

RAPID REPORT

Antagonism of rat orexin receptors by almorexant attenuates central chemoreception in wakefulness in the active period of the diurnal cycle

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Central chemoreception, the highly sensitive ventilatory response to small changes in CO₂/pH, involves many sites. Hypothalamic orexin neurons are CO₂ sensitive *in vitro*, prepro-orexin knockout mice have a reduced CO₂ response prominently in wakefulness, and focal antagonism of the orexin receptor 1 (OX₁R) in two central chemoreceptor sites, the retrotrapezoid nucleus (RTN) or the medullary raphé, results in a reduction of the CO₂ response predominately in wakefulness (–30% and –16%, respectively). Here we hypothesize that acute and selective inhibition of both orexin receptors (OX₁R and OX₂R) at all central locations by an orally administered dual orexin receptor antagonist, almorexant, will substantially attenuate the CO₂ response in a vigilance-state- and diurnal-cycle-dependent manner. We found that almorexant attenuated the CO₂ response by 26% only in wakefulness during the dark period of the diurnal cycle to a level observed during NREM sleep in the light period in controls suggesting that the sleep–wake difference in the CO₂ response can be in large part attributed to orexin. Almorexant also decreased wakefulness and increased NREM and REM sleep during the dark period, as previously reported, and unexpectedly decreased the number of sighs and post-sigh apnoeas during wakefulness in both the light and the dark period and during both wakefulness and NREM sleep in the dark period. The results support our hypothesis that the orexin system participates importantly in central chemoreception in a vigilance-state- and diurnal-cycle-dependent manner and indicate a role for orexin in the important process of sighing.

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Abbreviations aCSF, artificial cerebrospinal fluid; Almxt, almorexant; EEG, electroencephalogram; EMG, electromyogram; *f*, breathing frequency; NREM, non-rapid eye movement; NTS, nucleus of the solitary tract; ORX-KO, prepro-orexin knockout; OX₁R, orexin receptor-1; OX₂R, orexin receptor-2; REM, rapid eye movement; RTN, retrotrapezoid nucleus; RVLm, rostral ventrolateral medulla; \dot{V}_E , ventilation; \dot{V}_{O_2} , oxygen consumption; V_T , tidal volume; T_b , body temperature.

Introduction

The orexins consist of two neuropeptides, orexin A (hypocretin-1) and orexin B (hypocretin-2), both derived from prepro-orexin, which is expressed in the orexin neurons (de Lecea *et al.* 1998; Sakurai *et al.* 1998) exclusively located in the lateral and dorsal hypothalamus and perifornical region (Peyron *et al.* 1998; Sakurai *et al.* 1998). Orexin neurons have extensive projections throughout the central nervous system including basal forebrain, limbic structures and brainstem (Nixon & Smale, 2007; Tsujino & Sakurai, 2009) and they receive

cholinergic, serotonergic and GABAergic inputs from numerous brain nuclei (Peyron *et al.* 1998; Tsujino & Sakurai, 2009). The discharge of orexin neurons is synchronized with arousal states, being greater with increased arousal (Lee *et al.* 2005; Mileykovskiy *et al.* 2005), but the levels of orexin in cerebrospinal fluid (CSF) vary almost two-fold during the circadian cycle in rodents, monkeys and humans, with levels being highest at the end of the dark/active period and lowest toward the end of the light/inactive period (Yoshida *et al.* 2001; Desarnaud *et al.* 2004). There are two G-protein-coupled orexin receptors, orexin receptor 1 (OX₁R) and 2 (OX₂R) both of which are

widely distributed in the central nervous system (Sakurai *et al.* 1998; Tsujino & Sakurai, 2009), which indicates orexin participation in a variety of functions including the sleep–wake cycle, feeding and neuroendocrine system.

Our focus is on the role of orexin in the control of breathing and central chemoreception in different arousal states examined in both the dark/active and light/inactive periods of the diurnal cycle. Recent work supports the hypothesis that orexin plays a role in the control of breathing and in chemoreception. Orexin projections and receptors are found in brainstem regions involved in the control of breathing including the rostral ventrolateral medulla (RVLM), medullary raphé, locus coeruleus, Kölliker–Fuse nucleus, nucleus of the solitary tract (NTS), dorsal vagal motor nucleus, hypoglossal motor nucleus and phrenic motor nuclei (Marcus *et al.* 2001; Young *et al.* 2005; Nixon & Smale, 2007; Kuwaki, 2008; Tsujino & Sakurai, 2009).

In anaesthetized and vagotomized rats, microinjection of orexin A into the RVLM or phrenic nuclei increased the amplitude of diaphragm EMG activity without affecting frequency (Young *et al.* 2005), while in a perfused rat brainstem preparation, injection of orexin B into the pontine Kölliker–Fuse nucleus increased respiratory frequency and augmented preinspiratory hypoglossal nerve activity (Gestreau *et al.* 2008). *In vitro*, orexin neurons increase their discharge when stimulated by CO₂/H⁺ (Williams *et al.* 2007). *In vivo*, high CO₂ (10% CO₂ for 3 h) significantly increased c-fos expression in the orexin neurons in the perifornical region and the dorsomedial hypothalamus (Sunanaga *et al.* 2009). Also prepro-orexin knockout (ORX-KO) mice, with a deficiency of both orexin A and B, have a significantly attenuated hypercapnic ventilatory response (CO₂ response) in wakefulness but not in sleep, a defect that is partially restored by injection of orexin A and B via the cerebral ventricles (Nakamura *et al.* 2007; Kuwaki, 2008). Orexin-deficient mice also showed frequent spontaneous apnoeas in both NREM and REM sleep (Nakamura *et al.* 2007), and lack of ventilatory long-term facilitation induced by repetitive intermittent hypoxia (Terada *et al.* 2008).

