) was chosen for two reasons. First, according to the rat stereotaxic atlas of Paxinos and Watson (1998), each of the three subnuclei (RMg, RPa, ROb) covers a relatively large area. For example, the RMg includes a region of approximate 0.5-1 mm wide, centered at the middle, and extending from the base of the brain to 1 mm dorsal and extending ~2 mm from caudal to rostral. Previous studies have calculated that microinjection of a volume of 100 nL into the brainstem could spread as far as 1 mm (29, 37). Second, microinjection of this volume (100 nl) into the raphe
Microinjection of DAMGO was made purposely outside the cMRR in two rats to test the unique role of the cMRR. The microinjections were located at 1 mm left to midline and 7.5 mm and 9 mm ventral to the cerebellar surface, respectively, in one rat, and at 1 mm right to midline and 8.3 mm and 9.3 mm ventral to the cerebellar surface, respectively, in another one. To block µ-receptors in the cMRR, microinjections of CTAP (100 ng/100 nl containing 1% Chicago Sky Blue), a µ-receptor antagonist, were made in the cMRR (100 nl).

**Experimental Protocol** Our experiments were performed in seven groups of anesthetized and spontaneously breathing rats. Systemic administration of DAMGO alone was conducted in Group I (n = 6), in which animals were initially exposed to three degrees of hypoxia and hypercapnia to test the concentration-dependency of sigh responses on these challenges. Subsequently, the exposures to 10% O₂ and 7% CO₂ were repeated before, 5, and 30 min after intravenous injection of DAMGO. The effects of microinjection of DAMGO/CTAP into the MRR were evaluated in 5 groups of rats (n = 6 each group in Groups II-V, and n = 5 in Group VI). 10% O₂ and 7% CO₂ were performed before, and 5, 30, and 60 or up to 120 min after microinjection of DAMGO into the cMRR (Group II), mMRR (Group III), and rMRR (Group IV), respectively. Because only activation of cMRR µ-receptors inhibited sigh responses in our pilot studies, the role of blocking local µ-receptors in the systemic DAMGO-induced inhibition of sighs was studied in Group V. In these cases, sigh responses to hypoxia and hypercapnia were repeated four times. In other words, the exposures were applied before and after systemic DAMGO, and the same protocols repeated two hours later with exception that CTAP (100 ng/100 nl) was microinjected into the cMRR prior to systemic DAMGO. The two h interval
was chosen because systemic DAMGO has been reported to have an approximately 15-min half-life in mammals (52, 61). To verify the effect of local CTAP alone on sigh responses to hypoxia and hypercapnia, the same hypoxic and hypercapnic exposures were performed before and after microinjection of CTAP (100 ng/100 nl) into the cMRR in Group VI. Animals in Group VII (n = 11) served as sham-operation control, in which intravenous and local injection of vehicle instead of agents was conducted in nine animals, and microinjection of DAMGO was made outside of the cMRR in two rats.

Identification of Microinjection Sites

After completing the experiments, all animals were euthanized by an overdose of anesthetic. The brainstem was removed and fixed by soaking in 4% paraformaldehyde (pH 7.4) for at least 36 h at 4°C, and subsequently sectioned at a 40-µm thickness by a slicing machine (Leica, CM 1850, Microsystems GMbH, Nussioch, Germany). The area marked by the Chicago Sky Blue was identified under a microscope and the center of the stained area utilized as the microinjection location.

