Pflugers Arch - Eur J Physiol (2016) 468:763–774
DOI 10.1007/s00424-016-1793-x

INTEGRATIVE PHYSIOLOGY

# Orexinergic system in the locus coeruleus modulates the $CO_2$ ventilatory response

**Mariane C. Vicente**[1] · **Mirela B. Dias**[2] · **Elisa M. Fonseca**[1] · **Kênia C. Bícego**[1] · **Luciane H. Gargaglioni**[1]

Received: 22 October 2015 / Revised: 8 January 2016 / Accepted: 12 January 2016 / Published online: 1 February 2016
© Springer-Verlag Berlin Heidelberg 2016

**Abstract** The orexins are hypothalamic neuropeptides involved in an array of functions such as regulation of sleep/wake states and chemoreception to $CO_2$/pH. The locus coeruleus (LC) is a chemosensitive site and expresses an extensive population of orexin receptor 1 (OX1R). We tested the hypothesis that OX1Rs located in the LC participate in the ventilatory response to hypercapnia in a vigilance state and diurnal cycle-dependent manner. For this, we performed unilateral injections of SB-334867 (OX1R antagonist, 5 mM) into the LC of male Wistar rats and evaluated the ventilatory response to 7 % $CO_2$ during wakefulness and sleep in the dark and light phases of the diurnal cycle. Hypercapnia induced an increase in ventilation ($V_E$) in all groups compared to normocapnic values. However, during the dark phase, but not in the light phase, SB-334867 injection promoted an attenuation of the hypercapnic chemoreflex during wakefulness ($V_E$: vehicle, 1502.6 ± 100 mL kg$^{-1}$ min$^{-1}$ vs SB-334867, 1200.3 ± 70.0 mL kg$^{-1}$ min$^{-1}$) but not during sleep ($V_E$: vehicle, 1383.0 ± 113.9 vs SB-334687, 1287.6 ± 92.1 mL kg$^{-1}$ min$^{-1}$), due to changes in tidal volume ($V_T$). We suggest that projections of orexin-containing neurons to the LC contribute, via OX1Rs, to the hypercapnic chemoreflex during wakefulness in the dark phase.

✉ Luciane H. Gargaglioni
lucihel@fcav.unesp.br

[1] Department of Animal Morphology and Physiology, Sao Paulo State University—UNESP/FCAV at Jaboticabal, Jaboticabal, SP, Brazil

[2] Department of Physiology, Institute of Bioscience, São Paulo State University—UNESP, Botucatu, SP, Brazil

**Keywords** Awake · Chemoreception · Orexin · Hypercapnia · Sleep · Ventilation

## Introduction

Central chemoreception refers to the detection of $CO_2$/pH at multiple brain loci. The resultant effects on ventilation [37] and the contribution of each site varies in a state-dependent manner, so that vigilance and circadian period differentially modify the responses of distinct chemosensory loci [45]. One of the brain regions that is considered an important chemoreception site is the lateral hypothalamus/perifornical area [38], where the orexinergic neurons are located. These neurons produce a single polypeptide that is processed into two peptides orexin-A (Hcrt-1) and orexin-B (Hcrt-2) [12, 26]. Orexinergic neurons project throughout the whole central nervous system [43, 48, 53, 68] and bind to two distinct G protein-coupled receptors, orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). These widely distributed projections explain the various functions that are modulated by orexin, such as nociception, feeding behavior, control of energy homeostasis, sleep/wake states, stress response, and cardiovascular and respiratory control [9, 29, 34, 63, 69].

It has been suggested that orexinergic neurons play a key role in the vigilance state-dependent control of hypercapnic chemoreflex [17, 18, 27, 66]. One of the mechanisms by which orexin modulates the hypercapnic chemoreflex is related to its projections to other chemosensitive sites such as the retrotrapezoid nucleus (RTN) and medullary raphe. For instance, in the RTN, unilateral microdialysis of OX1R antagonist in rats during the light/inactive phase of the circadian

cycle resulted in a 30 % reduction of the ventilatory response to breathing, 7 % $CO_2$ during wakefulness, and 9 % during non-rapid eye movement (NREM) sleep [17]. In the medullary raphe, during wakefulness in the dark period, but not in the light period, OX1R antagonism caused a 16 % reduction of the ventilatory response to hypercapnia compared with the vehicle [18]. These results indicate that orexin neurons interact with chemosensitive neurons of other regions, perhaps augmenting their responses during wakefulness. Orexin levels in the brain vary according to the sleep–awake cycle; the highest levels in rodents occur during the dark-active phase and the lowest levels during the light-inactive phase [16]. Orexin levels increase just before awakening, remain high during wakefulness, and decrease during NREM sleep [58]. These arousal-promoting effects of orexin depend on its dense projections to the locus coeruleus (LC) [10, 14, 20, 22, 33, 40, 67], which is an important $CO_2$/pH chemosensitive site [6, 11, 25]. Orexin promotes depolarization in LC noradrenergic neurons in vitro and in vivo [33, 58, 65]. In addition, in situ hybridization and immunohistochemical studies demonstrate a high index of the mRNA of the orexin receptor 1 in the LC [28, 31, 64] with evidence of limited orexin receptor 2 expression [40].

Nevertheless, despite the anatomical and functional relationship between the orexinergic neurons and LC, the role of orexinergic inputs to LC in the modulation of hypercapnic chemoreflex remains to be elucidated. Based on these facts, we hypothesized that OX1R in the LC contribute to $CO_2$/pH chemoreception in a vigilance state and diurnal cycle-dependent manner.

## Material and methods

### Animals

The experiments were performed on unanesthetized adult male Wistar rats weighing 300–350 g. The animals had free access to water and food and were housed in a temperature-controlled chamber maintained at 24–26 °C (ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil) with a 12:12-h light/dark cycle, lights on at noon for the dark period group and at 07.00 a.m. for the light period group. The experiments were performed between 09.00 a.m. and 12.00 p.m. in the animals of the dark period group and between 01.00 p.m. and 04.00 p.m. in the animals of the light period group. The study was conducted in compliance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, MCT, Brazil) and with the approval of the Faculty of Agricultural and Veterinary Sciences and Animal Care and Use Committee (CEUA, FACV-UNESP, Jaboticabal campus; Protocol no. 010377/14).

### Drug and gas mixture

The OX1R antagonist SB-334867 (Sigma, St. Louis, MO, USA) was first dissolved in 4 % DMSO and then the solution was diluted using 35 % (2-hydroxypropyl)-β-cyclodextrin in artificial cerebrospinal fluid (aCSF) to prepare 5 mM SB-334867. For the vehicle, a solution of 4 % DMSO and 35 % (2-hydroxypropyl)-β-cyclodextrin in aCSF was used. The dose (5 mM) and method to dissolve the drug were selected as per pilot experiments and previous studies [15, 17, 18]. The gas mixtures used in this study were room air (normocapnia) and a hypercapnic gas mixture (7 % $CO_2$, 21 % $O_2$, balance $N_2$) purchased from White Martins Gases Industriais Ltda (Sertãozinho, SP, Brazil).

### Surgical procedure and microinjection

All surgical procedures were performed under anesthesia with an intraperitoneal injection of 100 mg/kg of ketamine (Union National Pharmaceutical Chemistry S/A, Embu-Guaçu, SP, Brazil) and 10 mg/kg of xylazine (Laboratories Calier S/A Barcelona, Spain).

