**Abstract**

Orexinergic (OX) neurons in the lateral hypothalamus (LH), perifornical area (PFA) and dorsomedial hypothalamus (DMH) play a role in the hypercapnic ventilatory response, presumably through direct inputs to central pattern generator sites and/or through interactions with other chemosensitive regions. OX neurons can produce and release orexins, excitatory neuropeptides involved in many functions, including physiological responses to changes in CO$_2$/pH. Thus, in the present study, we tested the hypothesis that different nuclei (LH, PFA and DMH) where the orexinergic neurons are located, show distinct activation by CO$_2$ during the light–dark cycle phases. For this purpose, we evaluated the Fos and OXA expression by immunohistochemistry to identify neurons that co-localize Fos + OXA in the LH, LPeF, MPeF and DMH in the light-inactive and dark-active phase in Wistar rats subjected to 3 h of normocapnia or hypercapnia (7% CO$_2$). Quantitative analyses of immunoreactive neurons show that hypercapnia caused an increase in the number of neurons expressing Fos in the LH, LPeF, MPeF and DMH in the light and dark phases. In addition, the number of Fos + OXA neurons increased in the LPeF and DMH independently of the phases of the diurnal cycle; whereas in the MPeF, this increase was observed exclusively in the light phase. Thus, we suggest that OX neurons are selectively activated by hypercapnia throughout the diurnal cycle, reinforcing the differential role of nuclei in the hypothalamus during central chemosensitivity.

**Keywords** Central chemoreflex · Hypercapnia · Hypothalamus · Orexins

**Introduction**

Central chemoreception is a homeostatic process in the central nervous system by which CO$_2$/pH changes are detected and appropriate ventilatory responses are activated to contribute to the maintenance of acid–base equilibrium. Central chemosensitive sites are represented by some traditionally recognized brain stem regions located on the ventral surface of the medulla (Mitchell et al. 1963; Loeschcke 1982; Guyenet et al. 2008), as well as other regions, such as the locus coeruleus (LC) (Nornhna-de-Souza et al. 2006; Biancardi et al. 2008), the medullary raphe (Wang and Richerson 1999; Nattie and Li 2001; Taylor et al. 2006) and even some hypothalamic nuclei, which have been considered to have chemosensory properties (Berquin et al. 2000; Deng et al. 2007; Williams et al. 2007; Sunanaga et al. 2009).

Electrophysiological and in vivo studies have reported that the lateral hypothalamus/perifornical area and medial hypothalamus are activated in response to alterations in CO$_2$/pH levels (Deng et al. 2007; Williams et al. 2007; Sunanaga et al. 2009; Li et al. 2013). In these regions, the ventilatory responses to hypercapnia have been attributed to the orexinergic (OX) neurons, also known as hypocretinergic neurons, located exclusively in the lateral hypothalamus/perifornical area and the dorsomedial hypothalamus (LH/PFA/DMH). The OX neurons produce two excitatory neuropeptides: orexin-A (OXA; 33 amino acids) and orexin-B (OXB; 28 amino acids) from the cleavage of a common precursor pre-pro-orexin mRNA. These neuropeptides can bind to specific G-protein-coupled receptors (OX1R or OX2R) in many regions of the CNS (Peyron et al. 1998; de Lecea et al. 1998; Sakurai et al. 1998) to regulate several functions, such as maintaining...
wakefulness, control of energy homeostasis, feeding behavior, stress response, motivation, nociception, modulation of breathing, heart rate, blood pressure and neuroendocrine systems (van den Pol and Trombley 1993; Elias et al. 1998; Sakurai et al. 1998; de Lecea et al. 1998;Date et al. 1999; Samson et al. 1999; Kayaba et al. 2003; Williams et al. 2007; Ohno and Sakurui 2008; Puskás et al. 2010; Li et al. 2013).

Regarding their chemosensitive function, OX neurons increase their firing rate in response to extracellular acidification and may influence breathing parameters by interacting with several brainstem nuclei from the respiratory network, such as the NTS, the hypoglossal nuclei and the pre-Bötzinger complex (Peyron et al. 1998; Deng et al. 2007; Williams et al. 2007; Gestreau et al. 2008; Kuwaki et al. 2010; Li et al. 2013; Yang and Ferguson. 2003; Rosin et al. 2006; Young et al. 2005). In OX knockout mice, there is a 50% reduction in the hypercapnic ventilatory response, despite the maintenance of respiratory parameters in normocapnia (Nakamura et al. 2007). Furthermore, the role of the OX system in the central chemoreflex appears to be related not only to its intrinsic sensitivity to CO$_2$/pH, but also to its interaction with other chemosensitive sites, such as the RTN (Lazarenko et al. 2011) and medullary raphe (Dias et al. 2009, 2010) modulating its activity in response to high levels of CO$_2$.

Furthermore, it is worth noting that OX neurons are directly involved in maintaining wakefulness (Abrahamson and Moore 2001; Kiyashchenko et al. 2002; Mileykovskiy et al. 2005; Lee et al. 2005; Takahashi et al. 2008; Gestreau et al. 2008), predominantly during the active phase of the animal’s diurnal cycle, which corresponds to the dark phase in nocturnal rodents. Temporal control of the photic information that stimulates or inhibits the activity of OX neurons comes from the suprachiasmatic nuclei (SCN) (Marston et al. 2008) and, as a result, the activity of OX neurons follows the biological circadian rhythms of the wake/sleep and light/dark cycles.

Thus, compelling evidence suggests that CO$_2$/H$^+$ sensitivity of OX neurons also occurs in a vigilance state-dependent manner and varies throughout the light/dark cycle (Dias et al. 2009, 2010; Li and Nattie 2010; Li et al. 2013; da Silva et al. 2018; Rodrigues et al. 2019). The antagonism of OX receptors by Almorexant attenuated the ventilatory response to CO$_2$ in 26% of rats during wakefulness, but not during sleep, in the dark-active phase of the diurnal cycle, while there was no change in the light phase, compared to the control group (Li and Nattie 2010). Moreover, blocking OX1R receptors in the medullary raphe by microdialysis of the OX1R antagonist, SB334867, caused a 16% reduction in the ventilatory response to CO$_2$ in awake rats in the dark-active phase, but not in the light-inactive phase of the diurnal cycle (Dias et al. 2010).

Despite evidence for the involvement of LH/PFA/DMH in central chemosensitivity and the OX system in the diurnal variations of the central chemoreflex, there are still many unanswered questions. One question concerns the possible differential activation of these hypothalamic nuclei by high levels of CO$_2$ in a diurnal cycle dependent manner. In fact, evidence points to the idea that different subsets of orexinergic neurons have different roles or different degrees of participation in certain functions (Harris et al. 2005, 2007; Zink et al. 2014). This could be applied to the response to CO$_2$/H$^+$. In this context, it was demonstrated, in mice, that OX neurons distributed mainly in the PFA and DMH region, but not in the LH, are activated by CO$_2$/pH (Sunanaga et al. 2009). Moreover, it has been demonstrated that LH and DMH present a different pattern of expression of TASK 1 and TASK 3 channels, which are channels supposedly involved with the CO$_2$/H$^+$ sensing mechanisms in OX neurons, with the expression of these channels being higher in LH than in DMH (Wang et al. 2021). Thus, our hypothesis is that the different OX nuclei (LH, PFA and DMH) present distinct activation by CO$_2$/pH during the light–dark cycle phases.