Data Acquisition and Statistical Analysis

Raw data of the airflow signal, BP, HR, P_{ET}CO_{2}, and rectal temperature were digitized, monitored, and recorded using a PowerLab/8sp (model ML 785; ADInstruments Inc., Colorado Springs, CO) connected to a computer employing the PowerLab Chart 5 software. The airflow signals were integrated to generate tidal volume (V_T), respiratory frequency (f), and minute ventilatory volume (V_E). Sighs were defined as an augmented spontaneous inspiration with V_T being at least two-fold greater than that of the preceding normal breath (21). Sigh amplitudes
were calculated by the augmented inspiration (phase II) above the preceding eupneic Vₜ (phase I), as phase II, unlike phase I, was relatively constant and largely independent of chemical stimuli (7). All data are presented as means ± standard error (SE). Student’s t-test was used to compare the difference between the hypoxia-induced and hypercapnia-induced respiratory variables and sighs’ responses, and the sigh responses before and after microinjection of CTAP alone into the cMRR. One-way analysis of variance (ANOVA) for repeated measures was employed to compare: 1) the number and amplitude of sighs during different degrees of hypoxic or hypercapnic exposures; 2) the number and amplitude of sighs during 10% O₂ and 7% CO₂ before and several time-points after administration of DAMGO; and 3) the hypoxia- or hypercapnia-evoked sigh responses (Δ% change from control, baseline value) before and after DAMGO alone and coupled with CTAP. The Fisher’s LSD post test was used if the overall ANOVA (an omnibus test) had a P value less than 0.05. STATISTICA 6.0 software (StatSoft, Inc., Tulsa, OK.) was employed for statistical analysis. Difference was considered significant at a P value < 0.05.
Results

*Hypoxia and Hypercapnia Increased the Number of Sighs in a Concentration-Dependent Manner*

Sighs were rarely observed during eupneic breathing, while various concentrations of hypoxia and hypercapnia increased the number, but not the amplitude, of sighs in a concentration-dependent manner (Fig. 1). Previous studies have indicated a stronger sigh response to hypoxia than hypercapnia in cats by comparing the number of sighs at similar VT levels (7). To estimate the differences in sigh responses to hypoxia and hypercapnia in rats, we compared the responses of sighs and respiratory variables between the two chemical challenges. Table 1 shows that the averaged VE and VT responses to the 1.5-min hypoxia were strikingly lower than those of the 4-min hypercapnia (P < 0.01); but the number of sighs induced by hypoxia was significantly greater than that of the hypercapnia. Additionally, the amplitude of sighs in response to hypoxia and hypercapnia was not markedly different.

*Systemic DAMGO Decreased the Sigh Responses to Hypoxia and Hypercapnia*

Intravenous administration of DAMGO decreased both responses of the number and amplitude of sighs to hypoxia and hypercapnia, but the effects were much greater on the number than on the amplitude. The typical recordings and the corresponding group data were exhibited in Figs. 2 and 3, respectively. As shown, systemic DAMGO significantly depressed the number and amplitude of sighs in response to hypoxia by 65% and 14%, respectively, and the responses to hypercapnia by 55% and 17%, respectively. These DAMGO-induced depressions lasted no
longer than 30 min. In sharp contrast, intravenous injection of vehicle did not change the responses of the number and amplitude of sighs to hypoxia and hypercapnia.

Microinjection of DAMGO into the cMRR Attenuated Sighs during Hypoxia and Hypercapnia

Similar to systemic DAMGO, microinjection of DAMGO into the cMRR attenuated both responses of sigh number and amplitude to hypoxia and hypercapnia with a greater effect on number (Fig. 4). Five minutes after microinjection, the number and amplitude of sighs in response to hypoxia were strikingly attenuated by 59% and 40%, respectively, and to hypercapnia by 68% and 30%, respectively. Microinjection of the same volume of vehicle into the cMRR had no effect on these responses (Table 2). Different from systemic DAMGO, microinjection of DAMGO-induced depression of sigh responses usually lasted longer (> 60 min). Particularly, hypercapnic-induced the responses of sigh number were abolished 30 min after microinjecting DAMGO, and the inhibitory effect lasted for 90 min. To test the unique role of the cMRR, DAMGO was administered into the regions outside the cMRR in two rats (Fig. 5). These microinjections did not make a remarkable difference in the number of sighs (4.02 ± 0.98 vs. 3.89 ± 0.92 during hypoxia; 4.16 ± 0.82 vs. 4.08 ± 0.94 during hypercapnia) or the amplitude of sighs (9.04 ± 0.79 vs. 8.83 ± 0.86 during hypoxia; 8.88 ± 0.82 vs. 8.62 ± 0.76 during hypercapnia).