The head was shaved and the skin was sterilized with betadine solution and alcohol. The rats were fixed to a Kopf stereotaxic frame and implanted with a stainless steel guide cannula as previously reported [7, 23, 59, 62]. The guide cannula (0.7 mm outer diameter (o.d.) and 15-mm long) was implanted 1 mm above the LC region (distance from lambda: anterior −3.4 mm; lateral −1.2 and 1.2 mm; and dorsal −5.8 mm deep from the skull and inclination of vertical stereotaxic bar at 15°), according to the Paxinos and Watson atlas [51]. The cannula was attached to the bone using stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion.

Three electroencephalogram (EEG) electrodes were screwed into the skull, and a pair of electromyogram (EMG) electrodes was inserted deep into the neck muscle. The skull wound was sutured. Postoperatively, the animals were treated with antibiotic (enrofloxacin, 10 mg/kg, intramuscular) and analgesic (flunixin meglumine, 2.5 mg/kg, subcutaneous) agents. The experiments were performed 7 days postoperatively.

A day before the experiments, the rats underwent a second surgery under ketamine/xylazine anesthesia for the implantation of a temperature datalogger (SubCue Dataloggers, Calgary, Canada) into the abdominal cavity through a midline laparotomy. The datalogger was programmed to acquire data every 5 min.

A 5-μL Hamilton syringe and a dental injection needle (Mizzy, 200 μm o.d.) connected to a PE-10 tube were used to perform the microinjections into the LC of unanesthetized rats. The injection needle was 1 mm longer than the guide cannula so that the LC was reached by the needle only at the

time of injection. A volume of 0.1 μL of the vehicle or drug solution was injected over a period of 30 s. Following the injection, the needle was removed from the guide cannula after an additional 30 s to avoid reflux. All injections were performed using a microinjector machine (model 310, Stoelting Co., IL, USA).

### Determination of pulmonary ventilation

Measurements of pulmonary ventilation ($V_E$) were performed using the whole body plethysmography method [4] as commonly used in our laboratory [6, 11, 50].

Freely moving rats were kept in a 5-L chamber ventilated with room air or a hypercapnic gas mixture containing 7 % $CO_2$ (White Martins, Sertãozinho, Brazil) in low ambient noise conditions. The flow rate of the inflow gas into the animal chamber was monitored by a flowmeter (model 822-13-OV1-PV2-V4, Sierra Instruments, Monterey, CA). During measurements, the flow was interrupted, and the chamber was sealed for short periods of time (approximately 2 min); the pressure oscillations due to respiration were monitored by a differential pressure transducer (TSD 160A, Biopac Systems, Santa Barbara, CA). The signals were fed into a differential pressure transducer (DA 100C, Biopac Systems), passed through an analog-to-digital converter, and digitized on a microcomputer equipped with data acquisition software (MP100A-CE, Biopac Systems). The sampling frequency was 1-kHz samples per second. The results were analyzed using the data analysis software Acqknowledge (v. 4.2.3 data acquisition system, Biopac Systems). Tidal volume ($V_T$) and respiratory frequency (fR) were calculated per breath to estimate ventilation per breath. $V_T$ was calculated by using an appropriate formula [4]. The vapour pressure of water vapour in the animal chamber was calculated indirectly using an appropriate table [13]. The calibration for volume was obtained during each experiment by injecting the animal chamber with 1 mL of air.

### EEG and EMG signals

The arousal state was determined by analyzing the EEG and EMG records as previously described [44]. The signals from the EEG and EMG electrodes were sampled at 1 kHz, filtered at 0.3–50 and 0.1–100 Hz, respectively, and recorded on the computer. Both wakefulness and NREM sleep states were observed consistently through the experiments, but periods of rapid eye movement (REM) sleep were short and were not present in every experiment; thus, REM sleep periods were excluded from the analysis.

### Experimental protocol

At 7 days after the unilateral implantation of guide cannulae, the animals were individually placed in a Plexiglass chamber (5 L) and allowed to move freely while the chamber was flushed with atmospheric air. After approximately 40 min of acclimatization period, the control $V_E$ was measured before the microinjection. Subsequently, 0.1 μL of vehicle or SB 334867 (5 mM) was microinjected into the rat LC. Respiratory measurements were performed 7, 14, 21, 28, 35, and 42 min after the microinjection under normocapnic conditions. Then, based on the sleep/wake cycle, for the $V_E$ measurements, we selected the period when the animals were in wakefulness or in sleep. The core body temperature was measured at 5 min intervals throughout the experiment. For the hypercapnia groups, after the control $V_E$ measurement, a hypercapnic gas mixture (7 % inspired $CO_2$ in air) was flushed through the chamber for 10 min, and then the rats received 0.1 μL of vehicle or SB 334867 into the LC, followed by subsequent measurements during the addition of 7 % $CO_2$. The experiments were performed in different groups of animals during the dark and light phases of the diurnal cycle.

### Histology

Upon completion of the experiments, the animals were deeply anesthetized with ketamine and xylazine and perfused intracardially with saline followed by 10 % formalin solution for 10 min (5 min for each solution). A needle injector (19.6-mm long) was inserted through the guide cannula and a 0.1-μL microinjection of Evan's blue was performed. The brain was removed and stored in 10 % formalin for at least 2 days. After fixation, the brainstem was embedded in paraffin, sectioned on a microtome (15-μm-thick coronal sections), and stained by the Nissl method for light microscopy determination of the region reached by the microinjection, according to the Paxinos and Watson atlas [51]. Only the rats with a positive site of microinjection into the LC were considered.

### Data processing and analysis

The results are expressed as mean ± SEM. The normocapnic and hypercapnic conditions were analyzed separately to determine the effect of drug microinjection. The effects of SB-334867 microinjection on the ventilatory variables, body temperature, and the percentage time spent in each arousal state were evaluated by two-way analysis of variance with phase of the day and drug as factors and a Bonferroni post hoc test to assess the differences between the groups. A $P$ value of $<0.05$ was considered to be statistically significant. All statistical analyses were performed using SigmaStat 274 (v3.5, Systat Software Inc., San Jose, CA, USA).

## Results

### Histology

Figure 1 is a representative photomicrograph of the transverse section of the rat brainstem through the region of LC injection.

### Effect of SB-334867 microinjection intra-LC on ventilation during room air and 7 % $CO_2$ during the dark period

During normocapnia, the microinjection of SB-334867 intra-LC had no effect on $V_E$, in wakefulness, or in sleep during the dark period (Fig. 2). Hypercapnia elicited an increase in ventilation in all the groups ($P<0.0001$, Fig. 2). However, the $CO_2$ response in the SB-334867 group was attenuated during wakefulness ($V_E$ vehicle, 1502.6 ± 100 mL kg$^{-1}$ min$^{-1}$ vs $V_E$ SB-334867, 1200.3 ± 70.0 mL kg$^{-1}$ min$^{-1}$, $P<0.05$) but not during sleep ($V_E$ vehicle, 1383.0 ± 113.9 vs $V_E$ SB-334687, 1287.6 ± 92.1 mL kg$^{-1}$ min$^{-1}$) (Fig. 2). The reduction in $V_E$ was due to a decreased $V_T$ ($V_T$ vehicle, 11.3 ± 0.7 vs $V_T$ SB-334687, 8.8 ± 0.3 mL kg$^{-1}$). The effect was specific for intra-LC injection since peri-LC injections did not have the same effect ($V_E$ peri-LC, 1760.2 ± 105.1 mL kg$^{-1}$ min$^{-1}$; $V_T$ peri-LC, 12.2 ± 0.6 mL kg$^{-1}$; data not shown in the graph).