Based on the above, here we intended to determine (1) whether hypercapnia activates orexin-containing neurons in a light–dark cycle dependent manner using immunohistochemistry, and (2) whether there is a difference in the activation of orexinergic neurons by hypercapnia between the different hypothalamic areas that express these neurons: LH, PFA and DMH.

**Materials and methods**

**Animals**

All experimental procedures were carried out under the principles and regulations for animal experimentation defined in the Brazilian guidelines on animal use—the National Council for Animal Experimentation Control (CONCEA, MCT, Brazil), and approved by the Animal Care and Use Committee for the Institute of Biosciences in Botucatu, Brazil (CEUA—IBB, UNESP, Botucatu campus; protocol nº. 597).

All experimental procedures were performed in male Wistar rats (280–400 g), obtained from the Central Animal Facility of UNESP—Botucatu. The animals were kept in a light- and temperature-controlled room (23 ± 2 °C), with free food and water, under a 12/12 h light/dark cycle (lights on at 2 p.m. for the dark period group, and at 7 a.m. for the light period group). For the dark period group, the animals were acclimatized to a partially reversed light/dark cycle for at least 4 weeks before the experiment.
Experimental protocol

To reduce non-specific expression of Fos, all animals were acclimatized for 1 h under normocapnic conditions for five consecutive days prior to the experiment, at the same time and under the same environmental conditions as the actual experiments would occur. On the sixth day, unanesthetized rats were placed inside the 5-L acrylic chamber, which was continuously flushed with room air or a hypercapnic gas mixture (7% CO₂, 21% O₂, N₂ balance; White Martins, Sertãozinho, Brazil), provided by a gas mixer (Pegas 4000F; Columbus Instruments, Columbus, OH, USA), for 3 h. The experimental protocols were performed during the light and dark phases of the diurnal cycle. Thus, we obtained four experimental groups: (1) normocapnia during the light period, (2) hypercapnia during the light period, (3) normocapnia during the dark period, and (4) hypercapnia during the dark period. In both phases of the cycle, the normocapnic groups represented the control group, while the animals subjected to hypercapnia were the treated animals.

Experiments in animals in the light period group were performed in the afternoon (1–5 PM), which represents the end of the light phase, when the activity of the OX neurons is supposedly minimal. On the other hand, the dark group experiments occurred in the morning (8–12 AM), which represents the end of the dark phase, when the activity of the OX neurons is supposedly maximal (Yoshida et al. 2001; Desarnaud et al. 2004).

Tissue preparation for immunohistochemistry

Immediately at the end of each experiment, the animals were deeply anesthetized by i.p. injection of an anesthetic compound blend of xylazine (0.5 mg/mL), ketamine (25 mg/mL) and acepromazine (1 mg/mL), diluted in purified water to the dose of 0.2 mL/100 g of their body weight. The rats were then transcardially perfused with 150 mL of calcium-free heparinized (0.3 ml) Ringer buffer (0.85% NaCl; 0.025% HCl; 0.02% NaHCO₃), pH 6.9, at 37 °C, followed by 1 L of paraformaldehyde (4%; pH 7.4; 0.1 M) at room temperature, applied with a peristaltic pump adjusted for a flow of 30 mL/min. The brains were removed by dorsal craniotomy and cryoprotected for 48 h at 4 °C in 30% sucrose (pH 7.4; 0.1 M). The hypothalamic regions of interest, delimited for a ~1 cm block (from optic chiasm to superior colliculus), were cut (30-μm sections) on a frozen cryostat (Reichert-Jung, Leica, Germany), and the serial coronal sections obtained were stored in an antifreeze solution (6.25% PBS, 0.25% NaCl, 15% sucrose, 35% ethylene glycol in aqueous solution).

Free-floating hypothalamic sections were pre-treated with PBS (phosphate-buffered saline; pH 7.4; 0.1 M) containing 10% hydrogen peroxide for 20 min to inactivate endogenous peroxidases, followed by normal donkey serum (2%; pH 7.6; 0.05 M) (Vector Laboratories) for 40 min to block non-specific labeling.

Double immunohistochemistry labeling for Fos protein and orexins was performed following sequential immunohistochemistry protocols: (1) primary antibodies directed against Fos protein, and then (2) primary antibodies directed against OXA.

Initially, sections were incubated with primary anti-Fos antibody (α-C-FOS Calbiochem CAT# PC38; 1:10,000) for 24 h at 4 °C, washed and incubated with their corresponding biotinylated secondary antibody (Biotin-SP-conjugated AffiniPure Donkey Anti-Rabbit IgG. #711–065-152; 1:100) for 2 h at room temperature. After the removal of the secondary antibody, sections were incubated with an avidin–peroxidase complex (ABC Kit, Vectastain Standard®, Vector Labs, #PK-4000; 1:200) for 2 h. Fos-immunoreactive neurons were visualized by reaction with 3,3′diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO, EUA) and intensified with nickel (Nickel ammonium sulfate, Sigma #A-1827), which generates a stable black product (Hsu et al. 1981; Adams. 1981; Hsu and Soban. 1982).

On the next day, the sections were washed in TBS-Tx (pH 7.6; 0.05 M) and incubated overnight in goat primary OXA antiserum (Santa Cruz-C-19; sc-8070; 1:3000) for 48 h at 4 °C under agitation. After PBS washes, the tissue was incubated in biotinylated secondary anti-goat IgG antiserum (Biotin-SP-conjugated AffiniPure Donkey Anti-Goat IgG; 1:200) for 2 h at room temperature, followed by a 2-h incubation with the ABC complex, and revealed with DAB as previously mentioned, but without nickel.

At the end of the immunohistochemical procedure, all sections were mounted sequentially from rostral to caudal on slides, dehydrated and coverslipped with Permount® (Fisher Scientific).

Image acquisition

The sections were photographed with a digital camera (axi-oCamHRc, Zeiss) connected to an optical microscope (Axioplan2, Carl Zeiss) in a light field. To obtain the number of neurons expressing Fos, OXA and Fos + OXA in the LH/PFA/DMH, we performed manual counts of photomicrographs taken with a 10× objective. Slight adjustments in the sharpness, contrast, brightness and clustering were made to compose panoramic bilateral images using Adobe.
Photoshop® (version 9.0; Adobe Systems Incorporated, San Jose, CA, USA).