Changes in Sigh Responses after Microinjecting DAMGO into the mMRR or rMRR Were Limited
The data presented in Fig. 6 indicated that microinjecting DAMGO into the mMRR only significantly attenuated the responses of sigh number to hypercapnia by 38% and 67% 5 and 30 min after microinjection, respectively. This microinjection failed to alter the responses of sigh number and amplitude to hypoxia and the amplitude responses to hypercapnia. Again, microinjecting the same volume of vehicle into this region did not lead to remarkable alterations in sigh responses (Table 2). When the microinjection was made in the rMRR (Fig. 7), no significant changes in the responses of sigh number or amplitude to hypoxia or hypercapnia were observed. The locations of the microinjections made in the mMRR and rMRR are also illustrated in Fig. 5, respectively.

Blocking cMRR µ-Receptors Diminished the Systemic DAMGO-induced Attenuation of Sigh Responses

This study was performed in two groups of rats. In Group V, we first tested the effects of systemic DAMGO on hypoxia- and hypercapnia-induced sighs and found that DAMGO significantly inhibited the responses of sigh number and amplitude to hypoxia and hypercapnia, similar to those mentioned above. Secondly, we examined whether locally blocking cMRR µ-receptors by microinjecting CTAP into the cMRR, using the same protocol described above, would affect this systemic DAMGO-induced change. As illustrated in Fig. 8A, the systemic DAMGO-induced attenuation of sigh number rather than amplitude in response to hypoxia was significantly diminished from 65% to 31% after CTAP microinjection. In other words, the systemic DAMGO-induced depression of the sigh number during hypoxia was diminished by 52% after blocking cMRR µ-receptors. Although local blockade of cMRR µ-receptors tended to decrease the systemic DAMGO-induced depression of the sigh number during hypercapnia, this
change did not reach significance (Fig. 8B). The effects of microinjection of CTAP alone into the cMRR on sigh responses were evaluated in Group VI. We found that the microinjection did not significantly affect the responses of sighs to hypoxia (3.93 ± 0.46 vs. 4.13 ± 0.64 for number; 8.85 ± 0.91 vs. 9.16 ± 0.89 for amplitude) and hypercapnia (4.01 ± 0.31 vs. 3.82 ± 0.53 for number; 8.79 ± 0.81 vs. 9.13 ± 0.86 for amplitude).
Discussion

Similar to previous studies reported in anesthetized cats (7) and awake dogs (45), we found that hypoxia and hypercapnia increased the number, but not amplitude of sighs, in a concentration-dependent manner in anesthetized rats. Though hypoxia induces sighs associated with hypotension, this transient hypotension is unlikely a major trigger of sighs. First, it was reported that hypotension remained while sighs disappeared in bilateral vagotomized rats exposed to hypoxia (3, 30). Second, hypercapnia is also able to increase sighs without decreasing the blood pressure (61), which is the same in the present study (see Fig. 2B). Third, systemic injection of DAMGO did not markedly alter hypoxia-induced hypotension, but dramatically reduced hypoxia-induced sighs (see Fig. 2A). To estimate whether sigh responses to hypoxia and hypercapnia differed, we compared sighs and respiratory variables responses to hypoxia and hypercapnia owing to the dependency of the number of sighs on VT (7). We found that compared with hypercapnia, hypoxia with a shorter exposure period (1.5 min vs. 4 min) produced lower VE and VT responses, but it generated a greater number of sighs, suggesting that hypoxia is much more powerful than hypercapnia in producing sighs, which is in agreement with previous studies in cats (7, 20). A stronger hypoxic stimulating effect than hypercapnia on sigh genesis may be due to that carotid chemoreceptors’ inputs play an important role in facilitating sighs (3, 20, 55).