In previous studies we have shown that, in the conscious rat, focal antagonism of the OX₁R in two central chemoreceptor regions, the RTN or the medullary raphé, by focal dialysis of an OX₁R antagonist resulted in a reduction of the CO₂ response predominately in wakefulness (Dias *et al.* 2009*a,b*). In this study we hypothesize that acute inhibition of both OX₁R and OX₂R at all central locations by an oral dual orexin receptor antagonist (almorexant) will significantly attenuate the CO₂ response in a vigilance-state- and diurnal-cycle-dependent manner. We administered either almorexant, a dual orexin receptor antagonist, or a vehicle control, in the same rats studied while breathing air and 7% CO₂ during wakefulness, NREM and REM sleep

in both the dark/active and light/inactive period of the diurnal cycle.

Methods

Ethical approval

All animal experimentation and surgical protocols were within the guidelines of the National Institutes of Health for animal use and care and the American Physiological Society's *Guiding Principles in the Care and Use of Animals* and approved by the Institutional Animal Care and Use Committee at the Dartmouth College Animal Resource Center. The authors have read and complied with guidelines for research in rodents outlined for *The Journal of Physiology* and UK regulations (Drummond, 2009).

Animals

A total of six adult male Sprague–Dawley rats (250–350 g) were used for the experiments and they were individually housed in a light- and temperature-controlled environment. Food and water were provided *ad libitum*. The general methods are those in common use in our lab (Nattie & Li, 2000; Li *et al.* 2006). Below they are described in brief. At the conclusion of the experiments, the rats were killed with an overdose of sodium pentobarbitone injected intraperitoneally (>75 mg kg⁻¹).

Diurnal cycles

All the rats are housed in 12 h light–12 h dark cycle. In order to efficiently study the same rats in both the light/inactive cycle and the dark/active cycle during the investigator's daylight hours, two different light settings were used for this study: (1) 07:00 h lights on and 19:00 h lights off for study in the morning during the early period of the light cycle; and (2) 12:00 h lights off and 00:00 h lights on for study in the afternoon during the early period of the dark cycle. The same rats were randomly assigned to either light cycle first. When both the control and drug treatment experiments were completed in the 1st assigned light cycle, the rats were moved to the opposite light cycle and acclimatized for at least 7 days before the second set of the experiments was conducted.

Surgery

All the rats were surgically implanted with EEG and EMG electrodes along with a telemetric temperature probe. The detailed surgical procedures have been described in our earlier publications (Nattie & Li, 2000; Li *et al.* 2006). In brief, sterile surgery was performed under general

anaesthesia induced with intramuscular administration of ketamine (100 mg kg^{-1}) and xylazine (15 mg kg^{-1}). Three EEG electrodes were screwed into the skull and two EMG electrodes were sutured onto the dorsal neck muscles, and all the electrode wires were connected to a six-prong plastic pedestal. A sterile telemetry temperature probe (TA-F20, Data Sciences, St Paul, MN, USA) was placed in the abdominal cavity. The incision was closed, and the animal was allowed to recover for at least 7 days. Following the surgery, the animals received two doses of buprenorphine (0.05 mg kg^{-1}) for post-surgical discomfort. The first dose was given at the conclusion of the surgery and the second 6–12 h later.

Dual orexin receptor antagonist

Almorexant (ACT-078573, (2*R*)-2-[(1*S*)-6,7-dimethoxy-1-[2-(4-trifluoromethylphenyl)-ethyl]-3,4-dihydro-1*H*-isoquinolin-2-yl]-*N*-methyl-2-phenyl-acetamide), an OX_1 – OX_2 receptor antagonist, is a tetrahydroisoquinoline derivative optimized by Actelion Pharmaceuticals Ltd. It is an orally available, potent, selective and competitive orexin receptor antagonist that is active at both rat and human OX_1 R and OX_2 R. It inhibited the increase in intracellular Ca^{2+} induced by 10 nM human orexin-A in Chinese hamster ovary cells overexpressing the rat or human OX_1 and OX_2 receptors (Brisbare-Roch *et al.* 2007). Almorexant was supplied generously by Actelion Pharmaceuticals, Ltd. An oral gavage was made by dissolving almorexant (300 – 400 mg kg^{-1}) in 0.25% methylcellulose solution in water with 0.25% methylcellulose solution in water alone used as the vehicle control. Each rat received either almorexant gavage or vehicle control gavage 2 h prior to any experimental recording.