**Image analysis**

Slides containing sections of the LH/PFA/DMH region were studied under a microscope according to the Stereotaxic Atlas of the Rat Brain (Paxinos and Watson 2005). For the quantitative analysis of OX neurons activated under normocapnic and hypercapnic conditions in the light and dark phases of the diurnal cycle, we selected the series obtained bilaterally along the 5 sections per brain in the LH/PFA/DMH comprehended in the region between −2.28 and −3.60 mm from bregma, with an interval of 150 μm.

The LH/PFA/DMH region was divided into four minor zones, adapted from previous studies (Sunanaga et al. 2009; Clifford et al. 2015; Li et al. 2016). A vertical line that passes through the center of the fornix divided the perifornical area (PFA) into the lateral perifornical area (LPeF) and the medial perifornical area (MPeF). Two thirds of the DMH region were demarcated, and a vertical line drawn from that point separated the DMH from the MPeF. One-third of the distances from the center of the fornix to the third ventricle, which corresponds to the MPeF drawn laterally from the center of the fornix, demarcate the width of the LPeF. The remaining ventrolateral region up to the internal capsule (ic) was defined as the lateral hypothalamic area (LH). The lower and upper limits of the five vertical lines traced were the lower edge of the fornix and the top of the third ventricle, respectively (see Fig. 1).

The image analysis consisted of counting the distinguishable neurons expressing: (1) Fos (black staining points), (2) OXA (brown cytoplasm), and (3) Fos + OXA (brown cytoplasm with black nucleus in the center) (Fig. 2).

**Statistical analyses**

The nomenclatures and abbreviations used in the description of the results were based on the The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2005). Statistical analyses were performed using the Statistica v10 program for each hypothalamic subnucleus (LH, LPeF, MPeF and DMH) with treatment (normocapnia or hypercapnia) and phases (light or dark phases) as categorical factors. The results were described in terms of mean and standard deviation. A statistically significant difference was considered when \( p < 0.05 \).

Initially, the data were subjected to normality and homoscedasticity tests (Shapiro–Wilk and Levene). In some cases (Fos-LH, Fos-LPeF and Fos + OXA groups), we used log-transformed data to increase the normality and homoscedasticity. Raw or log-transformed data were analyzed by two-way ANOVA. When we observed an isolated effect of the tested factors or when the factors interacted significantly, ANOVA was complemented by the Newman–Keuls test or, in some cases, by the LSD Fisher, if a potential type II error was detected.

Statistical analyses of Fos, OXA, and Fos + OXA neurons comparing the hypothalamic areas were performed using the three-way ANOVA, considering area (LH, LPeF, MPeF and DMH), treatment (normocapnia and hypercapnia) and phase boundaries. A p-value of less than 0.05 was considered statistically significant.

**Fig. 1** Representative photomicrograph showing immunoreactive neurons in the LH, LPeF, MPeF and DMH, delimited by the rectangles in green. LH lateral hypothalamic area, LPeF lateral perifornical area, MPeF medial perifornical area, DMH dorsomedial hypothalamus. Hatching lines indicates the anatomical structures of reference.
(light and dark phases) as categorical factors, followed by a post hoc test (Tukey’s multiple comparisons test). A statistically significant difference was considered when $p < 0.05$.

**Results**

Figure 2 shows representative photomicrographs of Fos immunoreactivity in the hypothalamic OX neurons during...
normocapnia and hypercapnia, in different hypothalamic rostro-caudal levels.

The region-specific difference in the responses

We performed a 3-way ANOVA analysis comparing each of the neuron counts (Fos, OXA or Fos + OXA) between the hypothalamic areas (Fig. 3) in normocapnia (black circles) and 7% CO₂ hypercapnia (opened circles), in both phases of the light–dark cycle (light and dark).

Regarding Fos expression (Fig. 3A), it was observed a significant effect of area ($p < 0.0001$; three-way ANOVA), treatment (normocapnia or hypercapnia; $p = 0.0117$; three-way ANOVA), cycle phase ($p = 0.0264$, three-way ANOVA) and interaction between treatment and area ($p < 0.0001$; three-way ANOVA).

The comparison between the OXA-ir neurons of the different hypothalamic regions (Fig. 3B) showed a significant effect of area ($p < 0.0001$; three-way ANOVA), interaction between treatment and area ($p = 0.0106$; three-way ANOVA) and interaction between treatment and cycle phase ($p = 0.0051$; three-way ANOVA).

Concerning the Fos + OXA-positive neurons (Fig. 3C), the analysis showed a significant effect of area ($p < 0.0001$; three-way ANOVA), treatment (normocapnia or hypercapnia; $p = 0.0094$; three-way ANOVA) and interaction between treatment and area ($p = 0.0008$; three-way ANOVA).

As can be seen, the hypothalamic area was always the most significant factor for the three cell types counted (Fos, OXA and FOS + OXA). For this reason, we performed two-way ANOVA analyzes for each cell type, in each area separately, as shown below.

Fos, OXA and Fos + OXA expression in the lateral hypothalamus (LH)

Figure 4 shows the number of LH neurons that express Fos, OXA and Fos + OXA during the light (left side) and dark (right side) phases in normocapnia (black bars) and

---

**Fig. 3** Number of immunolabelled neurons expressing (A) Fos, (B) OXA and (C) Fos + orexin-A in the lateral hypothalamus (LH), lateral perifornical area (LPeF), medial perifornical area (MPeF), and dorsomedial hypothalamus (DMH) after 3 h of room air (black circles) or hypercapnia (7% CO₂; opened circles) during the light and dark phases of the diurnal cycle ($n = 5$ per group).
Regarding the number of Fos-ir neurons, there was no significant interaction between phase and treatment \((p = 0.2882)\), but the phase of diurnal cycle and the CO\(_2\) exposure treatment had significant effects alone \((p = 0.0024\) and \(p = 0.0067,\) respectively). As observed in Fig. 4, there was an increase in the number of Fos-ir neurons during hypercapnia compared to normocapnia \((p = 0.0067,\) two-way ANOVA).

Concerning the number of OXA-ir neurons, there was a significant interaction between phase and treatment \((p = 0.0023)\), but no significant effects of these factors alone \((phase\ of\ cycle\ p = 0.1148;\ treatment\ p = 0.0607)\). The number of OXA-ir neurons in the dark phase was higher compared to the light phase in normocapnia \((178 \pm 52\) versus \(39 \pm 20;\ p = 0.0049,\) two-way ANOVA). However, in the light phase, but not in the dark, there was a greater number of OXA-ir cells in hypercapnia compared to normocapnia \((188 \pm 93\) versus \(39 \pm 20;\ p = 0.0053,\) two-way ANOVA). However, despite the increase in OXA-ir cells in the LH during hypercapnic stimulus, there was no interaction \((p = 0.1370)\) or significant effects alone \((phase\ of\ cycle\ p = 0.4149,\ treatment\ p = 0.0801)\) in neurons with co-localization of Fos + OXA.