### Effect of SB-334867 microinjection intra-LC on ventilation during room air and 7 % $CO_2$ during the light period

Under room air conditions, SB-334867 microinjection intra-LC did not change $V_E$, $V_T$, and fR during wakefulness and sleep in the light period (Fig. 3). Hypercapnia induced an increase in ventilation in all the groups ($P<0.0001$), although different from the dark period; the SB-334867 treatment caused no effect on the ventilatory response to $CO_2$ in wakefulness or in sleep (Fig. 3).

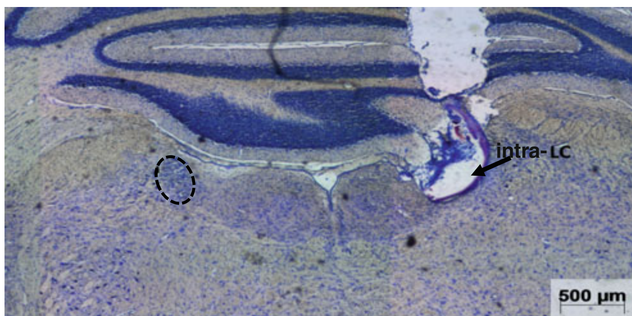


**Fig. 1** Representative photomicrograph of a microinjection into the locus coeruleus (*LC*) of rat. The figure shows a coronal section at the pons level illustrating the LC location (*dashed circle*) and a typical intra-LC microinjection (*black arrow*)

### Effect of SB-334867 microinjection intra-LC on Tb

No difference was observed between the OX1R antagonist-treated and vehicle-treated animals in terms of Tb during normocapnic or hypercapnic conditions in the dark (Fig. 4a) or in the light period (Fig. 4b). Hypercapnia caused a drop in Tb in all the groups in both periods ($P<0.05$).

### Effect of SB-334867 microinjection intra-LC on the time spent in each arousal state, during dark and light periods

Under room air condition, during the dark period (Fig. 5a), the rats spent more time awake, compared to NREM sleep, but the OX1R antagonist treatment decreased the total period of wakefulness compared with the vehicle-treated animals (1247.5 ± 173.2 s in the SB-334867 group vs 1941.8 ± 157.5 s in the control group; $P<0.05$; Table 1). This effect was due to a significant reduction in the length of the episodes of wakefulness, while the number of episodes did not change. $CO_2$ significantly increased the time the rats were awake (Fig. 5a), but the SB-334867 treatment shortened the wakefulness episodes ($P<0.05$; Table 1). However, this effect did not result in a significant difference in the total time spent in wakefulness.

During the light period (Fig. 5b), under normocapnia, the OX1R blockade did not change the time the rats spent in wakefulness, but during hypercapnia, the SB-334867-treated animals were less awake than the control group ($P<0.05$). This effect was due to a decreased duration of the wakefulness episodes and the number of episodes ($P<0.05$; Table 2).

## Discussion

In the present study, we suggest that orexin acting on OX1R of the LC neurons in unanesthetized adult rats contributes to the increase in ventilation in response to hypercapnia during wakefulness in the dark phase. The effect was specific in $V_T$ but not in fR. In addition, these receptors do not have a tonic role in ventilatory control, because no effect was observed under normocapnic conditions during wakefulness and during NREM sleep in the dark and light phases.

Previous studies have demonstrated that orexin stimulates breathing. Orexinergic projections and receptors are found in the brainstem regions involved in the control of breathing, including the rostral ventrolateral medulla (RVLM), Kölliker–Fuse nucleus, nucleus of the solitary tract (NTS), dorsal vagal motor nucleus, hypoglossal motor nucleus, and phrenic motor nuclei [39, 46, 67]. Orexin A injected into the RVLM or phrenic nucleus increased the amplitude of diaphragm EMG activity in anesthetized and vagotomized rats [67], while the injection of orexin B into the pontine Kölliker–Fuse nucleus, in a perfused rat brainstem preparation,

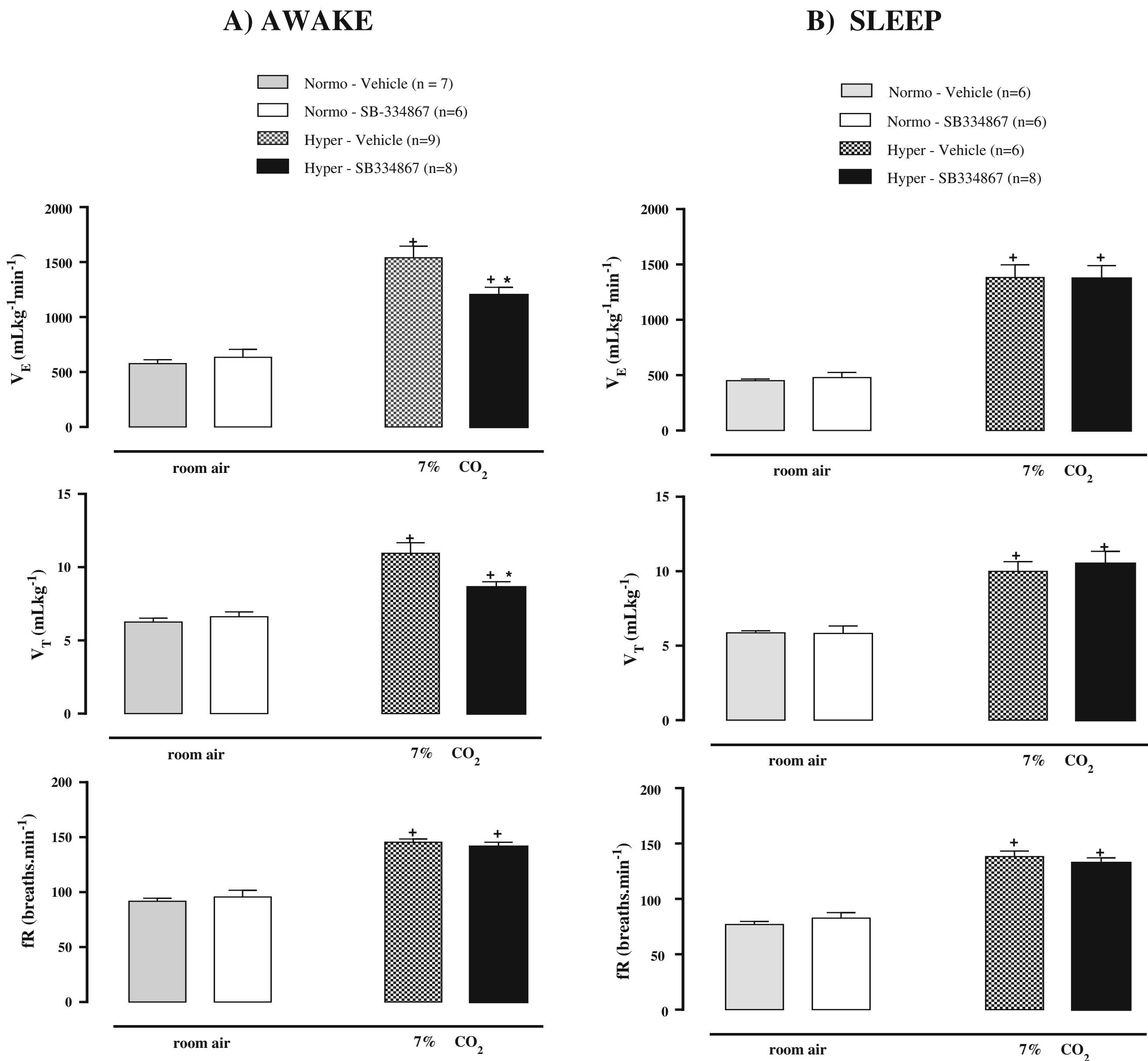


**Fig. 2** **a**, **b** Intra-LC microinjection of SB-334867 (5 mM) reduces hypercapnic response but not baseline respiratory activity in dark phase. Values are shown as mean ± S.E.M. *Asterisk* indicates significant differences between SB-334867 and vehicle groups. *Plus sign* indicates significant differences between normocapnic and hypercapnic groups

