Published in final edited form as:

Respir Physiol Neurobiol. 2014 August 15; 200: 118–125. doi:10.1016/j.resp.2014.03.014.

Effects of Orexin 2 Receptor Activation on Apnea in the C57BL/6J Mouse

Michael W. Moore¹, Afaf Akladios¹, Yufen Hu^{1,2}, Sausan Azzam^{1,3}, Pingfu Feng^{1,2}, and Kingman P. Strohl^{1,3}

¹Louis Stokes Cleveland DVA Medical Center, Division of Pulmonary, Critical Care, and Sleep Medicine, UH Case Medical Center, Cleveland OH, 44016

²Neogene Biosciences LLC, Cleveland OH

³Case Western Reserve University, Cleveland OH

Abstract

Background—The hypothesis was that an orexin 2 receptor (OX2R) agonist would prevent sleep-related disordered breathing.

Methods—In C57BL/6J (B6) mice, body plethysmography was performed with and without EEG monitoring of state (wakefulness, NREM and REM sleep). Outcome was apnea rate/hr during sleep-wake states at baseline and with an intracerebroventricular administration of vehicle, 4nMol of agonist OB^{DL}, and 4nMol of an antagonist, TCS OX2 29.

Results—A significant reduction ($p=0.035$; $f=2.99$) in apneas/hour occurred, especially with the agonist. Expressed as a function of the change from baseline, there was a significant difference among groups in Wake ($p=0.03$, $f=3.8$), NREM ($p=0.003$, $f=6.98$) and REM ($p=0.03$, $f=3.92$) with the agonist reducing the rate of apneas during sleep from 29.7 ± 4.7 (M $\pm$ SEM) to 7.3 ± 2.4 during sleep ($p=0.001$). There was also a reduction in apneas during wakefulness. Administration of the antagonist did not increase event rate over baseline levels.

Conclusions—The B6 mouse is a preclinical model of wake-and sleep-disordered breathing, and the orexin receptor agonist at a dose of 4nMol given intracerebroventricularly will reduce events in sleep and also wakefulness.

1.0 Introduction

Sleep-related breathing disorders accompanied by sleepiness are prevalent (9–14%) in the US general population. Although the epidemiology, risk factors, pathogenesis and consequences are increasingly refined (Dempsey et al., 2010), current treatments directed at apnea reduction are based on surgical or mechanical principles. Drug treatment would be a

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement. Dr. Feng is the President of Neogene Biosciences LLC. Mr. Miller and Drs. Afaf Akladios, Azzam, and Kingman Strohl have no declared conflicts, other than being supported by the NIH STTR sub-contract.

paradigm shift; however, evidence for success is mixed and targets are few (Conduit et al., 2007; Veasey, 2010).

The orexinergic system might be a promising pathway as it has direct connections to respiratory drive as well as to its better known effects on vigilance (Gestreau et al., 2008). Orexin immunoreactive fiber varicosities are found in respiratory-related motor neurons, including trigeminal and hypoglossal motor nuclei (Fung et al., 2001). Blocking the orexin A receptor prevents the phrenic burst frequency response normally associated with hypercapnia (Corcoran et al., 2010). The dual orexin 1 and 2 receptor antagonist, almorexant, attenuates the CO₂ response in wakefulness during the dark period of the diurnal cycle to a level observed during NREM (Li and Nattie, 2010; Nattie and Li, 2012). Orexin knock out animals exhibit an increased number of apneas during sleep (Han et al., 2010; Yamauchi et al., 2010; Yamauchi et al., 2008b). Orexin microinjections into the hypoglossal motor nucleus increase genioglossus muscle activity (Peever et al., 2003), and an injection of orexin B in the Koliker-Fuse nucleus will exert an excitatory effect through networks to hypoglossal motoneurons (Dutschmann et al., 2007). Taken together, these findings suggest that activation of the orexinergic system will have a facilitatory effect on respiratory drive not only in general but also in regard to upper airway patency. Thus, it is an appropriate to examine whether activation of OX2R might prevent sleep apnea.

The specific goal was to determine the effect of an OX2R agonists on the occurrence of apnea in sleep using the C57BL/6/J (B6) mouse, a preclinical model for irregular breathing and spontaneous apneas during wakefulness and sleep (Dias et al., 2009; Yamauchi et al., 2010). We compared vehicle to OX2R agonist peptide and to an antagonist modified endogenous peptide on sleep-wake behavior and the occurrence of apnea during wakefulness, and NREM and REM sleep. Administration of peptides systemically does not cross the blood brain barrier and this demonstration required intracerebroventricular (ICV) access and an experimental design to control for the effect of this mode of administration.