Ventilation, oxygen consumption and temperature measurement

The methods used to measure the ventilation, body temperature and oxygen consumption (\dot{V}_{O_2}) were those in common use in our lab (Nattie & Li, 2000; Li *et al.* 2006). In brief, to measure ventilation in conscious rats, we used the whole body plethysmograph technique. The volume of the plethysmograph was 7.6 l with a 3.5 l top to protect the head pedestal. It is connected to a similarly sized reference chamber by a high resistance port. Analog output from the pressure transducer was sampled at 150 Hz and converted into a digital signal by a computer using the DATAPAC 2K2 system (RUN Technologies, Laguna Hills, CA, USA). The rates of inflow and outflow to the plethysmograph were balanced to one another with a flow meter such that flow was maintained at $\geq 1.4 \text{ l min}^{-1}$ and the plethysmograph remained at

atmospheric pressure. CO_2 and O_2 fractions were sampled from the outflow line at $\sim 100 \text{ ml min}^{-1}$ by the CO_2 and O_2 gas analyser (Applied Electrochemistry). To calibrate the plethysmograph before each experiment, we obtained pressure data from four 0.3 ml air injections made with a 1 ml syringe. Breath-by-breath analysis was performed with the pressure deflections and the respiratory cycle time for each breath. Tidal volume (V_T) was calculated and breathing frequency (f_R) per breath to estimate ventilation (\dot{V}_E) per breath. Ventilatory parameters are expressed as mean values for quiet wakefulness, NREM and REM sleep during room air or 7% CO_2 conditions. Oxygen consumption (\dot{V}_{O_2}) was calculated by application of the Fick principle, using the difference in O_2 content between inflow air and outflow air and the flow rate as it was described previously (Nattie & Li, 2000; Li *et al.* 2006). It is important to note in the context of this study that our measure of \dot{V}_{O_2} is not a breath-by-breath measure but an average of many minutes of breathing as the time course of the expired gas measured reflects plethysmograph chamber size and the flow rate through the chamber as well as animal utilization. Core body temperature is measured continuously and recorded every 10 min using the signal from the telemetric temperature probe within the abdomen. The temperature of the experimental chamber is measured using a thermometer within the plethysmograph.

Sighs, post-sigh apnoeas and spontaneous apnoeas

Apnoea is defined as a respiratory pause or cessation lasting greater than 800 ms which represents at least two missed breaths (Stephenson & Horner, 2006). Apnoeas are divided into two categories: post-sigh apnoeas, in which the apnoeic interval occurred within 5 s following a 'sigh' or augmented breath (breath with tidal volume (V_T) more than 50% greater than those of preceding breaths), and spontaneous apnoeas in which the apnoea was not preceded by an augmented breath. Occasionally, sighs were observed without apnoea (Monti *et al.* 1995; Stephenson & Horner, 2006).

Determination of vigilance state

Raw EEG and EMG outputs from the skull and skeletal muscle electrodes were sampled at 150 Hz, filtered at 0.3–70 and 0.1–100 Hz, respectively, and were acquired continuously using the DATAPAC system. The vigilance states were determined by analysis of EEG and EMG records as previously described (Nattie & Li, 2000; Li *et al.* 2006). In brief, a fast Fourier transform was performed on the EEG and EMG signal at 4.0-s-long epochs using SleepSign software (Kissei Comtec Co. Ltd, Japan). The vigilance states were categorized as wakefulness, non-REM

(NREM) and REM sleep. The duration and percentage of time the animal spent in each state during the experimental hours were also analysed by SleepSign.

Statistics

We applied SigmaStat software for our statistical analysis. For the evaluation of treatment effects on \dot{V}_{O_2} and body temperature we applied a repeated measures (RM) ANOVA separately to the light/inactive and dark/active period data with application of the Tukey *post hoc* test when appropriate. For the analysis of treatment effects on arousal we categorized vigilance states into wakefulness, NREM and REM sleep, and evaluated the treatment effect as a RM separately within the light and dark periods,

applying a Tukey *post hoc* test as appropriate. For analysis of ventilation, we applied a RM ANOVA for a treatment effect separately in the light and dark periods with state and inspired gas as categorical variables, applying a Tukey *post hoc* test as appropriate. For the analysis of the treatment effect on the numbers of sighs, apnoeas following sighs and apnoeas unrelated to sighs we applied a RM ANOVA separately in the light and dark circadian periods only during air breathing with arousal state as the categorical variable. We ask if almorexant treatment affects these apnoea-related measures within each circadian period as a function of arousal state.

Experimental protocol

All six animals were studied in both the dark/active and light/inactive periods with both vehicle control and almorexant treatments. To study each animal in all these conditions two different diurnal cycles were used as described above: (1) for study in the light/inactive period, the animals were administered one dose of vehicle control or almorexant between 07:30–08:00 h, and the data collection started 2 h later; (2) for study in the dark/active period, the animals were administered one dose of vehicle control or almorexant between 11:30–12:00 h, and the data collection started 2 h later. Once both the vehicle control and almorexant treatment experiments for one light cycle were completed the rats were then moved into the other light cycle and acclimatized for at least 7 days before starting another set of the experiments. We allowed a minimum of 7 days recovery after almorexant and a minimum of 2 days for the vehicle control.