increased the respiratory frequency and the preinspiratory hypoglossal nerve activity [27]. In the current study, we showed that microinjection of SB-334867 intra-LC did not change the ventilation under room air conditions. Our result is consistent with previous data showing that spontaneous breathing was not different between wild-type and prepo-orexin knockout mice [15]. Similarly, selective inhibition of both the orexin receptors using a dual orexin receptor antagonist, administered orally, did not change breathing at rest [37], and the microdialysis of SB-334867 in the rostral medullary raphe and RTN did not affect ventilation during normocapnia, either in wakefulness or in sleep, during the dark and light phases of the diurnal cycle [17, 18]. Therefore, previous studies and the present results suggest that the orexinergic system acting on the LC, RTN, and rostral medullary raphe plays no tonic role during breathing at rest.

Hypercapnia augmented ventilation in all the groups, due to an increase in $V_T$ and fR. However, the microinjection of SB-334867 intra-LC attenuated the ventilatory response to hypercapnia by 25 %, during wakefulness in the dark but

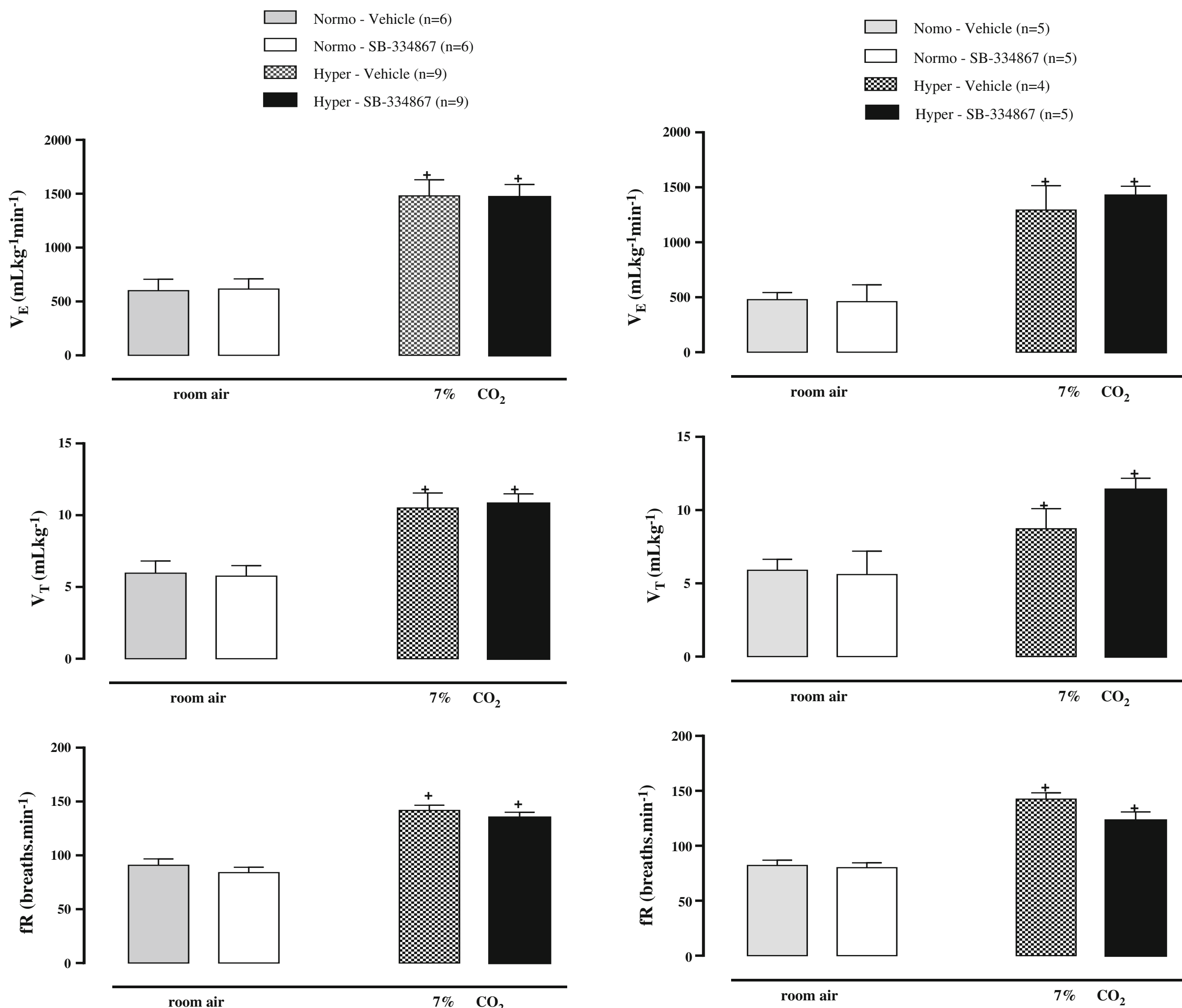


**Fig. 3** **a**, **b** Intra-LC microinjection of 5 mM SB-334867 does not change the ventilatory response to hypercapnia and normocapnia in light phase. Values are shown as mean ± SEM

not in the light period. This attenuation was due to a decreased $V_T$. Therefore, our data corroborate previous studies, which demonstrated that orexin plays an excitatory role in the hypercapnic ventilatory response during wakefulness [17, 18, 37, 38, 42, 66].

Orexins are important in the modulation of the central chemoreflex [15, 17, 18, 27, 42, 66]. In the present study, the ventilatory response to hypercapnia was attenuated after the injection of OX1R antagonist in the LC during wakefulness in the dark phase but not during sleep or in the light phase. The lack of effect of SB-334867 on respiratory variables during the light phase is consistent with orexin's circadian oscillation: it has little effect because there is little presynaptic orexin release [16]. Figure 6 depicts proposed LC orexinergic neurotransmission mechanisms based on the present data and evidence from the literature [10, 14, 20, 22, 33, 38, 40, 58, 65, 67]. During the dark phase, orexin A release is elevated and modulates the response of chemosensory signaling pathways in LC. Then, orexin A activates noradrenergic neurons that induce noradrenaline release which project to the

