Published in final edited form as:

Prog Brain Res. 2012 ; 198: 25–46. doi:10.1016/B978-0-444-59489-1.00004-5.

Respiration and Autonomic Regulation and Orexin

Eugene Nattie and Aihua Li

Department of Physiology, Dartmouth Medical School, One Medical Center Drive: Remsen 706E, Lebanon, N.H., 30756-0001

Abstract

Orexin, a small neuropeptide released from neurons in the hypothalamus with widespread projections throughout the central nervous system, has broad biological roles including the modulation of breathing and autonomic function. That orexin activity is fundamentally dependent on sleep-wake state and circadian cycle requires consideration of orexin function in physiological control systems in respect to these two state-related activity patterns. Both transgenic mouse studies and focal orexin receptor antagonism support a role for orexins in respiratory chemosensitivity to CO₂ predominantly in wakefulness, with further observations limiting this role to the dark period. In addition, orexin neurons participate in the regulation of sympathetic activity, including effects on blood pressure and thermoregulation. Orexin is also essential in physiological responses to stress. Orexin-mediated processes may operate at two levels: 1) in sleep-wake and circadian states; and 2) in stress, e.g., the defense or “fight or flight” response and panic anxiety syndrome.

Keywords

orexin; Almorexant; chemoreception; CO₂; blood pressure

Introduction

General

Orexin is a recently discovered small peptide of hypothalamic origin (see earlier Chapters in this volume). There are two orexins, orexin A and B, that arise from a common precursor, prepro-orexin, that is localized to neurons in the lateral (perifornical region) and dorsal hypothalamus and (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). At very widespread central nervous system projection sites of these neurons, orexins act via two G-protein coupled orexin receptors, OX₁R and OX₂R (Sakurai *et al.*, 1998; Tsujino and Sakurai, 2009). The orexin neurons themselves receive cholinergic, serotonergic and GABAergic afferents from many central nervous system (CNS) sites (Tsujino and Sakurai, 2009). Their focal hypothalamic source, wide projections and multiple inputs suggest key and broad biological functions (see Fig. 1). For example, orexins appear to have clear roles in the regulation of sleep-wake state, activity and metabolism (Adamantidis and de Lecea, 2008, 2009; Boutrel

et al., 2009; Carter *et al.*, 2009; Chiou *et al.*, 2010; de Lecea, 2009; Ganjavi and Shapiro, 2007; Jones, 2005; Kuwaki, 2011; Roecker and Coleman, 2008; Sakurai, 2002, 2003).

Functional studies indicate that orexins can also modulate breathing (Deng *et al.*, 2007; Dutschmann *et al.*, 2007; Gestreau *et al.*, 2008; Kuwaki, 2008, 2010; Kuwaki *et al.*, 2010; Lazarenko *et al.*, 2011; Nakamura *et al.*, 2007; Terada *et al.*, 2008; Toyama *et al.*, 2009; Williams and Burdakov, 2008; Williams *et al.*, 2007; Young *et al.*, 2005; Zhang *et al.*, 2005). The orexin neuron projection sites include brainstem regions with known involvement in the control of breathing and blood pressure including the rostral ventrolateral medulla (RVLM), medullary raphe, locus ceruleus (LC), Kolliker-Fuse nucleus, nucleus of the solitary tract (NTS), dorsal vagal motor nucleus, hypoglossal motor nucleus, and phrenic motor nucleus (Kuwaki, 2008; Marcus *et al.*, 2001; Nixon and Smale, 2007; Tsujino and Sakurai, 2009; Young *et al.*, 2005). Our lab has focused on the role of orexins in the control of breathing particularly central chemoreception, the physiological process by which changes in blood and brain PCO₂ and/or pH affect the level of alveolar ventilation (Dias *et al.*, 2010; Dias *et al.*, 2009; Kuwaki *et al.*, 2010; Li and Nattie, 2010; Nattie, 2011; Nattie and Li, 2010).

Orexins also affect sympathetic activity and blood pressure and its regulation (Antunes *et al.*, 2001; Bastianini *et al.*, 2011; Chen *et al.*, 2000; de Oliveira and Ciriello, 2003; de Oliveira *et al.*, 2003; Donadio *et al.*, 2008; Dun *et al.*, 2000; Hirota *et al.*, 2003; Huang *et al.*, 2010; Kannan *et al.*, 2007; Kayaba *et al.*, 2003; Liewellyn-Smith *et al.*, 2003; Lin *et al.*, 2002; Machado *et al.*, 2002; Matsumura *et al.*, 2001; Matsumura *et al.*, 2003; Samson *et al.*, 1999; Shahid *et al.*, 2011; Shirasaka *et al.*, 1999; Shirasaka *et al.*, 2003; Smith *et al.*, 2002; Smith *et al.*, 2007; Zhang *et al.*, 2005) as well as body temperature regulation (Mochizuki *et al.*, 2006; Plazzi *et al.*, 2011; Rusyniak *et al.*, 2011; Tupone *et al.*, 2011).

Circadian and sleep-wake state orexin rhythms

Orexin levels measured in cerebrospinal fluid (CSF) vary two-fold during the diurnal cycle with the highest levels in rodents occurring during the dark-active period and the lowest levels during the light-inactive period (Fig. 2) (Desarnaud *et al.*, 2004). In contrast, the firing rates of orexin neurons are correlated with arousal state, being greater in states of higher arousal (Lee *et al.*, 2005; Mileykovskiy *et al.*, 2005). The physiological functions of orexins then may have two state-related aspects: 1) they may vary as sleep-state varies, i.e., with a time constant of minutes and a peak effect during wakefulness, and 2) they may vary in a circadian manner, i.e., with a time constant of hours and a peak effect (in rodents) during the dark, active phase. Thus, wake periods during the dark, active phase of the rodent circadian rhythm would be the time of greatest orexin effects. We need to consider orexin function in physiological control systems in respect to these two state-related activity patterns. In addition, orexins have been proposed to function in stress responses, that of the 'fight or flight' or hypothalamic 'defense response' (Zhang *et al.*, 2006b), the vigilance response (Donadio *et al.*, 2008), and the panic anxiety response (Johnson *et al.*, 2010). Presumably the physiological stress responses would supersede the circadian and sleep-state related activities.