After gavage, the animals were placed into the whole body plethysmograph and allowed to recover from gavage and acclimatized for a 2 period prior to the experiment. All the data were collected continuously starting from 2 h post gavage. To obtain sufficient data from each vigilance state including wakefulness, NREM and REM sleep, all the animals were exposed to 1 h of air and followed by 1 h of 7% CO₂ (CO₂ response). We find that the 1 h time of exposure and measurement allows reasonable evaluation of ventilation in both sleep and wakefulness while minimizing the total exposure time to 7% CO₂.

Results

Metabolic rate

Compared to control, almorexant-treated rats had a significantly lower \dot{V}_{O_2} during air breathing in the dark, active period ($P < 0.02$, RM ANOVA applied within the dark period; $P < 0.05$; *post hoc* Tukey) (Fig. 1). There was no significant treatment effect within the light period. It is important to note that our measure of \dot{V}_{O_2} is averaged

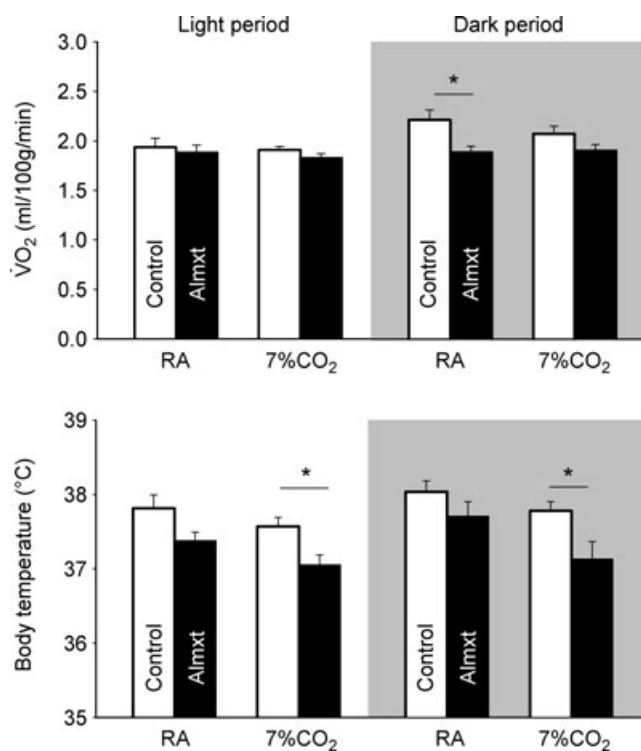


Figure 1. The effects of systemic treatment with almorexant or vehicle control on oxygen consumption and body temperature

The effects of systemic treatment with almorexant (Almxt) or vehicle control on oxygen consumption (top panel) and body temperature (bottom panel) are shown as mean \pm s.e.m. values during the period of exposure to air (RA) and 7% CO₂ in both the light/inactive and dark/active periods. We evaluated these data using a RM ANOVA applied to all data separately within the light/inactive and dark/active periods with application of the Tukey *post hoc* test when appropriate. Within the dark period, there was a significant RM effect to increase \dot{V}_{O_2} ($P < 0.02$) with *post hoc* analysis showing significance in RA ($P < 0.05$; Tukey). There was no significant effect within the light period. For body temperature, there was a significant overall RM effect to decrease body temperature in the light period ($P < 0.01$; Friedman Rank test) with no significant *post hoc* effects. In the dark period, there was a significant overall RM effect ($P < 0.001$) with a significant *post hoc* effect while breathing 7% CO₂ ($P < 0.05$; *post hoc* Tukey).

over one exposure period, air or CO₂; our method does not allow detection of changes in \dot{V}_{O_2} during rapid changes in arousal state.

Body temperature

Almorexant treatment decreased body temperature within the light period ($P < 0.01$; Friedman rank test; no significant *post hoc* effect) and within the dark period ($P < 0.001$, RM ANOVA) that was significant during 7% CO₂ breathing ($P < 0.05$, *post hoc* Tukey) (Fig. 1). Overall there was a tendency for body temperature to decrease during the 7% CO₂ inhalation compared to room air breathing in both control and almorexant-treated animals (this was not tested statistically).

Vigilance states

We categorized vigilance states into wakefulness, NREM and REM sleep, and evaluated the almorexant treatment effect as a RM separately within the light and dark periods (Fig. 2). In the light period there is a significant state effect ($P < 0.001$) but no RM treatment effect or interaction. The rats spent more time in NREM sleep compared to wakefulness ($P < 0.05$; Tukey) and to REM sleep ($P < 0.05$; Tukey) and more time in wakefulness than in REM sleep ($P < 0.05$; Tukey). In the dark period, there was no RM effect but there was a significant state effect ($P < 0.001$) and a significant interactive effect ($P < 0.01$) with significant effects of almorexant to decrease the percentage time in wakefulness (-56%) ($P < 0.05$) while increasing the percentage time in NREM