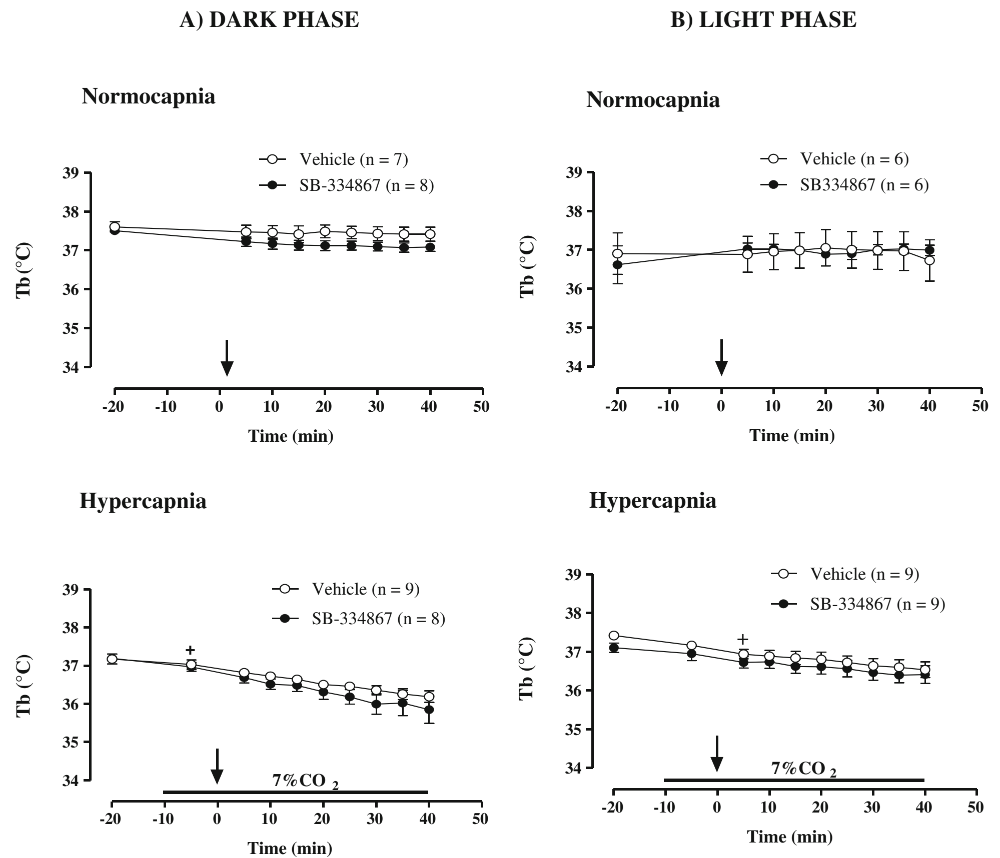


**Fig. 4** Effects of intra-LC microinjection of vehicle and SB-334867 (5 mM) on body temperature (Tb) of rats during normocapnia and hypercapnia in the dark phase (**a**) and light phase (**b**). Values are shown as mean ± SEM. The *arrow* indicates the time of the microinjection. *Plus sign* indicates the starting point of the significant drop of Tb during hypercapnia compared to normocapnia in both groups

central respiratory pattern generator (CPG) or directly to the respiratory premotoneurons, causing an increase in the activity of these neurons, promoting an increase in ventilation. Antagonism of ORX1 receptors may decrease the release of noradrenaline (NOR) from LC, leading to a reduction in the $CO_2$ drive to breath.

Similarly, Dias et al. [18], using microdialysis of SB-334867 in the medullary raphe, observed a decrease of 16 % in the ventilatory response to $CO_2$ during wakefulness in the dark phase. In another study, Dias et al. [17] demonstrated that the microdialysis of the same OX1R antagonist, but in the RTN, elicited a 30 % reduction in the ventilatory response to $CO_2$ during wakefulness and a reduction of 9 % during NREM sleep. These observations and the present study results support the hypothesis that the one mechanism by which orexinergic neurons modulate breathing is through OX1Rs located in different chemosensitive nuclei, such as the RTN, medullary raphe, and, as we have shown here, the LC.

The fact that the participation of orexin is dependent on the vigilance state is consistent with the well-established notion that the orexinergic neurons are essential to promote and consolidate wakefulness [27, 46]. Narcolepsy results from the loss of hypothalamic orexin/hypocretin neurons [12, 56]. Orexinergic neurons present inputs to the primary nuclei involved in the control of wakefulness [47, 53, 54]. Moreover, recordings of the activity of these neurons demonstrated that their discharge is state dependent [36, 41], because the activity of these neurons is high during wakefulness and decreases during NREM sleep [60]. Similarly, c-fos expression in orexinergic neurons is higher during wakefulness and lower during REM and NREM sleep [21].

In the present study, we limited the room air and hypercapnia periods to ~40 min each, which is appropriate to study the hypercapnic ventilatory responses but is not well suited to evaluate completely the characteristics of wakefulness and NREM sleep. To do so, it would be necessary many hours of EEG/EMG recordings along the experiment. However, even considering this limitation of recording time, we show that (1) the rats spent less time in sleep during hypercapnia compared with normocapnia, which was expected due to the arousal effect of $CO_2$ and (2) the OX1R blockade in the LC shortened the time the rats were awake during normocapnia in the dark but not in the light period. This effect was due to a reduction in the length of the awake episodes while the frequency of episodes did not change. (3) During hypercapnia, the inhibition of OX1Rs on the LC decreased the duration of awake episodes not only in the dark but also in the light period, but the total time spent in wakefulness was reduced just in the light period which is the inactive period of the rat diurnal cycle. Overall, these results are consistent with the arousal-promoting effect of orexin neurons and with their role in the maintenance of wakefulness, which could explain the fact that

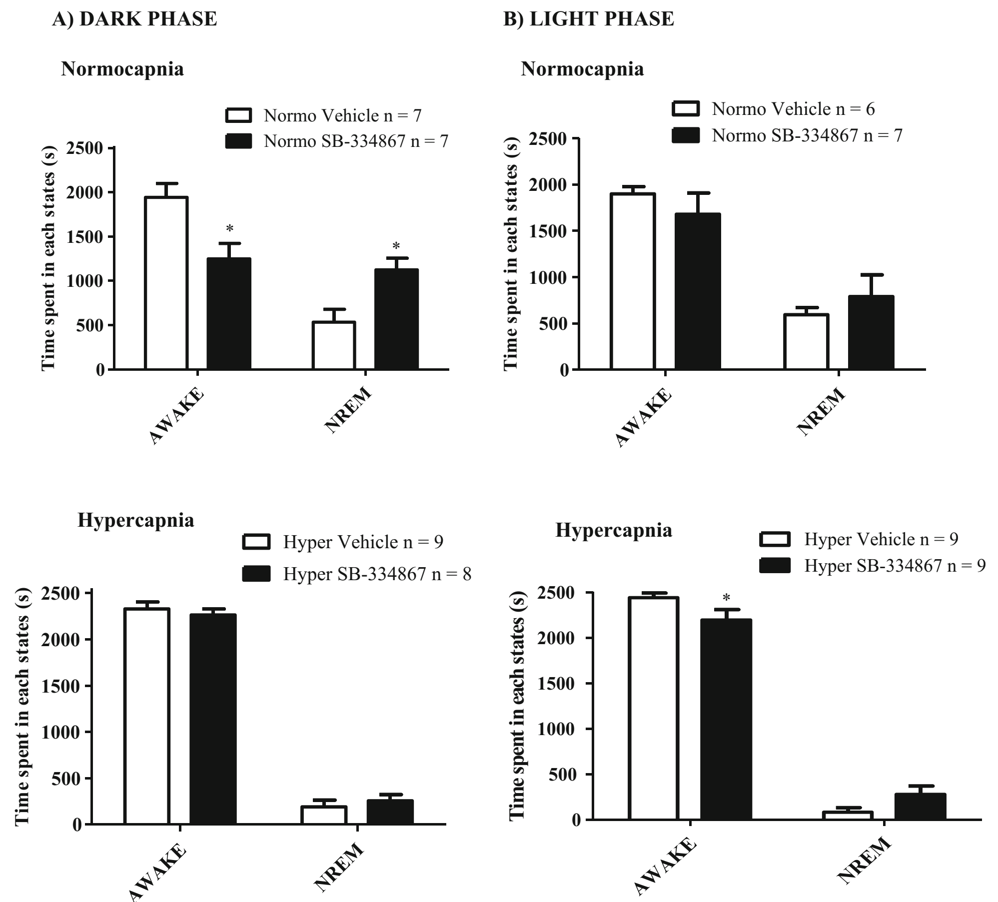


**Fig. 5** Effect of intra-LC microinjection of vehicle and 5 mM SB-334867 on sleep/wake states during normocapnia and hypercapnia in the dark phase (**a**) and light phase (**b**). Values are shown as mean ± SEM. *Asterisk* indicates significant differences between SB-334867 and vehicle groups

OX1R blockade shortened the duration of awake episodes. These results also corroborate with data from previous studies showing that the oral administration of a dual orexin receptor antagonist, almorexant, caused a 56 % decrease in the percentage time in wakefulness in rats [37], while it induced somnolence in rats, dogs, and humans [8].