Orexins and Breathing

The regulation of breathing and blood pressure change in ways that parallel the state and circadian variations in CSF orexin levels; and the defense response, attributed to orexin, includes changes in breathing and blood pressure. We shall first consider chemosensitivity, the ventilatory response to changes in CO₂.

Intrinsic chemosensitivity of orexin neurons

Extending earlier work showing that hypothalamic neurons as examined in a slice preparation are themselves responsive to CO₂ as well as hypoxic stimuli (Dillon and Waldrop, 1992), more recently investigators in the Burdakov lab have shown specifically that the orexin neurons themselves are excited by relatively small changes in CO₂ (Fig. 3) (Williams *et al.*, 2007). They used a mouse model with orexin neurons made visible by a targeted green fluorescent protein (GFP) thus facilitating the evaluation of a specific neuron type, here orexin neurons. Neurons so identified were excited by small increases in CO₂ via a leak-like K⁺ channel (Fig. 3). This evidence shows focal and specific CO₂ sensitivity in orexin neurons providing strong support for their role in central chemosensitivity.

Focal acidification of lateral hypothalamus

We have successfully applied the technique of reverse microdialysis of an artificial cerebrospinal fluid (aCSF) equilibrated with high levels of CO₂ to produce focal regions of acidosis (Feldman *et al.*, 2003; Li and Nattie, 2002; Nattie and Li, 2009, 2010). We used this approach to ask whether the site at which focal dialysis is applied participates in central chemoreception by monitoring the effect of the focal acidosis on breathing in conscious rats. By measuring the tissue pH adjacent to the dialysis probe at one site, the retrotrapezoid nucleus (RTN), we established that the degree of focal pH change was like that associated with a ~5 to 6 mm Hg increase in arterial PCO₂ as measured with systemic CO₂ application, which is a mild stimulus (Li and Nattie, 2002). This approach has identified the following as putative central chemoreceptor sites; the RTN, the rostral medullary raphe, the caudal NTS, the LC, the caudal ventral medulla, the pre-Bötzinger complex (da Silva *et al.*, 2010; Hodges *et al.*, 2004a; Hodges *et al.*, 2004b; Nattie, 2011; Nattie and Li, 2009, 2010; Solomon *et al.*, 2000), and the lateral hypothalamus/perifornical region (unpublished results; Ningjing Li, Aihau Li, and E. Nattie). The ventilatory response to focal acidification in the perifornical region of the lateral hypothalamus was more prominent in wakefulness. These observations are complementary to the *in vitro* sensitivity of identified orexin neurons to CO₂ (Williams *et al.*, 2007). This broad distribution of central chemoreceptor sites and their possible sleep-wake state modulation by orexin is summarized in Fig. 4.

Prepro-orexin null mice

The prepro-orexin knockout (KO) mouse is devoid of both orexin A and B and exhibits a phenotype like that of human narcolepsy with cataplexy and abnormal early onset of REM sleep at the beginning of the sleep period (Chemelli *et al.*, 1999). These mice are also moderately obese, have low blood pressure at rest, and have an abnormal fight-or-flight response (Kayaba *et al.*, 2003). Studies of the control of breathing in these mice revealed a remarkable phenotype (Nakamura *et al.*, 2007). The level of ventilation in baseline resting

conditions was not different from that of control mice, although oxygen consumption ($\dot{V}_{O_2}$) was not measured. During NREM and REM sleep, the prepro-orexin KO mice had significantly more apneas, which were defined as an absence of breathing over a period greater than two control respiratory cycle lengths (Nakamura *et al.*, 2007). These were central, not obstructive, apneas. Interestingly, the incidence of sighs and post-sigh apneas did not differ between control and prepro-orexin KO mice. Sighs are periodic deep breaths, which depend on vagal mechanisms, and provide lung inflation, allow surfactant redistribution and prevent atelectasis (Bartlett, 1973). Post-sigh apneas are thought to reflect the inhibitory influence mediated via chemoreceptors of the transient fall in arterial PCO_2 that occurs after the very large breath (Bartlett, 1973). In wakefulness, the arterial PCO_2 was normal in these mice indicating normal alveolar ventilation relative to metabolism. The ventilatory response to mild hypoxia, 15% O_2 , as measured during wakefulness was also unaffected. The big surprise in these mice is a large, significant decrease in the ventilatory response to CO_2 , an effect that is highly arousal state-specific (Kuwaki *et al.*, 2010; Kuwaki *et al.*, 2008; Nakamura *et al.*, 2007). In wakefulness but not NREM or REM sleep, these mice had a ~50% reduction in the ventilatory response to 5% and 10% CO_2 measured as the change in ventilation vs. the inspired CO_2 (Fig. 5). The use of inspired CO_2 as the measure of stimulus intensity is not a true reflection of the stimulus at the sites of chemoreception, which detect brain and/or blood PCO_2 or pH. In this case, the reduced ventilatory response, as measured, would contribute to a higher arterial PCO_2 in prepro-orexin KO mice and to a greater reduction in CO_2 sensitivity had the investigators been able to measure arterial PCO_2 . Of note is the use in this study of 5% and 10% inspired CO_2 , i.e., both a mild and strong stimulus intensity. These prepro-orexin KO mice exhibit two remarkable and seemingly independent respiratory abnormalities; 1) more frequent central apneas in sleep, and 2) a large reduction in the CO_2 response in wakefulness. As discussed by the authors (Kuwaki *et al.*, 2010; Kuwaki *et al.*, 2008; Nakamura *et al.*, 2007), orexin may contribute to the known sleep-wake state alterations in chemoresponse sensitivity (See Fig. 4). They suggest that the greater CO_2 sensitivity present in wakefulness as compared to NREM sleep arises from the orexin contribution. They also have proposed that orexin contributes to the defense response (Kuwaki, 2011; Zhang *et al.*, 2006b; Zhang *et al.*, 2009), a variant of the fight-or-flight response in which external or internal threats to organism well-being induce physiological responses that prepare the organism for active reaction. These two hypothetical explanations for a physiological function of orexins could both be accurate but represent two quite different levels of activation of the orexin system. If so it is an interesting paradigm in which the defense response would represent an exaggeration of the change in arousal between NREM sleep and wakefulness. This would be an approximate opposite of an analogous paradigm in which hibernation would represent an exaggerated NREM sleep state. To determine the conditions under which orexins contribute to the CO_2 response, we can ask what types of activities or stressors, other than wakefulness and activity, induce orexin release? Measurements of rat cerebrospinal fluid orexin A levels in response to a variety of stressors show no effects of short-term immobilization, sleep deprivation or cold exposure, all of which are certainly moderate to severe stressors (Martins *et al.*, 2004). But these investigators observed decreased orexin A levels after long-term immobilization and increased levels after short-term forced swimming (Martins *et al.*, 2004). In this regard it may be useful to try to categorize stresses. For example, as discussed