Our major finding is that intravenous and local injection of DAMGO into the cMRR decreased the number and amplitude of sighs induced by hypoxia or hypercapnia. Systemic DAMGO significantly depressed the responses of sigh number and amplitude to hypoxia by 65% and 14%, respectively, and the responses to hypercapnia by 55% and 17%, respectively. These
results agree with an early observation (14) in which morphine used systemically in patients for postoperative analgesia reduced the number of sighs. Because hypoxia and hypercapnia stimulate sighs primarily via elevating their number, it is understandable that DAMGO mainly affects the number of sighs. Given that our study was performed in anesthetized rats, we cannot rule out the possible interaction between the anesthetic and DAMGO on sighs. However, it was reported that the number of sighs was not strikingly influenced by anesthesia in rats (3). Similar to systemic DAMGO, microinjecting DAMGO into the cMRR attenuated both responses of the sigh number and amplitude to hypoxia and hypercapnia with a greater effect on the number. In sharp contrast, the same microinjections made into the mMRR or rMRR had no effect on sighs with exception that the former attenuated the response of sigh number to hypercapnia. Clearly, our results demonstrate that activating MRR µ-receptors, especially those within the cMRR, depresses chemical stimulation-induced sighs.

An interesting finding is that microinjecting CTAP, a µ-receptor antagonist, into the cMRR diminishes the systemic DAMGO-depressed responses of sigh number to hypoxia with no significant effect on the amplitude responses. Moreover, unexpectedly, the CTAP microinjection failed to significantly change the systemic DAMGO-depressed hypercapnia-induced sighs. The absence of a CTAP effect may be due to that at the same dose of the systemic DAMGO, the role of cMRR µ-receptors in inhibiting the hypercapnia-induced sighs is less important than in depressing the hypoxia-induced sighs although local high dose of DAMGO could diminished both chemical stimuli-induced sighs.

In the present study, systemic DAMGO-induced attenuation of sighs is not fully eliminated by blocking cMRR µ-receptors, which leads to a postulation that µ-receptors in other regions, in addition to the cMRR, also participate in the controlling sighs. Previous studies have pointed out
that both vagal mechanoreceptors’ and carotid chemoreceptors’ inputs are critical for evoking sighs (3, 7, 30, 31). These, along with the presence of heavy expression of µ-receptors on vagal afferents (nodose ganglion) (1, 27, 40) and the carotid body (35), raise a possible peripheral µ-receptors’ contribution to systemic DAMGO-induced depression of sighs. Another possibility is that other central µ-receptors may be involved owing to a wide distribution of µ-receptors in the brainstem (12, 44), including the preBötC that is thought to be critical for the genesis of sighs (28, 49). Because of the presence of the mutual projections between the MRR and the preBötC (15, 16), further studies are needed to define whether the MRR functions as a sigh generator and/or a relay station on the ascending or descending pathway of the sigh-generator. Hypoxia (42) and hypercapnia (19) could promote central release of opioids; however, microinjection of CATP alone into the cMRR did not significantly alter the sighs during hypoxia and hypercapnia in this study. These results allow us to believe that the endogenously-released opioids in cMRR by hypoxia and hypercapnia contribute little to modulate the sigh responses. Nevertheless, our results suggest that cMRR µ-receptors play a crucial role in modulating systemic DAMGO-induced attenuation of the response of sigh number to hypoxia.

Sighs are thought to maintain a healthy lung condition by reopening collapsed alveoli (3, 9, 20, 46), increasing lung compliance and preventing atelectasis (36). Opioids used in postoperative analgesia increased pulmonary morbidity, including atelectasis, pulmonary infection, and overall pulmonary complications (2, 47). More importantly, the majority of sighs during sleep are associated with electroencephalographic signs of arousal (43, 59) that is critical for breaking an apnea in obstructive sleep apnea syndrome or SIDS (25). Our finding that cMRR µ-receptors play an important role in depressing sighs during hypoxia and hypercapnia is consistent with the abnormality of the MRR and elevated endogenous cerebral opioid in SIDS.
Determining the role of cMRR u-receptors in hypoxic and hypercapnic modulation of sighs helps us understand participation of these receptors in atelectasis and respiratory disorders, including sleep apnea and breathing mechanisms in SIDS.