Similar to the orexinergic neurons, the firing rate of LC noradrenergic neurons is also state dependent, being active during wakefulness, reducing their activity during non-REM sleep, and almost silent during REM sleep [2, 3, 32]. Corroborating these data, Shouse et al. [57] demonstrated that there is a progressive decline of noradrenaline release from

**Table 1** Sleep–wakefulness analysis of animals injected with vehicle or SB-334867 in the dark phase during normocapnia and hypercapnia

| Dark phase | Normocapnia | | | Hypercapnia | | |
|---|---|---|---|---|---|---|
| Treatment | Time spent in each states (s) | Number of episodes | Duration of episodes (s) | Time spent in each states (s) | Number of episodes | Duration of episodes (s) |
| Awake | | | | | | |
| Vehicle | 1941.8 ± 157.5 | 5.7 ± 1.0 | 608.5 ± 306.7[c] | 2328.5 ± 73.6[c] | 2.3 ± 0.5[c] | 1500.8 ± 325.8[c] |
| SB-334867 | 1247.5 ± 173.2[a] | 6.4 ± 0.7 | 179.1 ± 41.5[a] | 2261.8 ± 66.62[c] | 3.5 ± 0.6[c] | 964.8 ± 296.7[a] |
| NREM | | | | | | |
| Vehicle | 533.1 ± 145.5[b] | 5.1 ± 1.2 | 95.2 ± 19.7[bc] | 191.4 ± 73.64[bc] | 1.6 ± 0.6[c] | 66.4 ± 33.2[b] |
| SB-334867 | 1125.5 ± 129.7[a] | 7.2 ± 1.3 | 144.1 ± 24.3 | 258 ± 66.5[bc] | 3.0 ± 0.7[c] | 46.2 ± 11.2[b] |

Values are shown as mean ± SEM

[a] Significant differences between SB-334867 and vehicle groups

[b] Significant difference from the awake state in the same group

[c] Significant difference between normocapnia and hypercapnia in the same group

**Table 2** Sleep–wakefulness analysis of animals injected with vehicle or SB-334867 in the light phase during normocapnia and hypercapnia

| Light phase | Normocapnia | | | Hypercapnia | | |
|---|---|---|---|---|---|---|
| Treatment | Time spent in each states (s) | Number of episodes | Duration of episodes (s) | Time spent in each states (s) | Number of episodes | Duration of episodes (s) |
| Awake | | | | | | |
| Vehicle | 1896.5 ± 79.2 | 5.6 ± 0.4 | 346.2 ± 28.9 | 2438.3 ± 51.4[c] | 1.2 ± 0.2[c] | 2260.7 ± 189.7[c] |
| SB-334867 | 1677.1 ± 229.8 | 6.2 ± 1.0 | 349.4 ± 90.4 | 2193.4 ± 114.3[ac] | 3.4 ± 0.8[ac] | 1077.2 ± 297.9[ac] |
| NREM | | | | | | |
| Vehicle | 593 ± 77.1[b] | 5.0 ± 0.3 | 110.0 ± 22.8[b] | 81.6 ± 51.4[bc] | 0.5 ± 0.3[c] | 72.4 ± 51.2[b] |
| SB-334867 | 788.2 ± 235.6[b] | 5.5 ± 0.9 | 115.5 ± 22.0[b] | 276.5 ± 93.2[bc] | 2.6 ± 0.8[ac] | 73.9 ± 17.8[b] |

Values are shown as mean ± SEM

[a] Significant differences between SB-334867 and vehicle groups

[b] Significant difference from the awake state in the same group

[c] Significant difference between normocapnia and hypercapnia in the same group

wakefulness to NREM sleep and reaches its lowest level during REM sleep. Moreover, pharmacological or electrical activation of LC promotes wakefulness and inhibits REM sleep [30, 39, 49]. Therefore, it is expected that the participation of orexin receptor 1 in the LC is specific during wakefulness, as suggested by the results of this study.

Regarding body temperature, the orexinergic neurons are located in the dorsomedial hypothalamus (DMH), which is involved in the integration and coordination of multiple thermoeffectors [19]. Because LC is also involved in thermogenesis, particularly during fever [1], we considered the possibility that LC OX1R antagonism affects body temperature. Our data demonstrated that SB-334867 intra-LC microinjection did not change the Tb of rats under euthermic conditions. Our data corroborate previous studies from our laboratory showing that specific lesions of LC noradrenergic neurons did not change the Tb of rats under euthermia in normocapnia or hypercapnia, suggesting that noradrenergic neurons of the LC play no role in Tb regulation under these euthermic conditions [6].

We observed that hypercapnia promoted a decrease in Tb during the light and dark phases in all the groups. $CO_2$ exposure causes a decrease in Tb due to heat loss during hyperpnea and vasodilatation [5, 35, 55]. Hypercapnia promotes an

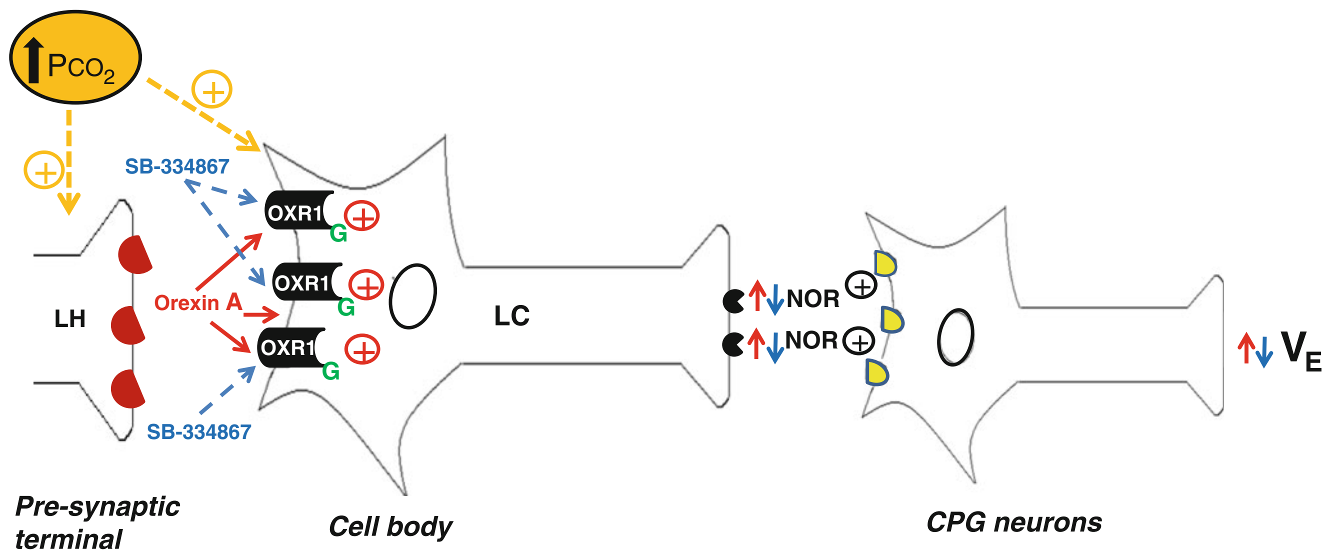


**Fig. 6** Schematic drawing depicting a possible mechanism of orexinergic signaling in the locus coeruleus (*LC*). During the dark phase, orexin A release from the lateral hypothalamus (*LH*) is elevated and modulates the response of chemosensory signaling pathways in LC. Orexin A binds on ORX1 receptors in the LC (*red arrows*) and induces depolarization of noradrenergic neurons and noradrenaline (*NOR*) release which project to the central respiratory pattern generator (*CPG*) or directly to the respiratory premotoneurons, causing an increase in the activity of these neurons, promoting an increase in ventilation. Antagonism of ORX1 receptors by SB-334867 (*blue arrows*) may decrease the release of NOR from LC, leading to a reduction in the $CO_2$ drive to breath