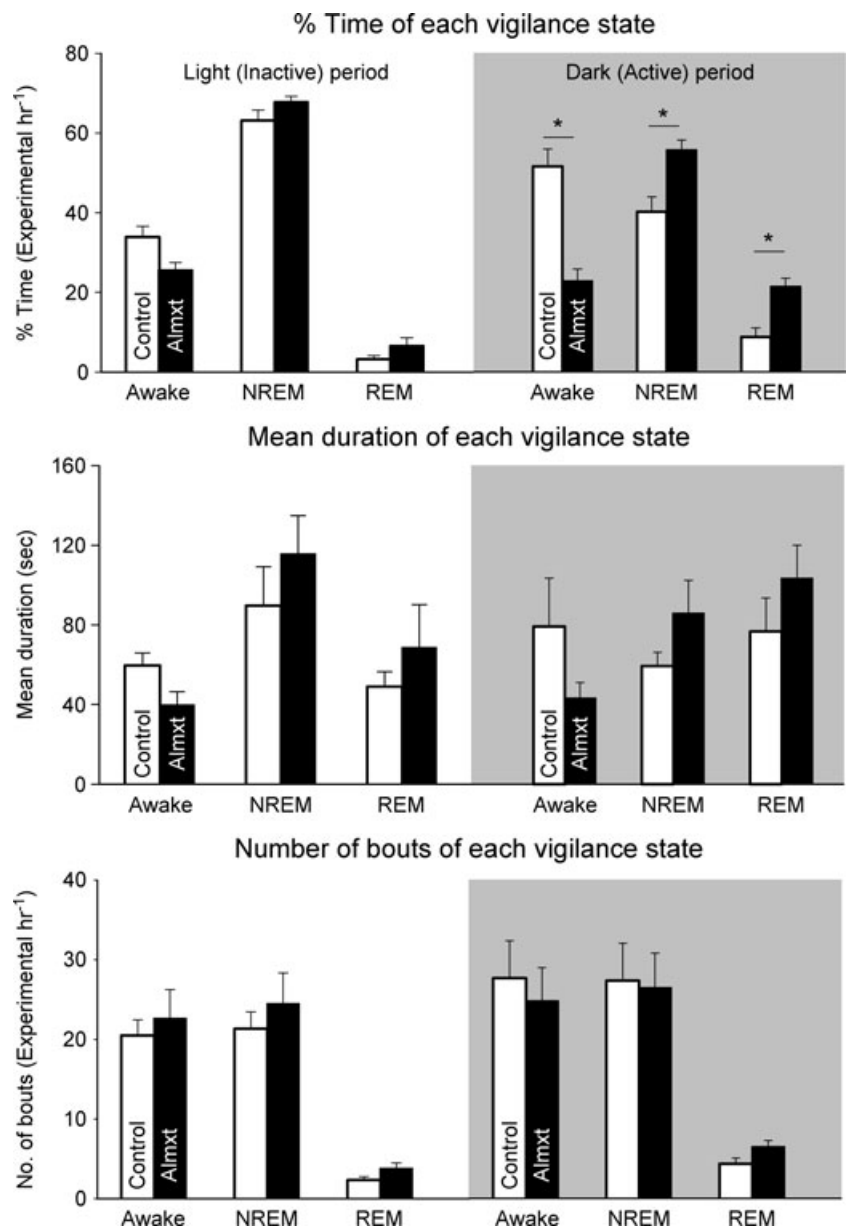


Figure 2. The effects of systemic treatment of Almx or vehicle control on the percentage time spent in each arousal state in animals exposed to either the light/inactive or dark/active cycle
 We show only the RA data and analyse the results separately by light and dark periods using Almx treatment as the RM. In the dark period, there was no RM effect but there was a significant state effect ($P < 0.001$) and a significant interactive effect ($P < 0.01$) with significant *post hoc* effects ($P < 0.05$) in wakefulness, NREM and REM sleep. Analysis of the number of bouts and duration of each state did not show any significant effects.

(+39%) ($P < 0.05$; Tukey) and REM (+146%) ($P < 0.05$; Tukey) sleep. Analysis of the number of bouts and duration of each state did not show any significant effects although the product of bout number and duration did. During the 7% CO₂ exposure the rats continued to cycle through wakefulness and NREM sleep with similar distributions of bout number, mean bout duration and total percentage time as in room air and similar effects of almorexant (data not shown).

Baseline ventilation and response to hypercapnia

For the analysis of the treatment effect on ventilation we applied a RM ANOVA separately for the light and dark periods with state (wakefulness, NREM, REM sleep) and

inspired gas (room air, 7% CO₂) as a categorical variables. In the light period, ventilation was significantly higher in CO₂ in both groups in all three states but there were no significant effects between control and almorexant treatment (Fig. 3). In the dark period, ventilation was significantly higher in CO₂ in both groups in all three states and there was a significant interaction between the RM and state. *Post hoc* analysis showed a significant difference between the ventilation breathing 7% CO₂ in control compared to almorexant treatment in the awake state ($P < 0.05$; Tukey). This was a ~26% decrease and was made up of decreases in both the tidal volume ($P < 0.05$; Tukey) and frequency ($P < 0.05$; Tukey) components of ventilation (data not shown). The ventilation breathing air in the dark period was slightly lower after almorexant treatment but was appropriate for the lower \dot{V}_{O_2} at that time, i.e. the \dot{V}_E/\dot{V}_{O_2} was unchanged.