Fos, OXA and Fos + OXA expression in the lateral perifornical area (LPeF)

Figure 5 shows the number of LPeF neurons that express Fos, OXA and Fos + OXA during the light and dark phases in normocapnia (black bars) and hypercapnia (7% CO\(_2\); gray bars).

Regarding Fos expression, there was no interaction between treatment and phase of the light/dark cycle \((p = 0.4961)\), but, in the same way as observed for the LH area, the phase of cycle and the CO\(_2\) exposure had significant effects alone \((p = 0.0028\) and \(p = 0.0020,\) respectively).

The analysis of the OXA-ir neurons in the LPeF area showed a significant interaction between phase and treatment \((p = 0.0016)\) and no significant effects of these factors alone \((phase\ of\ cycle\ p = 0.1931;\ treatment\ p = 0.7024)\). As shown, the number of OXA-ir neurons in the LPeF area, of the group exposed to hypercapnia in the light phase, was higher compared to the normocapnia group \((168 \pm 107\) versus \(68 \pm 43;\ p = 0.045,\) two-way ANOVA). In the dark phase, under normocapnic conditions, the number of OXA-ir neurons was higher compared to the light phase \((199 \pm 69\) versus \(68 \pm 43,\ p = 0.0113,\) two-way ANOVA).

Analyses of co-localization of Fos + OXA revealed no interaction between phase and treatment \((p = 0.0870)\) but treatment had significant effect alone \((p = 0.0118)\) with a greater number of neurons expressing Fos + OXA in hypercapnia compared to normocapnia \((p = 0.0120,\) two-way ANOVA).

Fos, OXA, Fos + OXA expression in the medial perifornical area (MPeF)

Figure 6 shows the number of neurons expressing Fos, OXA and Fos + OXA during the light (left side in white) and dark (right side in gray) phases in normocapnia (black bars) and hypercapnia (7% CO\(_2\); gray bars) in the MPeF.

The analysis of Fos expression in the MPeF showed no interaction between treatment and phase \((p = 0.9982)\), but
the phase and the treatment had significant effects alone ($p = 0.0107$ and $p = 0.0332$, respectively). As observed, there was a significant increase in the number of Fos-ir neurons during hypercapnia compared to normocapnia ($p = 0.033$, two-way ANOVA).

Concerning the OXA-ir in the MPeF, there was a significant interaction between phase and treatment ($p = 0.0051$) with no significant effects of these factors alone (phase $p = 0.2806$; treatment $p = 0.7762$). The number of OXA-ir neurons in the hypercapnia was greater ($213 \pm 117$) compared to the normocapnia ($95 \pm 62$; $p = 0.05$, two-way ANOVA) in the light phase, while in the dark phase, the number of OXA-ir cells was greater in the normocapnic group, compared to the group exposed to hypercapnia ($269 \pm 72$ versus $128 \pm 94$; $p = 0.024$, two-way ANOVA).

The analysis of Fos + OXA-positive neurons in the MPeF showed a significant interaction between phase and treatment ($p = 0.0080$) and a significant effect of treatment alone ($p = 0.013$). As observed in Fig. 6, the Fos + OXA-positive number of neurons in the MPeF during hypercapnia was higher compared to normocapnia in the light phase ($105 \pm 94$ neurons versus $10 \pm 5$ neurons; $p = 0.0009$, two-way ANOVA) but not in the dark.

**Fos, OXA and Fos + OXA expression in the dorsomedial hypothalamus (DMH)**

Figure 7 shows the number of DMH neurons expressing Fos, OXA and Fos + OXA during the light (left side in white) and dark (right side in gray) phases of normocapnia (black bars) and hypercapnia (7% CO$_2$; gray bars).

There was no interaction between phase and treatment in the analysis of Fos-positive neurons ($p = 0.5154$), but the phase and treatment had significant effects alone ($p = 0.039$ and $p = 0.0016$, respectively), with a significant increase of Fos-ir cells during hypercapnia compared to normocapnia ($p = 0.0017$, two-way ANOVA).

Regarding the number of OXA neurons, there was a significant interaction between phase and treatment ($p = 0.0172$) with a greater number of OXA-positive neurons in normocapnia during dark phase compared with the light phase ($p = 0.0399$, two-way ANOVA).

Fos + OXA-ir neurons analysis showed no interaction between phase and treatment ($p = 0.1068$) but treatment had a significant effect alone ($p = 0.0416$), with a significant increase in the number of Fos + OXA-ir during hypercapnia compared to normocapnia ($p = 0.0417$, two-way ANOVA).

**Discussion**

Our findings provide new insights into the activation of hypothalamic neurons in rats exposed to hypercapnia during the light and dark phases of the diurnal cycle. Our results show that OX neurons are selectively activated by CO$_2$ throughout the diurnal cycle, supporting the differential role of nuclei in the hypothalamus during central chemosensitivity.

The expression of Fos highlights intracellular neuronal events and can be used to investigate neuronal activation (Sagar et al. 1988; Draganow and Faull 1989; Herrera and Robertson 1996; Teppema et al. 1997; Berquin et al. 2000; Estabrooke et al. 2001). However, several factors should be carefully considered when assessing the expression of Fos in OX neurons in response to hypercapnia as the hypercapnic stimulus initially activates wakefulness and OX neurons are recognized for their function associated with arousal and locomotor activity (Kiyashchenko et al. 2002; Mileykovskiy et al. 2005; Lee et al. 2005). Thus, in order to avoid non-specific Fos labeling in OX neurons (due not only to the activation of these neurons by the mechanisms of chemoreception per se, but also to the awakening caused by hypercapnia), we chose to use the hypercapnic stimulus for 3 h. In our previous experience, hypercapnia at 7% CO$_2$ activates wakefulness in rats typically within the first 10–20 min of exposure, followed by a return to baseline levels of locomotor activity with subsequent sleep, especially in the light phase. Considering that, upon stimulation, Fos gene expression is rapidly induced in neurons, with peak mRNA production at 30 min and gene translation of the Fos protein reaching its maximum levels between 60 and 90 min (Morgan et al. 1987; Hoffman et al. 1993), surely the activation of OX neurons observed in our study occurred predominantly as a result of the CO$_2$ stimulus used during the 3-h experiment. In addition, we submitted the animals to experimental protocols only after 5 consecutive days of acclimatization.
which contributes to suppressing the environmental stress factors.

Some studies have highlight sex difference in lateral hypothalamic expression of orexins (Taheri et al. 1999; Jöhren et al. 2002), with females expressing more orexin mRNA and displaying more activation of orexin neurons as well as a higher concentration of the orexin-A peptide in cerebrospinal fluid (Grafe et al. 2017). In the current study, we have used only male rats, since most of the studies in CO2 chemosensitivity has been performed in males.