In conclusion, we found that hypoxia and hypercapnia selectively increased the number of sighs in a concentration-dependent manner. Systemic or local administration of DAMGO into the cMRR predominantly depressed the responses of sigh number to hypoxia and hypercapnia. These results suggest that cMRR µ-receptors play an important role in depressing sighs elicited by hypoxia and hypercapnia in anesthetized rats.
References


Fig. 1. Effects of different hypoxic and hypercapnic degrees on sighs. Panel A presents representative recordings of sighs induced by three different hypoxic (1.5 min) and hypercapnic exposures (4 min), respectively. To emphasize the sigh responses, the moving average of the VT is presented and the spikes reflect sighs. Panels B and C show the grouped responses of sigh number (left) and amplitude (right) to hypoxia (B) or hypercapnia (C), respectively. N= 6; data are presented as mean ± SE.

Fig. 2. Representative recordings exhibiting the impacts of systemic DAMGO (100 µg/kg) on the hypoxia- (A) or hypercapnia-induced (B) changes in sighs’ number and amplitude. In each panel, the chemical challenges (10% O2 for 1.5 min and 7% CO2 for 4 min) were applied before (left), 5 min (middle) and 30 min (right) after systemic DAMGO. Traces from the top to bottom are arterial blood pressure (BP), tidal volume (VT), end-tidal pressure of carbon dioxide (PETCO2), and the marks of stimulating durations of chemical challenges.

Fig. 3. The effect of systemic DAMGO (100 µg/kg) on the sighs’ number (left) and amplitude (right) induced by 10% O2 (A) or 7% CO2 (B). N= 6; data are presented as mean ± SE. * P < 0.05, ** P < 0.01 compared with before DAMGO. “0” on the X-axis indicates the onset of intravenous administration of DAMGO.

Fig. 4. The effect of microinjection of DAMGO into the cMRR on the number (left) and amplitude (right) of sighs induced by 10% O2 (A) or 7% CO2 (B). N= 6; data are presented as mean ± SE. * P < 0.05, ** P < 0.01 compared with before DAMGO. “0” on
the X-axis indicates the onset of intravenous DAMGO administration. Note: there was no amplitude response to 7% CO₂ at the 30-min point in B owing to the absence of a sigh at that time point.

Fig. 5. Diagram showing the sites where the microinjections occurred. The left of panel A shows a cartoon coronal slice, in which the symbols of “■” and “▲” represent the locations of the microinjections in the cMRR of 6 rats, while the symbols of “X” indicate four microinjections outside the cMRR in 2 rats. The right of panel A displays a representative slice containing the cMRR, in which the areas stained by Chicago Sky Blue are circled. The symbols of “■” in B and C show the locations of the microinjections in the mMRR and rMRR, respectively. Amb, ambiguus nucleus; IRt, intermediate reticular nucleus; LPB, lateral parabrachial nucleus; ml, medial lemniscus; Mo5, motor 5 nucleus; NTS, nucleus of solitary tract; PCrτA, parvicellular reticular nucleus alpha; Pr5VL, ventrolateral part of principal sensory 5 nucleus; ROb, raphe obscurus nucleus; RMg, raphe magnus nucleus; RPa, raphe pallidus nucleus; RVL, rostroventrolateral reticular nucleus; 7, facial nucleus; Sp5I, interpolar part of spinal 5 nucleus; Sp5O, oral part of spinal 5 nucleus.

Fig. 6. The influence of DAMGO microinjection into the mMRR on the responses of sigh number (left) and amplitude (right) to 10% O₂ (A) and 7% CO₂ (B). N = 6; data are presented as mean ± SE. * P < 0.05, ** P < 0.01 compared with before DAMGO. “0” on the X-axis indicates the onset of intravenous DAMGO administration.
Fig. 7. The effect of DAMGO microinjection into the rMRR on the responses of sigh number (left) and amplitude (right) to 10% O₂ (A) and 7% CO₂ (B). N= 6; data are presented as mean ± SE. * P < 0.05, ** P < 0.01 compared with before DAMGO. “0” on the X-axis indicates the onset of intravenous DAMGO administration.