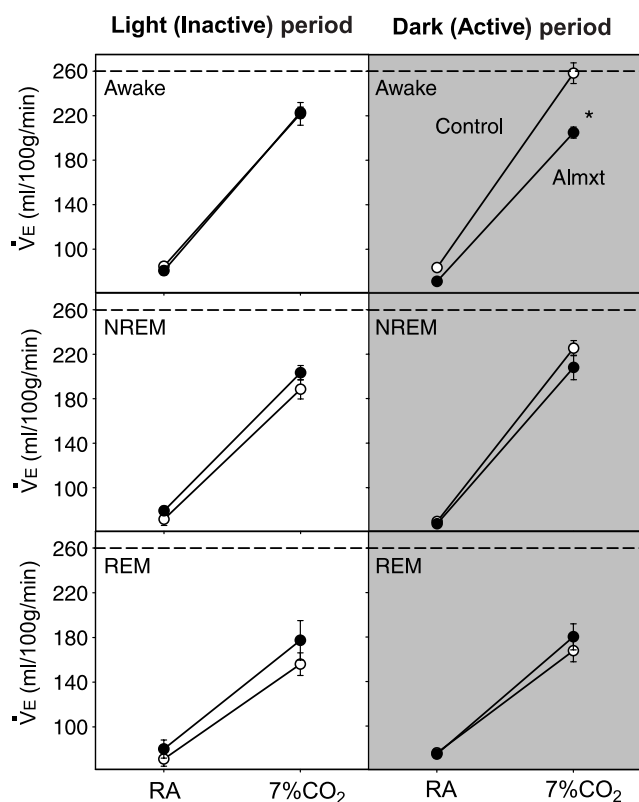


Figure 3. The effects of systemic treatment of Almx or vehicle control on ventilation normalized to body weight

For the analysis of the treatment effect on ventilation we applied a RM ANOVA separately for the light and dark periods with state (wakefulness, NREM, REM sleep) and inspired gas (room air (RA); 7% CO₂) as categorical variables. In the light and dark periods, ventilation was significantly higher in CO₂ in both groups in all three states but there were no significant effects between control and Almx treatment. In the dark period, there was a significant difference between the ventilation breathing 7% CO₂ in control compared to Almx 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 Almx in the awake dark period.

Sigh frequency, post-sigh apnoeas and spontaneous apnoeas

The effects of almorexant on sigh frequency, post-sigh apnoea and spontaneous apnoea are shown in Fig. 4 separately for light and dark periods. We analysed only the data obtained in air breathing. A typical sigh and the accompanying post-sigh apnoea are shown in the middle panel (Fig. 4). In the light period, almorexant treatment significantly decreased the number of sighs and post-sigh apnoeas ($P < 0.001$, RM ANOVA) with *post hoc* significance in wakefulness ($P < 0.05$, Tukey). In the dark period, almorexant significantly decreased the number of sighs and post-sigh apnoeas ($P < 0.001$, RM ANOVA) with *post hoc* significance in both wakefulness and NREM sleep ($P < 0.05$, Tukey). There are not enough numbers of sighs and post-sigh apnoeas in the REM sleep in either circadian period to make any comparison in our current experimental design. In respect to spontaneous apnoeas, during either the light or the dark period there was no significant treatment effect nor was there any interaction by state. While there is a suggestion for increased spontaneous apnoeas in REM sleep after almorexant treatment this did not reach statistical significance in this protocol.

Discussion

We find that acute and specific antagonism of both orexin receptors by systemic administration of almorexant in unanaesthetized and freely moving adult rats: (1) attenuates the respiratory response to CO₂ only in wakefulness and only during the dark period of the diurnal cycle, (2) decreases wakefulness and increases NREM and REM sleep during the dark period as reported previously

(Brisbare-Roch *et al.* 2007), and (3) decreases the number of sighs and post-sigh apnoeas during wakefulness in both the light and the dark period and during both wakefulness and NREM sleep in the dark period. We interpret the data to show that the orexin system (1) participates importantly in central chemoreception in a vigilance-state- and diurnal-cycle-dependent manner, (2) contributes to the sleep–wake difference in the CO₂ response, and (3) plays a role in the important process of sighing.

Orexin and breathing

Our almorexant treatment did not affect breathing at rest in any of our conditions, a finding that is congruent with observations in the ORX-KO mouse (Kuwaki, 2008). Resting ventilation in air exposure during wakefulness in the dark period is slightly decreased compared to the control (Fig. 3) but was appropriate for the lower \dot{V}_{O_2} at that time (Fig. 1), i.e. the V_E/\dot{V}_{O_2} was unchanged. This lower V_E and \dot{V}_{O_2} during wakefulness in the dark/active period may simply reflect less activity with dual orexin