by (Donadio *et al.*, 2008), the *defense reaction* or the ‘fight-or-flight’ response is associated with stimulation of the medial hypothalamus and periaqueductal gray and changes in cardiorespiratory function related to potential motor activity, e.g., increased cardiac output, blood pressure, and ventilation. In contrast, the *vigilance reaction* is associated with stimulation of the lateral hypothalamus and the ventrolateral periaqueductal gray and changes in cardiorespiratory and motor function related to vigilance and preparation for action, e.g., inhibition of movement, increased attention, increased blood pressure, apnea and bradycardia. These workers argue that the cataplexy of orexin deficiency has considerable overlap with the vigilance reaction. An additional category of stress response is that of panic-anxiety attacks (Johnson *et al.*, 2010). Here in rodent models an anxiety-like state can be induced by systemic infusion of sodium lactate along with chronic inhibition of GABA synthesis in the hypothalamus (Johnson *et al.*, 2010). Silencing of hypothalamic orexin neurons by focal injection of an siRNA for prepro-orexin blocks the panic-anxiety response in this rodent model. The vigilance, fight-or-flight, and panic-anxiety stress responses are similar in many respects and all are linked to orexin.

In contrast to the results obtained in prepro-orexin KO mice, data from humans with narcolepsy and variable orexin deficiency (Baumann and Bassetti, 2005; Peyron *et al.*, 2000; Thannickal *et al.*, 2000) indicate a slightly different phenotype (Han, 2011; Han *et al.*, 2010). Sleep apnea is more prevalent than in controls (Chokroverty, 1986; Han, 2011; Han *et al.*, 2010) as in the mice, but the ventilatory response to CO₂, decreased in prepro-orexin mice, is normal in man while that to hypoxic stimulation, normal in prepro-orexin mice, is reduced in man (Han, 2011; Han *et al.*, 2010). However there are variable phenotypes in different rodent models of orexin deficiency (Hara *et al.*, 2001; Hara *et al.*, 2005; Schwimmer *et al.*, 2010) and the ventilatory response to CO₂ has been tested only in the prepro-orexin KO mice (Nakamura *et al.*, 2007).

Focal OX₁R antagonism in retrotrapezoid nucleus (RTN)

Given the findings of 1) a 50% reduction of the CO₂ response in wakefulness in the prepro-orexin KO mice, 2) the known projections of orexin neurons to the RTN (Nambu *et al.*, 1999; Nixon and Smale, 2007), and 3) the presence of orexin receptors in the RTN (Ciriello and de Oliveira, 2003; Ciriello *et al.*, 2003), we measured ventilation in unanesthetized rats while exposed to air and 7% CO₂ before and after focal microdialysis of an OX₁R antagonist (SB334867; 5 mM) into the RTN region (Fig. 6) (Dias *et al.*, 2009). This treatment produced a significant decrease in the CO₂ response by 30% in wakefulness and 9% in NREM sleep without effect on basal ventilation, body temperature, V_{O₂}, or the frequency of sleep apneas (Dias *et al.*, 2009). Anatomical analysis after completion of the experiment demonstrated correct locations of the dialysis probe tips. The absence of any effect of this focal OX₁R antagonism in the RTN on ventilation at rest breathing air agrees with the results of a more general application via the cerebral ventricles of this antagonist (Deng *et al.*, 2007). Both data sets indicate that orexin does not contribute via OX₁R to the wakefulness drive that distinguishes ventilation in the awake state from that in NREM sleep. The larger effects on the CO₂ response in wakefulness suggest a role for the orexin-RTN pathway via OX₁R in the state-dependent modulation of CO₂ sensitivity. Of interest in this regard is our earlier observation that focal acidification of the RTN also has a greater

stimulatory effect in wakefulness (Li *et al.*, 1999). These complementary sets of data raise the possibility that the RTN functions in central chemoreception as a specific focal CO₂/pH detector that operates predominantly in wakefulness (Nattie, 2011; Nattie and Li, 2010) and is modulated by orexin inputs (Lazarenko *et al.*, 2011).