Fig. 8. Comparison of the systemic DAMGO-induced attenuation of the responses of sigh number (left) and amplitude (right) to 10% O₂ (A) or 7% CO₂ (B) before and after microinjection of CTAP into the cMRR. All variables are presented as ∆% change from control indicated as “0” on Y axis. N= 6; data are mean ± SE; * P < 0.05 and ** P < 0.01 compared with the control, while † P < 0.05 between DAMGO-induced changes before and after CTAP microinjection.
Fig. 1

A

Hypoxia

\[ V_T (\text{ml}) \]

\begin{tabular}{ccc}
15% & 10% & 5% \\
\end{tabular}

Hypercapnia

\[ V_T (\text{ml}) \]

\begin{tabular}{ccc}
25% O_2 & 10% O_2 & 15% O_2 \\
\end{tabular}

B

Number of sighs

\begin{tabular}{ccc}
15% O_2 & 10% O_2 & 5% O_2 \\
\end{tabular}

Amplitude of sighs (ml)

\begin{tabular}{ccc}
15% O_2 & 10% O_2 & 5% O_2 \\
\end{tabular}

C

Number of sighs

\begin{tabular}{ccc}
3% CO_2 & 7% CO_2 & 10% CO_2 \\
\end{tabular}

Amplitude of sighs (ml)

\begin{tabular}{ccc}
3% CO_2 & 7% CO_2 & 10% CO_2 \\
\end{tabular}
Fig. 2

A

<table>
<thead>
<tr>
<th>BP (mmHg)</th>
<th>V̅ (ml)</th>
<th>P_{ETCO}_2 (mmHg)</th>
<th>10% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>BP (mmHg)</th>
<th>V̅ (ml)</th>
<th>P_{ETCO}_2 (mmHg)</th>
<th>7% CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>10</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4

A

Number of sighs vs. Time (min)

B

Number of sighs vs. Time (min)

Amplitude of sighs vs. Time (min)
Fig. 5

A

cMRR (Bregma from -11.6 to -12.3 mm)

B

mMRR (from -10.04 to -10.52 mm)

C

rMRR (from -9.16 to -9.30 mm)
<table>
<thead>
<tr>
<th></th>
<th>Mean ± SE</th>
<th>$V_E$</th>
<th>$f$</th>
<th>$V_T$</th>
<th>Number of sighs</th>
<th>Amplitude of sighs (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ml/min)</td>
<td>(breaths/min)</td>
<td>(ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td>$485 \pm 24$</td>
<td>$127 \pm 3$</td>
<td>$3.84 \pm 0.45$</td>
<td>$4.75 \pm 0.57$</td>
<td>$9.45 \pm 0.48$</td>
<td></td>
</tr>
<tr>
<td>Hypercapnia</td>
<td>$674 \pm 47^{**}$</td>
<td>$130 \pm 6$</td>
<td>$5.16 \pm 0.38^{**}$</td>
<td>$3.43 \pm 0.32^{*}$</td>
<td>$8.66 \pm 0.85$</td>
<td></td>
</tr>
</tbody>
</table>

All data were averaged from three degrees of 1.5-min hypoxia (15%, 10% and 5% O$_2$) and 4-min hypercapnia (3%, 7%, and 10% CO$_2$). N = 6; * P < 0.05, ** P < 0.01 compared between hypoxia and hypercapnia; $V_E$ = minute ventilation; $f$ = respiratory frequency; $V_T$ = tidal volume.
TABLE 2. Sigh responses to hypoxia and hypercapnia before and after microinjection of vehicle into the caudal or middle medullary raphe region (cMRR or mMRR)

<table>
<thead>
<tr>
<th></th>
<th>10% O₂</th>
<th>7% CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of sighs</td>
<td>Amplitude of sighs</td>
</tr>
<tr>
<td></td>
<td>( n /1.5min)</td>
<td>(ml)</td>
</tr>
<tr>
<td>cMRR (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>3.83 ± 0.53</td>
<td>9.15 ± 0.52</td>
</tr>
<tr>
<td>After</td>
<td>4.08 ± 0.91</td>
<td>8.86 ± 0.85</td>
</tr>
<tr>
<td>mMRR (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>4.01 ± 0.58</td>
<td>9.01 ± 0.82</td>
</tr>
<tr>
<td>After</td>
<td>3.78 ± 0.73</td>
<td>9.26 ± 0.94</td>
</tr>
</tbody>
</table>