increase in the activity of neurons of the preoptic area of the anterior hypothalamus [61]. Moreover, a study in newborn mice showed that the rectal temperature decreases when the animals are exposed to 6 % $CO_2$ [24]. There was also a complete inhibition of thermogenesis in mice, when they were subjected to gas mixtures containing 10 % or more of $CO_2$ [52]. As observed during normocapnic conditions, SB-334867 intra-LC microinjection did not affect the drop in Tb during $CO_2$ challenge, suggesting that orexin acting in OX1R receptors in LC does not modulate the hypercapnia-induced hypothermia. However, we cannot exclude the possibility that ORX1 receptors in the LC may be involved in Tb regulation during specific types of thermal stress, such as exposure to cold or heat. Future studies are needed to clarify this issue.

In conclusion, we suggest that projections of orexin-containing neurons to the LC contribute, via OX1Rs, to the hypercapnic chemoreflex during wakefulness in the dark phase, indicating that this pathway may be involved in the vigilance state and diurnal cycle-dependent control of $CO_2$ drive to breathing.

**Acknowledgments** This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP - 2012/19966-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 442560/2014-1). The authors thank Euclides Seccato for his technical assistance and Luis Gustavo Patrone for helping with the statistics.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

## References

1. Almeida MC, Steiner AA, Coimbra NC, Branco LG (2004) Thermoeffector neuronal pathways in fever: role of the locus coeruleus. J Physiol 558:283–294
2. Aston-Jones G (2005) Brain structures and receptors involved in alertness. Sleep Med Suppl 1:S3-7
3. Aston-Jones G, Bloom FE (1981) Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci 8:876–886
4. Bartlett D, Tenney SM (1970) Control of breathing in experimental anemia. Respir Physiol 10:384–395
5. Barros RCH, Branco LGS, Carnio EC (2004) Evidence for thermoregulation by dopamine D1 and D2 receptors in the anteroventral preoptic region during normoxia and hypoxia. Brain Res 1030(2):165–171
6. Biancardi V, Bícego KC, Almeida MC, Gargaglioni LH (2008) Locus coeruleus noradrenergic neurons and CO2 drive to breathing. Pflugers Arch 455(6):1119–1128
7. Biancardi V, Bícego KC, Gargaglioni LH (2014) ATP in the locus coeruleus as a modulator of cardiorespiratory control in unanaesthetized male rats. Exp Physiol 99(1):232–247
8. Brisbare-Roch C, Dingemanse J, Koberstein R, Hoever P, Aissaoui H, Flores S, Mueller C, Nayler O, van Gerven J, de Haas SL, Hess P, Qiu C, Buchmann S, Scherz M, Weller T, Fischli W, Clozel M, Jenck F (2007) Promotion of sleep by targeting the orexin system in rats, dogs and humans. Nat Med 13(2):150–155
9. Bourgin P, Huitrón-Résendiz S, Spier AD, Fabre V, Morte B, Criado JR, Sutcliffe JG, Henriksen SJ, de Lecea L (2000) Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. J Neurosci 20(20):7760–7765
10. Burlet S, Tyler CJ, Leonard CS (2002) Direct and indirect excitation of laterodorsal tegmental neurons by Hypocretin/Orexin peptides: implications for wakefulness and narcolepsy. J Neurosci 22(7):2862–2872
11. De Carvalho D, Bícego KC, Castro OW, Da Silva GSF, Cairasco NG, Gargaglioni LH (2010) Role of neurokinin-1 expressing neurons in the locus coeruleus on ventilatory and cardiovascular responses to hypercapnia. Respir Physiol Neurobiol 172:24–31
12. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1996) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 5:322–327
13. Dejours P (1981) Principle of comparative respiratory physiology, 2nd edn. Elsevier, New York
14. Del Cid-Pellitero E, Garzón M (2011) Hypocretin1/OrexinA-containing axons innervate locus coeruleus neurons that project to the rat medial prefrontal cortex. Implication in the sleep-wakefulness cycle and cortical activation. Synapse 65(9):843–857
15. Deng BS, Nakamura A, Zhang W, Yanagisawa M, Fukuda Y, Kuwaki T (2007) Contribution of orexin in hypercapnic chemoreflex: evidence from genetic and pharmacological disruption and supplementation studies in mice. J Appl Physiol 103(5):1772–1779
16. Desarnaud F, Murillo-Rodriguez E, Lin L, Xu M, Gerashchenko D, Shiromani SN, Nishino S, Mignot E, Shiromani PJ (2004) The diurnal rhythm of hypocretin in young and old F344 rats. Sleep 27(5):851–856
17. Dias MB, Li A, Nattie EE (2009) Antagonism of orexin receptor-1 in the retrotrapezoid nucleus inhibits the ventilatory response to hypercapnia predominantly in wakefulness. J Physiol 587(9):2059–2067
18. Dias MB, Li A, Nattie EE (2010) The orexin receptor 1 (OX1R) in the rostral medullary raphe contributes to the hypercapnic chemoreflex in wakefulness, during the active period of the diurnal cycle. Respir Physiol Neurobiol 170(1):96–102
19. Dimicco JA, Zaretsky DV (2007) The dorsomedial hypothalamus: a new player in thermoregulation. Am J Physiol Regul Integr Comp Physiol 292(1):47–63
20. Eggermann E, Serafin M, Bayer L, Machard D, Saint-Mleux B, Jones BE, Mühlethaler M (2001) Orexins/hypocretins excite basal forebrain cholinergic neurones. Neuroscience 108(2):177–181
21. Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, Saper CB, Scammell TE (2001) Fos expression in orexin neurons varies with behavioral state. J Neurosci 21(5):1656–1662
22. España RA, Reis KM, Valentino RJ, Berridge CW (2005) Organization of hypocretin/orexin efferents to locus coeruleus and basal forebrain arousal-related structures. J Comp Neurol 481(2):160–178
23. Fabris G, Steiner AA, Anselmo-Franci JA, Branco LG (2000) Role of nitric oxide in rat locus coeruleus in hypoxia-induced hyperventilation and hypothermia. Neuroreport 11(13):2991–2995
24. Farkas M, Donhoffer S (1975) The effect of hypercapnia on heat production and body temperature in the new-born guinea pig. Acta Physiol Acad Sci Hung 46(3):201–217
25. Gargaglioni LH, Hartzler LK, Putnam RW (2010) The locus coeruleus and central chemosensitivity. Respir Physiol Neurobiol 173(3):264–273