Focal OX₂R antagonism in the RTN

In experiments similar in design but using dialysis of an OX₂R antagonist (TCS OX 29) into the RTN region, we observed a lower V_{O_2} while the rat was exposed to 7% CO₂ and, in respect to the CO₂ response, a greater increase in by 9% in V_E/V_{O_2} wakefulness and 22% in NREM sleep (Li, Li, and Nattie; Soc for Neurosci, 2011.) The results from both studies suggest that RTN neurons have OX₁R mediated modulation of central chemoreception and OX R mediated modulation of V_{O_2} .

Focal OX₁R antagonism in medullary raphe

The rostral medullary raphe contains a group of serotonergic neurons that are intrinsically chemosensitive *in vitro* (Corcoran *et al.*, 2010; Hodges and Richerson, 2010a, b) and participate in central chemoreception *in vivo* (Corcoran *et al.*, 2010; Hodges and Richerson, 2010a, b; Li *et al.*, 2006; Nattie, 2011; Nattie and Li, 2009, 2010; Ray *et al.*, 2011). The most compelling data in this regard is the recent study in which serotonin neurons specifically are transgenically modified to express a foreign receptor, based on the synthetic G protein-coupled receptor Di, that responds acutely and reversibly to an injected ligand, clozapine-N-oxide (CNO), which hyperpolarizes and ‘silences’ the neurons (Ray *et al.*, 2011). With acute ‘silencing’ of serotonergic neurons these mice demonstrate a 50% reduction in the response to systemic hypercapnia (5% CO₂) (Ray *et al.*, 2011).

Neurons in this rostral medullary raphe region receive orexin containing projections (Nambu *et al.*, 1999; Nixon and Smale, 2007; Tupone *et al.*, 2011) and express OX₁Rs (Hervieu *et al.*, 2001; Marcus *et al.*, 2001). Focal microdialysis of an OX₁R antagonist (SB334867; 5 mM) into the rostral aspect of the medullary raphe in unanesthetized rats decreased the ventilatory response to 7% CO₂ by 16% but only during wakefulness in the dark, active period of the rat diurnal cycle. There was no effect on baseline ventilation, body temperature, or V_{O_2} (Dias *et al.*, 2010). These results complement those obtained with focal inhibition of OX₁Rs in the RTN discussed above (Dias *et al.*, 2009) although the degree of inhibition of the CO₂ response is less with OX₁R antagonism in the rostral medullary raphe (–16%) than in the RTN (–30%). This difference may, in part, reflect the recently described interaction between the medullary raphe and the RTN in respect to chemoreception (Li *et al.*, 2006) in which simultaneous inhibition of both sites had a much greater inhibitory effect on the CO₂ response than did inhibition of each site alone. It is clear that in both cases the effect of OX₁R inhibition was greater in wakefulness, a response consistent with a) the notion that orexin levels in CSF rise during the dark, active period when periods of wakefulness are more prominent in rodents (Desarnaud *et al.*, 2004) and b) the activity of orexin neurons is greater in wakefulness (Lee *et al.*, 2005; Mileyskiy *et al.*, 2005).

Systemic antagonism of both OX₁R and OX₂R

A dual orexin receptor antagonist, Almorexant (ACT-078573, (2R)-2-((1S)-6, 7-dimethoxy-1-[2-(4-trifluoromethylphenyl)-ethyl]-3,4-dihydro-1H-isoquinolin-2-yl)-N-methyl-2-phenyl-acetamide), is available from Actelion Pharmaceuticals Ltd. as an oral agent effective in rat and man (Brisbare-Roch *et al.*, 2007). Oral treatment with Almorexant decreased alertness and increased both REM and NREM sleep in rats, dogs, and man but only in the active period of the circadian cycle (Brisbare-Roch *et al.*, 2007). We have studied the effect of this dual OX₁R antagonist on breathing and chemoreception in unanesthetized rats (Li and Nattie, 2010). We observed no effect on baseline ventilation but a decreased ventilatory response to 7% CO₂ by 26% only in wakefulness in the dark, active period of the circadian cycle (Fig. 7). The CO₂ response in wakefulness in the dark period after Almorexant was similar to that during NREM sleep in the light period in controls (Fig. 7). This suggests the possibility that the increased CO₂ response commonly observed in wakefulness compared to NREM sleep could be due to orexin. We observed the decrease in time spent in wakefulness and increase in time spent in NREM and REM sleep during the dark, active period, as reported (Brisbare-Roch *et al.*, 2007). An unexpected finding was an Almorexant induced decrease in the frequency of sighs and in the prevalence of post-sigh apneas during wakefulness in both the light and dark period and during both wakefulness and NREM sleep in the dark period (Fig. 8) (Li and Nattie, 2010).

Orexin and Long Term Facilitation (LTF)

Ventilatory long term facilitation refers to a prolonged increase in ventilatory activity (phrenic nerve activity in anesthetized animals; breathing or diaphragmatic activity in unanesthetized animals) that is induced by exposure to a small number of acute intermittent hypoxic episodes and involves serotonin mechanistically acting at phrenic motor neurons (Feldman *et al.*, 2003; McGuire *et al.*, 2002; Nakamura *et al.*, 2010; Olson *et al.*, 2001; Terada and Mitchell, 2011). The physiological function of LTF is unclear although it has been hypothesized that it may be a form of physiological ‘learning’ that protects against subsequent hypoxia (Mahamed and Mitchell, 2008). The finding that prepro-orexin KO mice have exaggerated apneas in sleep when orexin neuronal activity is low (Nakamura *et al.*, 2007) raised the question as to the role of orexin in LTF. Surprisingly, prepro-orexin KO mice do exhibit a failure to demonstrate LTF in both sleep and wakefulness (Terada *et al.*, 2008). This study was remarkable in showing very clear LTF in unanesthetized control mice. However, contrary to the investigator’s hypothesis, the prepro-orexin KO mice with an inability to show LTF still had an apnea index similar to controls. In support of a link between orexin lack and apneas the authors speculate on a relationship between an inability to express LTF and an enhanced incidence of sleep apnea in patients with narcolepsy (Chokroverty, 1986). They also point out that some patients with obstructive sleep apnea have low levels of orexin in the plasma (Busquets *et al.*, 2004) suggesting an interaction between orexin levels and the propensity for sleep apnea. These observations add to the panoply of physiological roles that can be attributed to orexin.