We analyzed the activation of OX neurons under hypercapnia in rats during the light and dark periods since orexin neurons demonstrate diurnal variations in their activity, with the highest levels occurring in the dark-active phase in nocturnal animals (Estabrooke et al. 2001; Yoshida et al. 2001; Desarnaud et al. 2004). Moreover, in vivo studies have suggested that the role of OX neurons in the central chemoreflex is predominant during the dark phase of the light/dark cycle, although it is not exclusive to this phase (Dias et al. 2010; da Silva et al. 2018). Here, we show that CO2 exposure increased the Fos+OXA labeling during both the light and dark periods, which is consistent with the suggestion that the participation of OX neurons in the central chemoreception occurs in both light and dark periods (Li et al. 2013).

It is worth mentioning that we chose to analyze only the interference of the light cycle and not the state of the animals with Fos expression in OX neurons during the chemoreflex due to the fact that changes in sleep and the vigilance state in rodent are rapid and moments of sleep and vigilance are inevitably present in both phases of the light/dark cycle. Moreover, the insertion of electrodes to measure cortical activity and determine sleep or wakefulness could be another stimulus for Fos expression.

It has been suggested that OX neurons can be divided into functionally distinct subpopulations: lateral neurons would be especially involved with motivated behaviors, whereas medial neurons would be more involved with wakefulness and stress (Harris et al. 2005, 2007; Zink et al. 2014). Therefore, we considered the possibility that hypercapnia would cause a differential activation of these subpopulations of OX neurons. To make this determination, we evaluated each region separately. Thus, a quantitative analysis of the expression of immunoreactive neurons was performed in four distinct regions (LH/LPeF/MpF/DMH) encompassing the entire hypothalamic region containing OX neurons (LH/PFA/DMH) (Fig. 1).

Our results demonstrate that hypercapnia caused an expressive activation of neurons of the LH, LPeF, MPeF and DMH in rats, which was highlighted by significant expression of Fos under the hypercapnic stimulus in all these regions. Since the cell bodies of OX neurons are located exclusively in the LH/LPeF/MpF/DMH and have a recognized chemosensitive function (Deng et al. 2007; Williams et al. 2007; Sunanaga et al. 2009; Li et al. 2013), the neuronal activation of these regions by CO2 could be related to OX activation. Thus, we also assessed the number of doubly labeled neurons (Fos+OXA). We found that the number of neurons that co-localized Fos+OXA was higher in the group of animals exposed to hypercapnia compared to normocapnia in three regions verified in the present study: LPeF, MPeF and DMH (Figs. 5, 6, 7). In the LPeF and DMH, this difference was observed independently of the phase of the diurnal cycle (Figs. 5, 7), whereas in the MPeF, the increased number of co-locations occurred only in the group exposed to hypercapnia in the light-inactive phase, but not in the dark-active phase (Fig. 6).

It has been demonstrated that the Fos expression in neurons is related to an augmented neuronal activity (Hoffman et al. 1993; Krukoff 1999) and, in OX neurons, Fos expression may be related to the release of orexins (Marston et al. 2008). Consequently, the increase in neuronal Fos+OXA co-localization in the LPeF and DMH, independently of the phase of the diurnal cycle, reinforces previous studies suggesting that OX neurons are active during extracellular acidification (Deng et al. 2007; Williams et al. 2007; Sunanaga et al. 2009; Li et al. 2013), and demonstrates, for the first time, by immunohistochemical methods that subpopulations of OX neurons in the LPeF and DMH under CO2 exposure are activated independently of the phase of the diurnal cycle.

In the MPeF, the increased expression of Fos+OXA in hypercapnia compared to normocapnia occurred only in the light phase (Fig. 6), suggesting that the OX neurons in this region are activated by the hypercapnic stimulus, especially during the light-inactive phase of the diurnal cycle. In the dark phase, we observed a high number of co-localizations in normocapnia without marked differences in the hypercapnia, demonstrating that this area is not significantly responsive to the hypercapnic stimulus in the dark-active phase or the same neurons activated by dark have been also activated by hypercapnia. Thus, the present study provides unprecedented data on a putative subpopulation of OXA neurons, in the medial part of the perifornical area, especially activated by hypercapnia in the light-inactive phase of the diurnal cycle. Moreover, it is important to note that regardless of the stimulus, the number of neurons in medial regions is greater than the lateral regions, which can explain, to some extent, the higher number of OXA-ir neurons in the dark-active phase during normocapnic conditions, even exceeding the values during hypercapnia (Fig. 6). Consequently, we suppose that orexin neurons located in the MPeF region are especially important in other functions of the orexinergic system, other than CO2 chemosensitivity, during the dark phase.

It is also relevant to consider that the number of neurons expressing Fos during hypercapnia was higher than the neurons expressing Fos+OX, which would indicate that other neurons with different phenotypes, in addition to
OX neurons, can be activated by the hypercapnic stimulus. Therefore, it is reasonable to think that CO₂/pH might activate a broad range of hypothalamic neurons in the LH/PFA/DMH to modulate the physiological responses to hypercapnia during the light and dark phase of the diurnal cycle. Other neurons, such as (1) leptin receptor-expressing neurons (LepRb) in the LH involved in energetic homeostasis (Leinninger et al. 2011), (2) tyrosine hydroxylase-positive neurons in the DMH involved in wakefulness, motivated behavior, and catabolic and anabolic processes (Horjaless-Araujo et al. 2014), and (3) MCH neurons involved in the antagonistic modulation of OX neurons during the activation of the central chemoreflex (Li et al. 2014), are set in intimate synaptic contact with OX neurons. Thus, by reciprocal connections, OX neurons may activate these neighboring neurons during hypercapnia (7% CO₂).

Furthermore, we should also consider that the number of Fos-ir neurons was greater in the DMH (Fig. 7) than in other sites analyzed in the present study (LH, LPeF and MPeF). Situated in the medial zone of the tuberal hypothalamus, the DMH is a hypothalamic region of integration involved in pressor and defense responses associated with stress, wakefulness, thermogenesis and autonomic functions, resulting from wide projections of DMH neurons to the nucleus of solitary tract, rostral ventral lateral medulla, pallid raphe nuclei, periaqueductal gray matter, paraventricular nuclei and limbic prefrontal cortex (de Leca et al. 1998; Peyron et al. 1998; Nambu et al. 1999; Kuwaki. 2008; Kataoka et al. 2014). In addition, this region shows massive connections with the perifornical area, nucleus Kölliker-fuse, and the lateral and medial parabrachial nucleus (Papp and Palkovits. 2014), which are regions associated with modulation of ventilatory responses. Therefore, it would be reasonable to expect that the DMH may be activated during a hypercapnic stimulus, which was observed in the present study via high expression of Fos, regardless of the phase of the diurnal cycle (Fig. 7).

Absence of increased Fos + OXA-ir labeling was observed in the LH region during hypercapnia in the light and dark phase of the diurnal cycle. This result is in agreement with a previous study in mice that showed that the number of double-labeled cells (Fos + OX) in the PFA/DMH significantly increased by the hypercapnic stimulation whereas that did not change in the LH (Sunanaga et al. 2009), which is a hypothalamic region that is supposedly mostly involved with the regulation of food behavior and recompense circuits (Harris et al. 2005, 2007; Zhang et al. 2006; Harris and Aston-Jones 2006; Arrigoni et al. 2019).