26. Gautvik KM, de Lecea L, Gautvik VT, Danielson PE, Tranque P, Dopazo A, Bloom FE, Sutcliffe JG (1996) Overview of the most prevalent hypothalamus-specific mRNAs, as identified by directional tag PCR subtraction. Proc Natl Acad Sci U S A 93(16): 8733–8738
27. Gestreau C, Bevengut M, Dutschmann M (2008) The dual role of the orexin/hypocretin system in modulating wakefulness and respiratory drive. Curr Opin Pulm Med 14(6):512–518
28. Greco MA, Shiromani PJ (2001) Hypocretin receptor protein and mRNA expression in the dorsolateral pons of rats. Brain Res Mol Brain Res 88:176–182
29. Grimaldi D, Silvani A, Benarroch EE, Cortelli P (2014) Orexin/hypocretin system and autonomic control: new insights and clinical correlations. Neurology 82(3):271–278
30. Gottesmann C (2008) Noradrenaline involvement in basic and higher integrated REM sleep processes. Neurobiol 82:237–272
31. Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA (2001) Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 103(3): 777–797
32. Hobson JA, McCarley RW, Wyzinski PW (1975) Sleep cycle oscillation: reciprocal discharge by two brainstem neuronal groups. Science 189:55–58
33. Horvath TL, Peyron C, Diano S, Ivanov A, Aston-Jones G, Kilduff TS, van Den Pol AN (1999) Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. J Comp Neurol 415(2):145–159
34. Kuwaki T (2011) Orexin links emotional stress to autonomic functions. Auton Neurosci 161:20–27
35. Lai YL, Lamm JE, Hildebrandt J (1981) Ventilation during prolonged hypercapnia in the rat. J Appl Physiol Respir Environ Exerc Physiol 51(1):78–83
36. Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. J Neurosci 25(28):6716–6720
37. Li A, Nattie E (2010) Antagonism of rat orexin receptors by almorexant attenuates central chemoreception in wakefulness in the active period of the diurnal cycle. J Physiol 588(15):2935–2944
38. Li N, Li A, Nattie E (2013) Focal microdialysis of $CO_2$ in the perifornical-hypothalamic area increases ventilation during wakefulness but not NREM sleep. Respir Physiol Neurobiol 185:349–355
39. McCarley WR, Christopher MS (2004) Neurophysiological mechanisms of sleep and wakefulness: a question of balance. Semin Neurol 24(3):211–223
40. Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol 435(1): 6–25
41. Mileykovskiy BY, Kiyashchenko LI, Siegel JM (2005) Behavioral correlates of activity in identified hypocretin/orexin neurons. Neuron 46(5):787–798
42. Nakamura A, Zhang W, Yanagisawa M, Fukuda Y, Kuwaki T (2007) Vigilance state-dependent attenuation of hypercapnic chemoreflex and exaggerated sleep apnea in orexin knockout mice. J Appl Physiol 102:241–248
43. Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K (1999) Distribution of orexin neurons in the adult rat brain. Brain Res 827:243–260
44. Nattie EE, Li A (2002) CO2 dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness. J Appl Physiol 92:2119–2130
45. Nattie E, Li A (2010) Central chemoreception in wakefulness and sleep: evidence for a distributed network and a role for orexin. J Appl Physiol 108(5):1417–1424
46. Nattie EE, Li A (2012) Respiration and autonomic regulation and orexin. Prog Brain Res 198:25–46
47. Nixon JP, Smale L (2007) A comparative analysis of the distribution of immunoreactive orexin A and B in the brains of nocturnal and diurnal rodents. Behav Brain Funct 3:28
48. Novak CM, Albers HE (2002) Localization of hypocretin-like immunoreactivity in the brain of the diurnal rodent, Arvicanthis niloticus. J Chem Neuroanat 23(1):49–58
49. Pal D, Mallick BN (2007) Neural mechanism of rapid eye movement sleep generation with reference to REM-OFF neurons in locus coeruleus. Indian J Med Res 125(6):721–739
50. Patrone LG, Bicego KC, Hartzler LK, Putnam RW, Gargaglioni LH (2014) Cardiorespiratory effects of gap junction blockade in the locus coeruleus in unanesthetized adult rats. Respir Physiol Neurobiol 190:86–95
51. Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates, 3rd edn. Academic, San Diego
52. Pepelko WE, Dixon GA (1974) Elimination of cold-induced nonshivering thermogenesis by hypercapnia. Am J Physiol 227: 264–267
53. Peyron C, Tighe DK, Van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1988) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18(23): 9996–10015
54. Ohno K, Sakurai T (2008) Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. Front Neuroendocrinol 29:70–87
55. Saiki C, Mortola JP (1996) Effect of CO2 on the metabolic and ventilatory responses to ambient temperature in conscious adult and newborn rats. J Physiol 491(1):261–269
56. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemilli RM, Tanaka H, Clay Williams S, Richardson JA, Kozlowski GP, Wilson S, Arch JRS, Buckingham RE, Haynes AC, Carr SA, Annan RS, Mcnulty DE, Liu WS, Terret JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behaviour. Cell 92:573–585
57. Shouse MN, Staba RJ, Saquib SF, Farber PR (2000) Monoamines and sleep: microdialysis findings in pons and amygdala. Brain Res 860(1-2):181–189
58. Soffin EM, Evans ML, Gill CH, Harries MH, Benham CD, Davies CH (2002) SB-334867-A antagonises orexin mediated excitation in the locus coeruleus. Neuropharmacology 42(1):127–133
59. Souza Moreno V, Bícego KC, Szawka RE, Anselmo-Franci JA, Gargaglioni LH (2010) Serotonergic mechanisms on breathing modulation in the rat Locus coeruleus. Pflugers Arch 459(3):357–368
60. Takahashi K, Lin JS, Sakai K (2008) Neuronal activity of orexin and non-orexin waking-active neurons during wake-sleep states in the mouse. Neuroscience 153(3):860–870
61. Tamaki Y, Nakayama T (1987) Effects of air constituents on thermosensitivities of preoptic neurons: hypoxia versus hypercapnia. Pflugers Arch 409:1–6
62. Taxini CL, Puga CC, Dias MB, Bícego KC, Gargaglioni LH (2013) Ionotropic but not metabotropic glutamatergic receptors in the locus coeruleus modulate the hypercapnic ventilatory response in unanaesthetized rats. Acta Physiol (Oxf) 208(1):125–135
63. Thakkar MM, Ramesh V, Strecker RE, McCarley RW (2001) Microdialysis perfusion of orexin-A in the basal forebrain increases wakefulness in freely behaving rats. Arch Ital Biol 139(3):313–328
64. Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LHT, Guan XM (1998) Distribution of orexin receptor mRNA in the rat brain. FEBS Lett 438(1-2):71–75
65. Van den Pol AN, Ghosh PK, Liu RJ, Li Y, Aghajanian GK, Gao XB (2002) Hypocretin (orexin) enhances neuron activity and cell synchrony in developing mouse GFP-expressing locus coeruleus. J Physiol 541:169–185

66. Williams RH, Burdakov D (2008) Hypothalamic orexins/hypocretins as regulators of breathing. Expert Rev Mol Med 10:e28
67. Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, Wang QP, Tominaga M, Goto K, Shioda S, Sakurai T (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. Biochem Biophys Res Commun 290(4):1237–1245
68. Young JK, Wu M, Manaye KF, Kc P, Allard JS, MACK SO, Haxhiu MA (2005) Orexin stimulates breathing via medullary and spinal pathways. J Appl Physiol 98(4):1387–1395
69. Xi MC, Morales FR, Chase MH (2001) Effects on sleep and wakefulness of the injection of hypocretin-1 (orexin-A) into the laterodorsal tegmental nucleus of the cat. Brain Res 901(1-2):259–264