Orexins and Blood Pressure

Orexin knock out mice

Prepro-orexin KO mice have a lower than normal blood pressure at rest in wakefulness, an effect that is more pronounced during the dark, active period of the diurnal cycle (Kayaba *et al.*, 2003). Sympathetic pharmacological blockade lowered blood pressure in wild type controls and normalized the difference in blood pressure between wild type and prepro-orexin KO mice indicating a role of decreased sympathetic outflow in the hypotension of the prepro-orexin KO (Kayaba *et al.*, 2003). As discussed below there is evidence of orexinergic innervation of neurons in brainstem sites involved in the regulation of sympathetic activity, e.g., the RVLM (Chen *et al.*, 2000; Ciriello *et al.*, 2003; Dun *et al.*, 2000) via effects mediated by OX₂Rs (Huang *et al.*, 2010). Such orexin-mediated sympathetic excitation also seems to participate in the defense response activated by hypothalamic disinhibition (Kayaba *et al.*, 2003) and in the response to acute hemorrhage (Jochem *et al.*, 2006).

There are numerous papers attributing a role for orexin in the defense response, an active strategy accompanied by excitation of sympathetic output with increased blood pressure and heart rate also labeled as the 'fight-or-flight' response (Kuwaki, 2011; Zhang *et al.*, 2006b). Prepro-orexin KO mice compared to wild type have a lessened response in terms of increased blood pressure, heart rate and respiration when tested by disinhibition of the perifornical region by focal injection of bicuculline, a GABA_A receptor antagonist, in anesthetized mice or by exposure of unanesthetized mice to a resident intruder test (Fig. 9) (Kayaba *et al.*, 2003; Zhang *et al.*, 2006b). Similar responses to hypothalamic disinhibition in anesthetized animals and, in this case, air jet stress in unanesthetized animals, were observed in orexin/ataxin-3 transgenic mice with 99% loss of orexin neurons (Zhang *et al.*, 2006a). This study also showed a reduced skeletal muscle vasodilation and a loss of baroreceptor resetting in the mice with absence of orexin neurons (Zhang *et al.*, 2006a). As mentioned earlier, orexin also seems essential for other stress responses, i.e., that of vigilance (Donadio *et al.*, 2008) and panic-anxiety (Johnson *et al.*, 2010).

Brainstem injections of orexins and cardiovascular effects

The administration of orexins via the cerebral ventricles increases mean arterial pressure (MAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and plasma catecholamine levels in conscious rats indicating that central orexins can affect sympathetic nerve activity and cardiovascular function. (Shirasaka *et al.*, 1999). In conscious rabbits, intracerebroventricular injection, but not intravenous injection, of orexin A also produced dose-related increases in MAP and RSNA as well as plasma epinephrine, vasopressin and glucose concentrations, effects that were blocked by pretreatment with an alpha adrenergic receptor antagonist. These results suggest that intracerebroventricular orexin A acts in the central nervous system and activates sympathoadrenal outflow, resulting in increases in arterial pressure and plasma glucose levels in conscious rabbits. (Matsumura *et al.*, 2001). Similar results have been reported by others in conscious rats. Both orexin A and orexin B given via the cerebral ventricles into conscious, unrestrained rats stimulated significant long lasting elevations in MAP (Samson *et al.*, 1999). Injections of orexins A or B via the cisterna magna also increased MAP and HR in urethane-anesthetized rats, with orexin A

being more effective in this regard. Microinjection of orexin A into the rostral ventrolateral medulla also increased MAP and HR suggesting an effect on medullary neurons. (Chen *et al.*, 2000).

Chronic intracerebroventricular administration of orexin A, but not orexin B, for 14 days in conscious rats elicited a significant increase in systolic blood pressure on the third day (+16 mm Hg) that returned to the baseline levels at day 14. There was no effect on urinary catecholamine excretion or on food and water intake. These results suggest that central orexin A participates in the short-term regulation of blood pressure but not in the long-term regulation of blood pressure (Lin *et al.*, 2002).

Orexin-containing neurons in the lateral hypothalamus also project to the intermediolateral cell column (IML) of the thoracolumbar spinal cord and intrathecal injection of orexins A or B at different doses increased MAP and HR in urethane-anesthetized rats, effects that were diminished by systemic treatment with antagonists of alpha and beta-adrenergic receptors (Antunes *et al.*, 2001). These effects involved orexin receptors on sympathetic preganglionic neurons as identified by antidromic activation in studies of spinal cord slices from 12- to 16-day old rats. These spinal cord effects were confirmed and extended in a more recent study in urethane-anaesthetized, vagotomized and artificially ventilated male Sprague-Dawley rats (Shahid *et al.*, 2011). Here intrathecal orexin A caused a prolonged dose-dependent sympathoexcitation with a pressor response and tachycardia, effects that were attenuated by the OX₁R antagonist SB334867. Intrathecal orexin A also significantly increased baroreflex sensitivity, the amplitude of the pressor response and the effect of hypoxia on sympathetic nerve activity. Thus, activation of orexin receptors in the rat spinal cord can augment sympathetic outflow, alter cardiorespiratory function and differentially modulate sympathetic reflexes. (Shahid *et al.*, 2011).