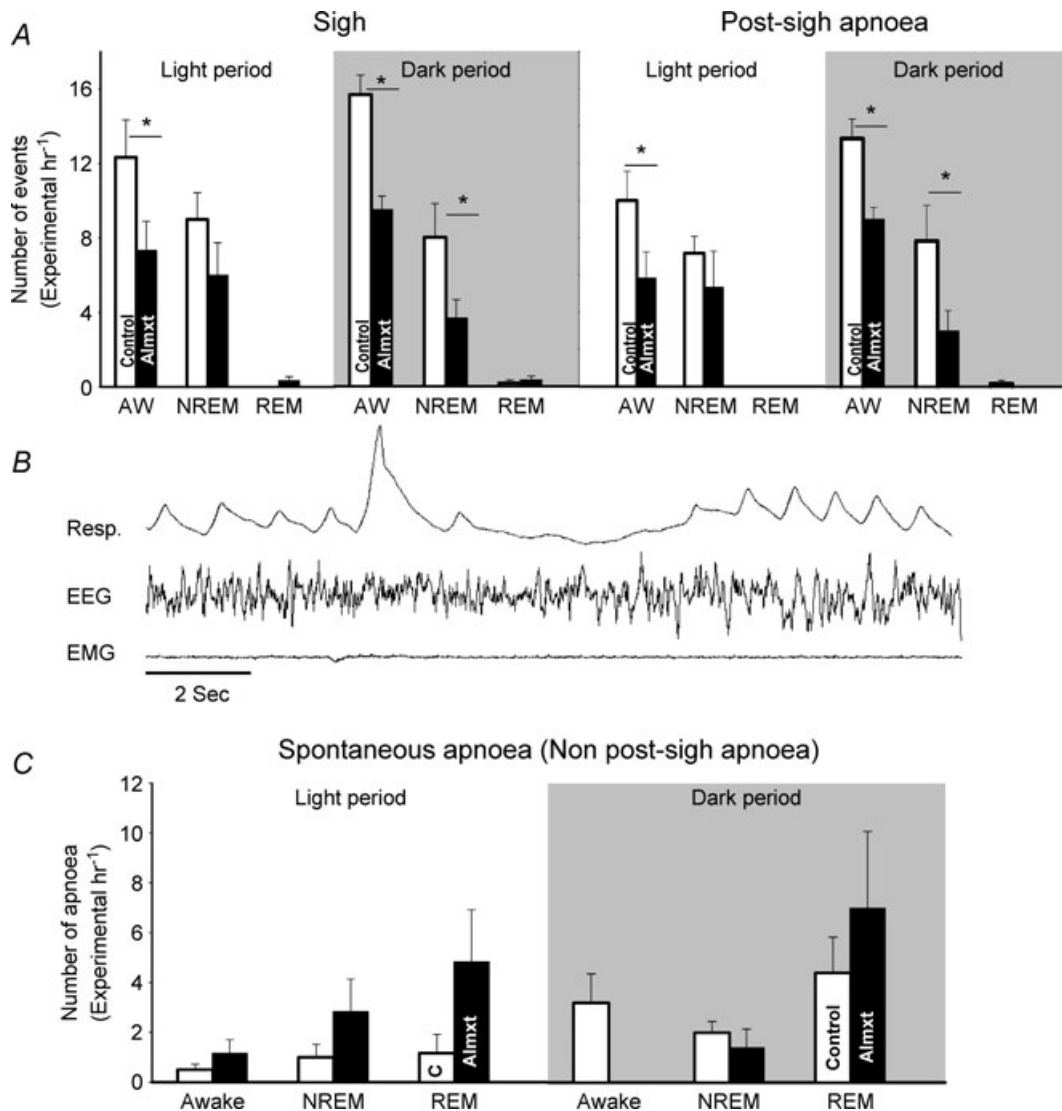


Figure 4. The effect of systemic Almx treatment or vehicle control on the frequency of sighs and both post-sigh and spontaneous apnoeas

The effect of systemic Almx treatment or vehicle control on the frequency of sighs and both post-sigh (A) and spontaneous (C) apnoeas are shown. A typical sigh with post-sigh apnoea is shown in B. In the light period, Almx treatment significantly decreased the number of sighs and post-sigh apnoeas in wakefulness ($P < 0.05$, Tukey). In the dark period, Almx significantly decreased number of sighs and post-sigh apnoeas in both wakefulness and NREM sleep ($P < 0.05$, Tukey). For spontaneous apnoeas, there was no significant treatment effect nor was there any interaction by state.

receptor antagonism. If so, this could contribute to the tendency for a lower body temperature as well.