In addition, using a similar approach in female rats, Tenorio-Lopes et al (2020) proposed that, by comparison with OX neurons from PFA and DMH, OX neurons from the LH do not contribute to stress-related increase in the ventilatory response to CO₂, which is in accordance with our results.

Maintenance of wakefulness, feeding behavior, release of hormones and muscle tone, characteristics of the active phase of the animals occurs in response to orexin circadian release (Estabrooke et al. 2001; Takahashi et al. 2008). Indeed, levels of orexins vary over 24 h in the CSF (Desarnaud et al. 2004) and in the hypothalamus of rats (Yoshida et al. 2001; Kiyashchenko et al. 2002), and are higher during the dark-active phase and lower in the light-inactive phase (Yoshida et al. 2001; Takahashi et al. 2008). These variations correlated positively with the labeling of OXA-ir neurons observed in the present study, which was higher in the dark phase compared to the light phase in normocapnic conditions in the LH, LPeF and MPeF.

We also observed an increased number of non-Fos-positive OXA neurons in the LH, LPeF and MPeF during hypercapnia in the light phase (Figs. 4, 5, 6). The fact that these neurons do not express Fos suggests that they are not directly associated with hypercapnic stimulation but are certainly related to other functions. Studies show that orexin is involved in the modulation of responses in neural circuits of autonomic control with numerous functions such as: maintenance of wakefulness, control of energy homeostasis, stress response, nociception and cardiovascular control (Sakurai et al. 1998b; Zhang et al. 2005; Samson et al. 2005; Chiu et al. 2010; Li et al. 2013). These functions possibly undergo several alterations, directly and indirectly, during the phases of the circadian cycle and may also suffer indirect influences from the CO₂/pH content during the experiment. As it is an indirect, later effect, this could be related to the absence of Fos labeling in these other positive OXA neurons and to the variation in their number, as we observed in our results.

Our results agree with early studies that identified the orexinergic chemosensitivity to CO₂ (Williams et al. 2007; Deng et al. 2007; Sunanaga et al. 2009; Li et al. 2013). Moreover, the present data show evidence that orexin neurons located especially in the DMH, MPeF and LPeF are involved with neural circuits activated by the hypercapnic chemoreflex independently of the phase of the light–dark cycle in rats, which suggests that the CO₂/H⁺ response of OX neurons differs between subpopulation of neurons. In fact, the comparison of the labeled neurons counts (Fos, OXA and Fos + OXA) between the four different hypothalamic areas (Fig. 3) showed there was a significant interaction between treatment (CO₂ exposure) and hypothalamic area, which confirms that there is a region-specific difference in the CO₂ response of hypothalamic neurons. The heterogeneity of subpopulations of orexinergic neurons, demonstrated by neuroanatomical studies, supports our data. The projections from the region encompassing the DMH, PFA and LH, to the lower brainstem have been investigated using an anterograde tracer in rats (Papp and Palkovits 2014) and the results demonstrated great heterogeneity regarding the targets of these projections. Likewise, a previous
anterograde tracing study suggests that different subgroups of orexin neurons may respond to different stimuli. Projections from hypothalamic regions preferentially reach orexin neurons in the medial and perifornical area, but most projections from the brainstem target the lateral part of the orexin field (Yoshida et al. 2006). These data suggest that homeostatic drives influence arousal by acting on orexin neurons in the medial part of the field while motivated behaviors and autonomic activity influence orexin neurons in the lateral part of the field. Moreover, not all OX neurons project to neural structures that regulate breathing. For instance, it has been demonstrated that a small percentage of orexin neurons directly projects to the pre-Bötzinger region and the phrenic nucleus: 0.5–2.9%, respectively (Young et al. 2005). This may be due to the possibility that only a subset of orexin cells participates in breathing control. However, part of the orexinergic neurons may regulate breathing through indirect projections and this must also be considered.

Conclusion

In summary, our data show new evidence that CO2 activates OX neurons located mainly in the medial hypothalamic regions (DMH and PFA) and this activation is independent of the phases of the diurnal cycle.

Acknowledgements The authors would like to thank Rodrigo E. Barreto for helping with the statistical analyses.

Author contributions All authors contributed to the study conception and design. The experimental protocols were performed by ENS. Data analysis, interpretation and writing of the manuscript was performed by ENS, JACH, LHG and MBD. All authors provided critical and intellectual input during the preparation of the manuscript and approved the final version.

Funding This work was supported by the Sao Paulo Research Foundation—Brazil (FAPESP, 2013/04216-8, 2019/17693-5, and 2016/13136-6) and by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), in the scope of the Program CAPES-PrInt, process number 88887.194785/2018-00. E.N.S. was a recipient of a master’s scholarship from the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES)—Finance Code 001.

Data availability The datasets generated during the current study are available in the figshare repository, https://doi.org/10.6084/m9.figshare.20518497.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Animal Care and Use Committee for the Institute of Biosciences at Botucatu, Brazil (CEUA—IBB, UNESP, Botucatu campus; protocol no. 597).

References

Abrahamson EE, Moore RY (2001) The posterior hypothalamic area: chemoarchitecture and afferent connections. Brain Res 889:1–22
Adams JC (1981) Heavy metal intensification of DAB-based HRP reaction product. J Histochem Cytochem 29:775
Arrigoni E, Chee MJS, Fuller PM (2019) To eat or to sleep: that is a lateral hypothalamic question. Neuropharmacology 154:34–49. https://doi.org/10.1016/j.neuropharm.2018.11.017
Berquin P, Bodineau L, Gros F, Larnicol N (2000) Brainstem and hypothalamic areas involved in respiratory chemoreflexes: a Fos study in adult rats. Brain Res 857:30–40. https://doi.org/10.1016/S0006-8993(99)02304-5
Biancardi V, Rícego KC, Almeida MC, Gargaglioni LH (2008) Locus coeruleus noradrenergic neurons and CO2 drive to breathing. Pflügers Arch 455:1119–1128. https://doi.org/10.1007/s00424-007-0338-8
Chiu L-C, Lee H-J, Ho Y-C et al (2010) Orexins/hypocretins: pain regulation and cellular actions. Curr Pharm Des 16:3089–3100
Clifford L, Dampney BW, Cattive P (2015) Spontaneously hypertensive rats have more orexin neurons in their medial hypothalamus than normotensive rats. Exp Physiol 100:388–398. https://doi.org/10.1113/epjphotol.2014.084137
da Silva EN, de Horta-Júnior J, AC, Gargaglioni LH, Dias MB, (2018) ATP in the lateral hypothalamus/perifornical area enhances the CO2 chemoreflex control of breathing. Exp Physiol. https://doi.org/10.1113/EP087182
Date Y, Ueta Y, Yamashita H et al (1999) Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci USA 96:748–753
de Lecce L, Kilduff TS, Peyron C et al (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci 95:322–327. https://doi.org/10.1073/pnas.95.1.322
Deng B-S, Nakamura A, Zhang W et al (2007) Contribution of orexin in hypercapnic chemoreflex: evidence from genetic and pharmacological disruption and supplementation studies in mice. J Appl Physiol 103:1772–1779. https://doi.org/10.1152/japplphysiol.00075.2007
Desarnaud F, Murillo-Rodriguez E, Lin L et al (2004) The diurnal rhythm of hypocretin in young and old F344 rats. Sleep 27:851–856. https://doi.org/10.1093/sleep/27.5.851
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 587:2059–2067. https://doi.org/10.1113/jphysiol.2008.168260
Dias MB, Li A, Nattie E (2010) The orexin receptor 1 (OX(1)R) in the rostral medullary raphe contributes to the hypercapnic chemoreflex in wakefulness, during the active period of the diurnal cycle. Respir Physiol Neurobiol 170:96–102. https://doi.org/10.1016/j.resp.2009.12.002
Dragunow M, Faul R (1989) The use of c-fos as a metabolic marker in neuronal pathway tracing. J Neurosci Methods 29:261–265. https://doi.org/10.1016/0165-0270(89)90150-7
Elias CF, Saper CB, Maratos-Flier E et al (1998) Chemically defined projections linking the mediodorsal hypothalamus and the lateral hypothalamic area. J Comp Neuro 402:442–459
Estabrooke IV, McCarthy MT, Ko E et al (2001) Fos expression in orexin neurons varies with behavioral state. J Neurosci 21:1656–1662. https://doi.org/10.1152/jn.00927.2005