Experiments using focal injections of orexins within the brainstem have identified other sites that participate in blood pressure effects. Orexin-immunoreactive axons have been demonstrated within the NTS suggesting that it may be a site at which orexin acts to influence cardiovascular control. Large microinjections (500 nl; 10⁻⁹M) of orexin A into the commissural or medial NTS of urethane anesthetized rats increased blood pressure and heart rate in a dose-dependent manner. Microinjection of orexin B into NTS also increased blood pressure suggesting specific actions at the OX₂R (Smith *et al.*, 2002). In contrast, smaller injections at lower doses (10–20 nl; 0.5–5.0 pmol) within the lateral and medial subnuclei of the caudal NTS elicited depressor and bradycardic effects and significantly potentiated the reflex bradycardia induced by activation of arterial baroreceptors (de Oliveira *et al.*, 2003). These highly specific focal injection effects suggest that orexin A in the NTS can activate neuronal circuits that increase vagal activity to the heart, inhibit sympathetic activity to the heart and vasculature, and alter the excitability of NTS neuronal circuits that reflexly control the circulation (de Oliveira *et al.*, 2003). While it is not precisely clear why one NTS study reported pressor and tachycardic effects of focally applied orexin while a second study reported the opposite, it seems reasonable to ascribe some of the difference in results to injection volume and the site of injection (de Oliveira *et al.*, 2003). In a similar study, mapping of the distribution of orexin receptor-immunoreactivity showed much of it to be localized in the region of the nucleus ambiguus. Microinjections of orexin into this region

elicited a dose-related decrease in HR with little or no change in MAP and significantly potentiated the reflex bradycardia elicited by activation of the baroreflex (Ciriello and de Oliveira, 2003). The interpretation of these data is that orexin in the nucleus ambiguus alters the excitability of central circuits that reflexly control the circulation through the activation of vagal preganglionic cardioinhibitory neurons. (Ciriello and de Oliveira, 2003). Microinjections of orexin A directly into the subfornical organ (SFO), a circumventricular structure implicated in autonomic control, decreased blood pressure and heart rate via a reduction in sympathetic activity in urethane anaesthetised male Sprague-Dawley rats (Smith *et al.*, 2007). However, it remains difficult to understand the physiological significance of the depressor and bradycardic effects of these SFO, NTS and nucleus ambiguus microinjections given the wide array of results supporting an excitatory (pressor, tachycardic, increased sympathetic activity) role for orexins. If the orexin system when strongly activated participates in the defense response, why are there brainstem pathways that can induce depressor and bradycardiac responses? It may simply be that these focal orexin injection studies uncovered hindbrain orexin pathways that subserve less general functions, e.g., aspects of digestion (de Oliveira *et al.*, 2003). Or it could be that the presence of anesthesia alters the cardiovascular responses to orexin injections at these sites.

Microinjections of orexin into the rostral ventromedial medulla (RVMM), within the magnocellular reticular nucleus and the adjacent nucleus paragigantocellularis lateralis, increased HR, with little effect on blood pressure. One interpretation is that orexin in the RVMM region activates neuronal circuits that both inhibit vagal activity and increase sympathetic activity to the heart, and that it alters the excitability of central circuits that reflexly control the circulation (Ciriello *et al.*, 2003).

Immunohistochemical studies have demonstrated the presence of orexin A-immunoreactive fibers in the rostral ventrolateral medulla (RVLM) in close proximity with TH-immunoreactive neurons, some of which may be barosensitive, bulbospinal neurons. Microinjection of orexin A into the RVLM increased MAP and HR in awake rats (Huang *et al.*, 2010; Machado *et al.*, 2002). *In vitro* recordings of neuronal activity within the RVLM showed that orexins depolarized RVLM neurons predominantly via OX₂Rs (Huang *et al.*, 2010). Orexins directly excite RVLM neurons, which include bulbospinal vasomotor neurons, and regulate cardiovascular function by increasing sympathetic outflow to heart and blood vessels mainly via the OX₂R, with a smaller contribution from the OX₁R (Huang *et al.*, 2010; Machado *et al.*, 2002).

Orexin deficiency and blood pressure

In 15 orexin-deficient male narcoleptic patients compared to controls matched for age and body mass, spectral power analysis revealed greater heart rate and blood pressure variability, suggesting reduced sympathetic tone (Fronczek *et al.*, 2008).

In orexin-ataxin3 transgenic mice with genetic ablation of hypocretin neurons (TG) and orexin gene knock-out mice (KO), blood pressure is higher than in wild-type controls (WT) during non-rapid eye movement sleep (NREM) and rapid eye movement sleep (REM), while their blood pressure did not differ significantly from controls during wakefulness. Thus the decrease in blood pressure that occurs between sleep and wakefulness was significantly

blunted in TG and KO with respect to WT. Chronic lack of orexin signaling may affect blood pressure adversely among wake-sleep states. (Bastianini *et al.*, 2011). This possible sleep-state related role for orexin in the regulation of blood pressure is of particular interest given the evidence that the blood pressure during sleep and the difference in blood pressure between sleep and wakefulness carry prognostic information.

Orexins and Thermoregulation

An altered ability to thermoregulate has been suggested to be present in patients with narcolepsy, who often have lowered body temperature (Plazzi *et al.*, 2011). While altered thermoregulation has not been reported in mouse models of orexin deficiency, recent results in rodents indicate a specific neural pathway from hypothalamic regions rich in orexin neurons to brainstem sites, the raphe pallidus and parapyramidal region, known to be involved in brown adipose tissue (BAT) thermogenesis (Tupone *et al.*, 2011). This study further showed that direct injection of orexin in these medullary regions increased BAT thermogenesis via increased BAT sympathetic outflow, observations that were duplicated by direct chemical excitation of hypothalamic sites (Tupone *et al.*, 2011). In these experiments the response to orexinergic activation BAT thermogenesis depended on the baseline state of the system being elicited when the animal was in a cooled state (Tupone *et al.*, 2011).