In respect to central chemoreception, our prior studies showed that focal inhibition of OX₁R by microdialysis of an OX₁R antagonist (SB-334867) in the RTN decreased the CO₂ response substantially in wakefulness (−30%), with a smaller (−9%) but significant effect in sleep (Dias *et al.* 2009a), and in the medullary raphé region decreased the CO₂ response in wakefulness (−16%) only during the dark/active period (Dias *et al.* 2009b). ORX-KO mice with no orexin exhibit an attenuated CO₂ response only in wakefulness, with ~15–20% reduction when tested with 5% CO₂ and ~50% reduction with 10% CO₂ (Kuwaki, 2008). Here, with acute antagonism of both orexin receptors throughout the brain by systemic administration of almorexant, we observed a 26% reduction in ventilation with 7% CO₂ but only during wakefulness in the dark/active period. The degree of inhibition that we observed is qualitatively comparable to that reported in the ORX-KO mice with absence of orexin. It is also comparable to that produced by the focal inhibition of OX₁R only in the RTN region (Dias *et al.* 2009a). The relatively greater degree of CO₂ response inhibition observed with focal OX₁R antagonist (SB-334867) in the RTN may be explained by a greater concentration of drug being delivered to the RTN receptors by focal dialysis than by oral administration of almorexant. Using reported tissue almorexant data (Brisbare-Roch *et al.* 2007), we can estimate brain tissue values in our protocol as being in the range of 60 nM. The concentration of OX₁R antagonist SB-33467 in the dialysate used for the RTN focal inhibition was 5 mM. Considering that in general the concentration delivered to the tissue is 10 times lower than the dialysate (Nattie & Li, 2000), then the concentration of antagonist in tissue adjacent to the dialysis probe would be in the range of 0.5 mM, quite a high value compared to the estimate for almorexant. We consider that the comparable inhibition of the CO₂ response produced both by almorexant and by the absence of orexin in the knockout mouse suggests that the almorexant effect was at many sites involved in central chemoreception. The relatively large effect observed by focal inhibition of OX₁R could reflect the higher dose delivered or perhaps a greater role for this single site in the overall orexin-mediated effect.

Orexin as a circadian modulator

The discharge rate of orexin neurons varies with arousal state, being highest during active waking, lower during quiet waking, and virtually absent during sleep, including REM sleep (Lee *et al.* 2005; Milevskiy *et al.* 2005; Takahashi *et al.* 2008). These arousal-state-related changes in firing rate seem to occur within both the light/inactive and dark/active circadian periods. In contrast, orexin levels in cerebrospinal fluid and in the extracellular fluid

within the lateral hypothalamus vary almost two-fold during the circadian cycle in rodents, monkeys and humans, with levels being highest at the end of the dark/active period and lowest toward the end of the light/inactive period (Yoshida *et al.* 2001; Desarnaud *et al.* 2004). In conscious cat (Kiyashchenko *et al.* 2002) the orexin-A concentration in dialysate from the hypothalamus is significantly higher during active waking than NREM sleep but these state-related changes were much smaller in comparison to the circadian difference (Yoshida *et al.* 2001; Kiyashchenko *et al.* 2002). These data suggest that the orexin system is a powerful circadian modulator in addition to its arousal-state-related effects. Our observation that the effects of almorexant on the CO₂ response are more potent during the dark period of the diurnal cycle when the level of orexin in the central nervous system is higher supports this view of orexin as a potent diurnal modulator. Studies of the diurnal variation of the ventilatory response to CO₂ support this interpretation (Stephenson *et al.* 2000; Mortola, 2004).

Orexin and the sleep–wake differences in the CO₂ response

The largest CO₂ response that we observed in these experiments was in controls in wakefulness during the dark/active period (open symbol; dashed line; top panel of Fig. 3). We have placed a dashed line at this ventilation value in the other panels. This shows that the CO₂ response during NREM sleep in the light/inactive period, that of deepest sleep, is the same after vehicle and almorexant and is much lower than observed in wakefulness in the dark/active period in controls (the dashed line). We interpret this to indicate that (1) the sleep–wake difference in the CO₂ response may be optimally examined by comparison of wake in the dark/active period and NREM sleep in the light/inactive period, and (2) this sleep–wake difference in the CO₂ response may be largely attributable to orexin.

Sighs, apnoeas and sleep disorders

Almorexant significantly decreased the number of sighs and post-sigh apnoeas in both parts of the circadian cycle. Sighs or augmented deep breaths serve to prevent atelectasis and enhance surfactant turnover, which maintains normal pulmonary compliance and prevents hypoxaemia (Bartlett, 1973; Nicholas *et al.* 1982; Patroniti *et al.* 2002). Sighs can be abolished by vagotomy or carotid body ablation in anaesthetized rats (Bartlett, 1973). Our results suggest a role for orexin in these sigh-inducing reflexes perhaps at the NTS.

We also observed a trend towards an increase in spontaneous apnoeas after almorexant in all vigilance

states in the light period and in REM sleep in the dark period.

We note that: (1) the dose used in this study was much higher (300–400 mg kg⁻¹) than that used in the original studies of almorexant effects on sleep and wakefulness (20–100 mg kg⁻¹) (Brisbare-Roch *et al.* 2007); and (2) our study was conducted for 1 h of room air breathing in each diurnal cycle. To make any definitive conclusion on apnoeas requires study with more hours of sleep–wake cycle recording. It is noteworthy to mention, however, that the ORX-KO mice did have a 2–3 times higher incidence of spontaneous apnoeas in both NREM and REM sleep than that in the wild-type control mice but no significant decrease in number of sighs or post-sigh apnoeas (Nakamura *et al.* 2007). It is possible that a certain level of orexin is necessary to maintain the excitability of respiratory neurons and prohibit the genesis of sleep apnoea during both NREM and REM sleep as well as promote reflexes that contribute to sighs.

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Author contributions

Both authors contributed to the conception and design of the experiments and data interpretation, to the drafting and revision of the article and to the final approval of the paper.

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