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 14:512–518. https://doi.org/10.1097/MCPB.0b013e3283131d3

Grafe LA, Cornfeld A, Luz S et al (2017) Orexins mediate sex differences in the stress response and in cognitive flexibility. Biol Psychiatry 81:683–692. https://doi.org/10.1016/j.biopsych.2016.10.013

Guyenet PG, Sornetta RL, Bayliss DA (2008) Retrotrapezoid nucleus and central chemoreception. J Physiol 586:2043–2048. https://doi.org/10.1113/jphysiol.2008.150870

Harris GC, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. Nature 437:556–559. https://doi.org/10.1038/nature04071

Harris GC, Wimmer M, Randall-Thompson JF, Aston-Jones G (2007) Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. Behav Brain Res 183:43–51. https://doi.org/10.1016/j.bbr.2007.05.025

Herrera DG, Robertson HA (1996) Activation of c-fos in the brain. Prog Neurobiol 50:83–107. https://doi.org/10.1016/S0301-0082(96)00021-4

Hoffman GE, Smith MS, Verbalis JG (1993) c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Front Neuroendocrinol 14:173–213

Horjales-Araujo E, Hellysaz A, Broberger C (2014) Lateral hypothalamic thyrotropin-releasing hormone neurons: distribution and relationship to histochemically defined cell populations in the rat. Neuroscience 277:87–102. https://doi.org/10.1016/j.neuroscience.2014.06.043

Hsu SM, Soban E (1982) Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. J Histochem Cytochem 30:1079–1082

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580. https://doi.org/10.1177/29.4.616661

Jöhren O, Neidert SJ, Kummer M, Dominiak P (2002) Sexually dimorphic expression of prepro-orexin mRNA in the rat hypothalamus. Peptides 23:1177–1180. https://doi.org/10.1016/S0196-9781(02)00052-9

Kataoka N, Iioki H, Kaneo T, Nakamura K (2014) Article psychological stress activates a dorsomedial hypothalamic-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia. Cell Metab 20:346–358. https://doi.org/10.1016/j.cmet.2014.05.018

Kayaba Y, Nakamura A, Kasuya Y et al (2003) Attenuated defense response and low basal blood pressure in orexin knockout mice. Am J Physiol Regul Integr Comp Physiol 285:R581–R593. https://doi.org/10.1152/ajpregu.00671.2002

Kiyashchenko LI, Mileyskovskiy BY, Maidment N et al (2002) Release of hypocretin (orexin) during waking and sleep states. J Neurosci 22:5282–5286. 20026541

Krukoff TL (1999) c-fos expression as a marker of functional activity in the brain. In: Boulton AA, Baker GB, Bateson AN (eds) Cell neurobiology techniques. Humana Press, Totowa, NJ, pp 213–230

Kuwaki T (2008) Orexinergic modulation of breathing across vigilance states. Respir Physiol Neurobiol 164:204–212. https://doi.org/10.1016/j.resp.2008.03.011

Kuwaki T, Li A, Nattie E (2010) State-dependent central chemoreception: a role of orexin. Respir Physiol Neurobiol 173:223–229. https://doi.org/10.1016/j.resp.2010.02.006

Lazarenko RM, Sornetta RL, Bayliss DA, Guyenet PG (2011) Orexin activates retrotrapezoid neurons in mice. Respir Physiol Neurobiol 175:283–287. https://doi.org/10.1016/j.resp.2010.12.003

Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. J Neurosci 25:6716–6720. https://doi.org/10.1523/JNEUROSCI.1887-05.2005

Leimninger GM, Opland DM, Jo Y-H et al (2011) Leptin action via neurotransin neurons controls orexin, the mesolimbic dopamine system and energy balance. Cell Metab 14:313–323. https://doi.org/10.1016/j.cmet.2011.06.016

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 589:2935–2944. https://doi.org/10.1113/jphysiol.2010.191288

Li A, Roy SH, Nattie EE (2016) An augmented CO2chemoreflex and overactive orexin system are linked with hypertension in young and adult spontaneously hypertensive rats. J Physiol 594:4967–4980. https://doi.org/10.1113/JP272199

Li N, Li A, Nattie E (2013) Focal microdialysis of CO2 in the perifornical-hypothalamic area increases ventilation during wakefulness but not NREM sleep. Respir Physiol Neurobiol 185:349–355. https://doi.org/10.1016/j.resp.2012.09.007

Li N, Nattie E, Li A (2014) The role of melanin concentrating hormone (MCH) in the central chemoreflex: a knockdown study by siRNA in the lateral hypothalamus in rats. PLoS ONE. https://doi.org/10.1371/journal.pone.0103585

Loeschke HH (1982) Central chemosensitivity and the reaction theory. J Physiol 332:1–24. https://doi.org/10.1113/jphysiol.1982.sp014397

Marston OJ, Williams RH, Canal MM et al (2008) Circadian and dark-pulse activation of orexin/hypocretin neurons. Mol Brain 1:19. https://doi.org/10.1186/1756-6606-1-19

Mileyskovskiy BY, Kiyashchenko LI, Siegel JM (2005) Behavioral correlates of activity in identified hypocretin/orexin neurons. Neuron 46:787–798. https://doi.org/10.1016/j.neuron.2005.04.035