Development of the orexin system

The postnatal development of the orexin system has been described in rats using *in situ* hybridization for the mRNAs of prepro-orexin, the OX₁R and the OX₂R as well as immunohistochemistry for orexin A and orexin B (Yamamoto *et al.*, 2000). These striking data can be summarized as in Fig. 10, which shows that levels of prepro-orexin mRNA in the lateral hypothalamus are low from P0 to P10 then increase dramatically at P15 reaching adult-like levels at P20 (Yamamoto *et al.*, 2000). It is at this age in development that orexin might participate to a greater extent in sleep regulation. This postnatal development of orexin, and with it, of a presumed enhanced ability to manifest physiological responses to stress, e.g., increased CO₂, fear, is of particular interest since it occurs at an age that is approximately that at which the peak incidence of the sudden infant death syndrome occurs (Kinney *et al.*, 2009; Kinney and Thach, 2009). Recently, based on analysis of young type 1 diabetes patients who die suddenly while asleep, an orexin malfunction has been proposed, and hypothetically extended, to the sudden infant death syndrome (Parekh, 2009a, b). The postnatal maturation of orexin would certainly lend support to this conjecture. Of additional interest is the observation that serotonergic neurons also become responsive to CO₂/pH at about this same developmental age in rodents (Corcoran *et al.*, 2010; Richerson, 2004) and deficiencies in serotonin are postulated as present in the sudden infant death syndrome (Kinney *et al.*, 2009; Kinney and Thach, 2009).

Concluding remarks

Orexin can be thought of as an activity related hormone. It functions in daily routine life to enhance or enable cardiorespiratory function when one is awake, especially in the active period of the circadian cycle. It also serves to further enhance cardiorespiratory function when one is stressed as in the fight-or-flight response and the panic-anxiety syndrome.

Orexin was discovered in relation to a remarkable phenotype ascribed to its absence; narcolepsy. It seems reasonable to suggest that disorders may yet be ascribed to excess orexin.

Acknowledgments

The authors gratefully acknowledge the generous support of the National Institutes of Health through NIH Program Project grant HD36379 (NICHD, PI, H. C. Kinney and Project 2 PI, E. Nattie) and NIH grant HL28066, (PI, E. Nattie). Andrea Corcoran helped with editing.

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Fig. 1. Schematic showing the broad hindbrain connections of orexin neurons

The figure summarizes how orexins may modulate both state-dependent behavioral regulation and stress-induced responses (left side of figure) and cardiorespiratory regulation (right side of figure). Known connections between orexinergic neurons in the hypothalamus and selected brain nuclei involved in cardiorespiratory regulation are depicted (thick lines). Arrows indicate a probable excitatory connection and circles indicate an inhibitory connection. Connections shown in thin lines are either direct or indirect. Abbreviations: AMG, amygdala; BNST, bed nucleus of the stria terminalis; DR, dorsal raphe; LC, locus coeruleus; MLR, medullary locomotor region; MR, medullary raphe; NTS, nucleus tractus solitarius; PAG, periaqueductal gray; PVN, paraventricular nucleus; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla where sympathetic premotor neurons are located; SCN, suprachiasmatic nucleus; TMN, tuberomammillary nucleus; VLPO, ventrolateral preoptic nucleus. Figure taken from (Kuwaki *et al.*, 2010) by permission.

Fig. 2. The diurnal rhythm of cerebrospinal fluid (CSF) orexin (hypocretin-1; HCRT-1) concentrations in rats of different ages

Data from young, middle-aged, and old F344 rats are shown. Rats were entrained to a 12-hour lights-on/lights-off cycle. In each rat, CSF was extracted at 4-hour intervals, and radioimmunoassay measured HCRT-1. The data represent mean $\pm$ SEM orexin (HCRT-1) concentrations at 4-hour intervals for each group of rats. Each data point represents orexin (HCRT-1) levels during the preceding 4-hour interval. Asterisks denote significant difference compared to young rats. The black moon icon represents the lights-off period. One young rat died during the CSF extraction and was excluded from study. Figure taken from (Desarnaud *et al.*, 2004) by permission.

Fig. 3. Acidosis directly stimulates orexin neurons

Effects of H⁺ and CO₂ on orexin and nonorexin cells in brain slices of the lateral hypothalamus of transgenic mice that express GFP only in orexin neurons. Current-clamp whole-cell recordings with K-gluconate intracellular solution. To monitor membrane conductance, cells were injected with periodic hyperpolarizing current pulses. The size of the resulting downward voltage deflections is inversely proportional to membrane conductance. (A) Effect of CO₂-induced changes in pH_e on an orexin neuron. Firing rate was 0.8 ± 0.3 Hz in 5% CO₂ and 3.7 ± 0.4 Hz in 10% CO₂ ($n = 5$; $P < 0.01$, two-tailed t test). (B) Effect of HEPES-buffered changes in pH_e on an orexin neuron. Firing rate was 4 ± 0.6 Hz at pH 6.9 and 0.5 ± 0.2 Hz at pH 7.4 ($n = 15$; $P < 0.001$, two-tailed t test). (C) No effect of changes in pH_e on firing of a nonorexin neuron. Figure taken from (Williams *et al.*, 2007) by permission.

System model: chemoreception in **WAKEFULNESS**:
unshaded areas are CO₂ responsive by focal acidification in vivo

Fig. 4 (top)

System model: chemoreception in **NREM SLEEP**:
unshaded areas are CO₂ responsive by focal acidification in vivo

Fig. 4 (bottom)

Fig. 4. Schematic model for central chemoreception in wakefulness (top) and NREM sleep (bottom) that represents a current working hypothesis

The unshaded areas represent sites at which focal acidification by dialysis with artificial cerebrospinal fluid (aCSF) equilibrated with high CO₂ produced an increase in ventilation in wakefulness. The areas in gray represent sites at which we anticipate a response to focal acidification in wakefulness. Solid lines show established functional connections related to chemoreception, e.g., dialysis of an orexin receptor 1 (OX₁R) antagonist at the retrotrapezoid nucleus (RTN) decreased the CO₂ response in wakefulness. Dashed lines show likely connections that remain to be established. That linking the caudal medullary raphe (MR) to the pre-Bötzinger complex (PBC) reflects observations obtained in a slice preparation (Ptak *et al.*, 2009). The gray line between the caudal MR and the RTN reflects a CO₂-linked connection, i.e., focal acidification of the caudal MR enhances the response to focal acidification of the RTN. LH, a designation that includes orexin neurons in lateral

hypothalamus, dorsomedial hypothalamus, and perifornical area; LC, locus ceruleus; CB, carotid body; NTS, nucleus tractus solitarius; VLM, ventrolateral medulla. See text for references. In NREM sleep (4B), orexinergic excitation of CCR is absent. The main sources of currently known chemoreception at the stimulus levels obtained by the in vivo focal dialysis approach are the caudal NTS, rostral MR, and CB. The role of the LC in sleep is unknown. It is not known if there is any synergy between CCR sites during sleep. Taken from (Nattie and Li, 2010) by permission.

Fig. 5. Prepro-orexin null mice have a reduced ventilatory response to CO₂ in wakefulness
Hypercapnic responses of respiratory minute volume in wild-type (WT) mice and prepro-orexin knockout mice (ORX-KO) during quiet awake (upper left panel) and sleep (upper right panel) periods. (Lower panel). Hypercapnic responses are shown as the slope of the relationship between inspired CO₂ concentration and respiratory minute volume. Data are presented as means $\pm$ SEM of 5 WT mice and 5 ORX-KO mice. Abbreviations: SWS, slow-wave sleep; REM, rapid-eye-movement sleep. Figure taken from (Kuwaki *et al.*, 2008) by permission.

Fig. 6. Focal inhibition of OX₁Rs in the retrotrapezoid nucleus (RTN) inhibits the CO₂ response in wakefulness

Effects of dialysis of vehicle (filled circles; $n = 6$) and an OX₁R antagonist (SB334867; 5 mM; open circles; $n = 6$) into the RTN on ventilation ($\dot{V}_E$), tidal volume (V_T) and frequency (f) while the rats were breathing air and 7% CO₂ during wakefulness (left) and NREM sleep (right). Mean $\pm$ S.E.M. values are shown. Indicates values that are significantly different comparing vehicle to SB-334867 treatment during 7% CO₂ breathing ($P < 0.01$ *post hoc* comparison). Figure taken by permission from (Dias *et al.*, 2009).

Fig. 7. The effects of systemic treatment with a dual orexin receptor antagonist, almorexant, (Almxt) on ventilation and the CO₂ response

In the light and dark periods, ventilation was significantly higher in CO₂ in both the control and Almxt treatment groups in all three states (wakefulness, NREM, REM) but there were no significant effects between control and Almxt treatment (RM ANOVA with state and gas as categorical variables). In the dark period, there was a significant difference between the ventilation breathing 7% CO₂ in control compared to Almxt treatment in the awake state ($P < 0.05$; Tukey). The dashed line shows the level of response to 7% CO₂ in wakefulness in the dark period, the greatest response. Note that in controls the level of response during NREM sleep in the light period is much less and similar to the response after Almxt in the awake dark period. Figure used by permission from (Li and Nattie, 2010).

Fig. 8. The effects of systemic treatment with a dual orexin receptor antagonist, almorexant, (Almxt) on the frequency of sighs and both post-sigh (A) and spontaneous (C) apneas in conscious rats

A typical sigh with post-sigh apnea is shown in *B*. In the light period, Almxt treatment significantly decreased the number of sighs and post-sigh apneas in wakefulness ($P < 0.05$, Tukey). In the dark period, Almxt significantly decreased number of sighs and post-sigh apneas in both wakefulness and NREM sleep ($P < 0.05$; Tukey). For spontaneous apneas, there was no significant treatment effect nor was there any interaction by state. Figure used by permission from (Li and Nattie, 2010).

Fig. 9. Orexin is required for the cardiovascular and behavioral responses during the resident–intruder test

Shown are results from radio telemeter-indwelled freely moving wild-type (WT) and orexin knockout (KO) mice. The presence of an intruder is indicated by the horizontal solid bar. Right side panels are the changes in blood pressure, heart rate, and activity expressed as area under the curve (AUC) during five minutes when an intruder was present in the same cage. Data are presented as mean $\pm$ SEM; 6 mice in each genotype. * $P < 0.05$ vs. WT mice. Figure used by permission from (Zhang *et al.*, 2006b).

Fig. 10. The postnatal development of prepro-orexin mRNA expression in neurons of the lateral hypothalamus
 (A) The *prepro-orexin* mRNA from postnatal days 0 to 10. (B) The *prepro-orexin* mRNA from postnatal days 10 to 25. Values represent the mean $\pm$ SEM. ($n=53$). *** $P = 0.01$ compared with postnatal day 10 in (A) and (B). Figure used by permission from (Yamamoto *et al.*, 2000).