Mitchell RA, Loeschcke HH, Massion WH, Severinghaus JW (1963) Respiratory responses mediated through superficial chemosensitive areas on the medulla. J Appl Physiol 18:523–533. https://doi.org/10.1152/jappl.1963.18.3.523

Morgan JJ, Cohen DR, Hempstead JL, Curran T (1987) Mapping patterns of c-fos expression in the central nervous system after seizure. Science 237:192–197

Nakamura A, Zhang W, Yanagisawa M et al (2007) Vigilance state-dependent attenuation of hypercapnic chemoreflex and exaggerated sleep apnea in orexin knockout mice. J Appl Physiol 102:241–248. https://doi.org/10.1152/japplphysiol.00679.2006

Nambu T, Sakurai T, Mizukami K et al (1999) Distribution of orexin neurons in the adult rat brain. Brain Res 827:243–260. https://doi.org/10.1016/S0006-8993(99)01336-0

Nattie EE, Li A (2001) CO2 dialysis in the medullary raphe of the rat increases ventilation in sleep. J Appl Physiol 90:1247–1257. https://doi.org/10.1152/jappl.2001.90.4.1247

Noronha-de-Souza CR, Bícego KC, Michel G et al (2006) Locus coeruleus is a central chemoreceptive site in toads. Am J Physiol Integ Comp Physiol 291:R997–R1006. https://doi.org/10.1152/ajpregu.00900.2006

Ohno K, Sakurai T (2008) Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. Front Neuroendocrinol 29:70–87. https://doi.org/10.1016/j.yfrne.2007.08.001

Papp RS, Palkovits M (2014) Brainstem projections of neurons located in various subdivisions of the dorsolateral hypothalamic area-an
anterograde tract-tracing study. Front Neuroanat 8:34. https://doi.org/10.3389/fnana.2014.00034

Paxinos G, Watson C (2004) The Rat Brain in Stereotaxic Coordinates - The new coronal set, 5th edn. Academic Press

Peyron C, Tighe DK, van den Pol AN et al (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015

Puskás N, Papp RS, Gallatz K, Palkovits M (2010) Interactions between orexin.immunoreactive fibers and adrenaline or noradrenaline-expressing neurons of the lower brainstem in rats and mice. Peptides 31:1589–1597. https://doi.org/10.1016/j.peptides.2010.04.020

Rodrigues LTC, da Silva EN, Horta-Júnior JAC, Gargaglioni LH, Dias MB (2019) Glutamate metabotropic receptors in the lateral hypothalamus / perifornical area reduce the CO2 chemoreflex. Respir Physiol Neurobiol 260:122–130. https://doi.org/10.1016/j.resp.2018.11.007

Rosin DL, Chang DA, Guyenet PG (2006) Afferent and efferent connections of the rat retrotrapezoid nucleus. J Comp Neurol 499(1):64–89. https://doi.org/10.1002/cne.21105

Sagar S, Sharp F, Curran T (1988) Expression of c-Fos protein in brain: metabolic mapping at the cellular level. Science 200(240):1328–1331. https://doi.org/10.1126/science.3131879

Sakurai T, Amemiya A, Ishii M et al (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573–585. https://doi.org/10.1016/S0092-8674(00)80949-6

Samson WK, Taylor MM, Ferguson AV (2005) Non-sleep effects of estradiol regulation of orexin neurons: a novel mechanism in non-orexin waking-active neurons during wake–sleep states in the mouse. Neuroscience 1331. https://doi.org/10.1016/j.neuroscience.2005.11.005

Sakurai T, Amemiya A, Ishii M et al (1998) Orexin and orexin receptors that regulate feeding behavior. Cell 92:573–585. https://doi.org/10.1016/S0092-8674(00)80949-6

Taylor NC, Li A, Nattie EE (2006) Ventilatory effects of muscimol microdialysis into the rostral medullary raphe region of conscious rats. Respir Physiol Neurobiol 153:203–216. https://doi.org/10.1016/j.resp.2005.11.005

Tenorio-Lopes L, Fournier S, Henry MS et al (2020) Disruption of estradiol regulation of orexin neurons: a novel mechanism in excessive ventilatory response to CO2 inhalation in a female rat model of panic disorder. Transl Psychiatry 10:394. https://doi.org/10.1038/s41398-020-01076-x

Teppema LJ, Veening JG, Kranenburg A et al (1997) Expression of c-fos in the rat brainstem after exposure to hypoxia and to normoxic and hyperoxic hypercapnia. J Comp Neurol 388:169–190. https://doi.org/10.1002/(SICI)1096-9861(19971117)388:2<3e169::AID-CNE1%3e3.0. CO;2-#

van den Pol AN, Trombley PQ (1993) Glutamate neurons in hypothalamus regulate excitatory transmission. J Neurosci 13(2829):2836

Wang W, Richerson GB (1999) Development of chemosensitivity of rat medullary raphe neurons. Neuroscience 90:1001–1011. https://doi.org/10.1016/S0306-4522(98)00505-3

Wang X, Guan R, Zhao X et al (2021) TASK1 and TASK3 in orexin neuron of lateral hypothalamus contribute to respiratory chemoreflex by projecting to nucleus tractus solitarius. FASEB J 35:1–14. https://doi.org/10.1096/fj.202002189R

Williams RH, Jensen LT, Verkrhatsky A et al (2007) Control of hypothalamic orexin neurons by acid and CO2. Proc Natl Acad Sci U A 104:10685–10690. https://doi.org/10.1073/pnas.0702676104

Yoshida K, McCormack S, España RA et al (2006) Afferents to the orexin neurons of the rat brain. J Comp Neurol 494:845–861. https://doi.org/10.1002/cne.20859

Yoshida Y, Fujiki N, Nakajima T et al (2001) Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep–wake activities. Eur J Neurosci 14:1075–1081. https://doi.org/10.1046/j.0953-816X.2001.01725.x

Young JK, Wu M, Manaye KF et al (2005) Orexin stimulates breathing via medullary and spinal pathways. J Appl Physiol 98:1387–1395. https://doi.org/10.1152/japplphysiol.00914.2004

Zhang S, Blache D, Vercoe PE et al (2005) Expression of orexin receptors in the brain and peripheral tissues of the male sheep. Regul Pept 124:81–87. https://doi.org/10.1016/j.regpep.2004.07.010

Zhang W, Shimoyama M, Fukuda Y, Kuwaki T (2006) Multiple components of the defense response depend on orexin: evidence from orexin knockout mice and orexin neuron-ablated mice. Autism Neurosci Basic Clin 126–127:139–145. https://doi.org/10.1016/j.autneu.2006.02.021

Zink AN, Perez-Leighton CE, Kotz CM (2014) The orexin neuropeptide system: physical activity and hypothalamic function throughout the aging process. Front Syst Neurosci 8:1–10. https://doi.org/10.3389/fnsys.2014.00211

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under applicable law.

Springer or its licensor holds exclusive rights to this article under applicable law.