2. Material and Methods

Experiments were performed using male and female (B6 mice, originally obtained from Jackson Laboratory (Bar Harbor, ME), and maintained as a breeding colony in the Louis Stokes Cleveland DVA Medical Center (LSDVAMC) Animal Resource Center. Animals were housed in the same room of the Animal Resource Center for at least 4 weeks before investigation (food and water ad libitum; with a 6:00 AM to 6:00 PM and 6:00 PM to 6:00 AM light-dark cycle). Animals were studied between 3–4 months of age, and in each protocol male and female animals were randomly assigned to groups and each group randomly assigned to study days over the period of the study. All injections and studies were performed at the same time of day. The experimental protocols were approved by the Animal Care and Use Committee (IACUC) of the LSDVAMC and were in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The overall order of this investigation is shown in Table 1. There was an initial screening of animals for a significant number of apneas per hour (>6/hr) of recording. One group of screened animals meeting criteria was selected for a study to determine dose effects on

apneas. A larger group of screened animals were entered into the main study of sleep-wake monitoring and apnea event rate. The rationale for screening is based on our experience that there can be a variability among B6 animals in regard to the expression of pauses on any given study day (Han et al., 2001; Yamauchi et al., 2008a; Yamauchi et al., 2007; Yamauchi et al., 2008b). As the longest time in prior studies of baseline breathing in the B6 was usually 2 hours, screening accomplished the goal of determining: a) the distribution of apneas (pauses >2.5 respiratory cycles) and apneas/hour in male vs. female animals of the same age over a 7 hour period, and b) select those with a moderate to high level (>6/hour), to provide opportunity to observe increases or decreases in event number as a result of instrumentation or drug interventions.

The rationale for the dosing protocol was to compare low and moderate doses of peptide prior to the main study and estimate the effect of a given dose on behavior and overall apnea rate. The main study was the effect of a single dose on event rate, a study where animals were instrumented to determine sleep and breathing.

2.1 Collection of Data

Ventilatory behavior was measured by placing animals in a 600 ml Lucite cylindrical plethysmography chamber, modified to permit ICV injections and sleep monitoring. Briefly, we measured breathing data via pressure change across a pressure transducer (Validyne DP45, Validyne Engineering, Northridge, CA, 91324 USA) attached to a reference chamber of 600 ml. For ICV catheter and measurements of sleep, we modified this method by adding a small tube (a “chimney” of 100 ml volume) on the top of the conventional chamber (Friedman et al., 2004). The bottom of this tube is open to the main testing chamber, and the top of this tube is closed with a commutator mounted on the inside ceiling. Mice monitored for sleep and breathing had ICV catheters and/or implanted electrodes ready to be connected to the commutator (see below). Thus, a PSG recording could be conducted simultaneously with the ventilatory monitoring, and injects could be made without removing the animals from the main chamber.

2.2 Surgical implantation of guide cannula for ICV injection and electrodes for polysomnographic (PSG) recording

Under anesthesia with 3.5% isoflurane, the mouse was shaved with an electric clipper and restrained with a stereotaxic instrument. Betadine and alcohol was applied to the shaved portion of the head to disinfect the incision site. Marcaine, a local anesthetic, was then injected around the skin margins of the skull. A 1.5 cm incision was made along the midline of the head roughly 1 – 1.5 cm from the brow ridge to expose the skull. A small hole was drilled at AP= -0.3, L=1.0 using a mouse atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). A guide cannula (0.5 mm o.d. hollow stainless steel tube) which guides an internal cannula to the specific injection site (H=2.5) was implanted. The internal cannula, inserted only when performing injections, was connected by injection tubing to a micro-syringe so the agents can be delivered. When not injecting through the guide cannula, a dummy cannula was inserted to cover the guide cannula. The guide cannula was cemented using C&B Metabond bonding agent. For animals those need to have sleep recording, three holes (3 EEG electrodes for one channel of parietal EEG (offsets theta signals too) and one

channel for frontal cortical EEG) was drilled into the skull in the frontal and parietal regions to accommodate three stainless steel jewel screws serving as EEG electrodes. These electrodes were bonded to the dorsal surface of the skull with C&B Metabond bonding agent. This bonding agent adheres extremely well to bone, enamel, and dentin. Three electrodes, created from teflon-coated stainless steel wires, each end stripped of 0.15 cm of teflon, were stitched onto the surface of the nuchal muscles immediately posterior to the dorsal area of the skull. The skin overlaying the skull and posterior muscles were re-opposed, allowing for a cable to extend through the skin and connect to the PSG. The animal was monitored continuously until recovery from the anesthesia, every 2 hours for the 1st day and at least twice a day thereafter.

The placement of the ICV catheter was assessed by observing the drinking response to angiotensin delivered through the ICV catheter, and animals observed immediately drinking water in >5 minutes proceeded into the study. The ICV was kept patent by the injection of Dulbecco's solution every fourth day during the study, but not on a baseline or study day. Instrumented animals were observed daily for 14–18 days before entering the protocol, and entry into the dosing or sleep monitoring protocol was planned for gender equivalence, which was not achieved for technical reasons (see below).

Recordings of all mice started at the same time daily. During the testing periods, the mice were provided with water and food inside the chamber. Between 8:00 and 8:30am animals were moved from their housing containers and into the testing chambers. In the screening phase, animals were recorded for 7 hours in the plethysmograph starting ~9am to ~4pm. In the dosing and sleep studies, there was an initial adaptation day (Day 1) without any recording. Day 2 was the baseline recording, without any injection. On Day 3, the animals were injected with either various doses of drug (OB^{DL}, OX2R agonist, [Ala11,D-Leu15]-Orexin-B, OB^{DL} cat# 2142; OX2R antagonist, TCS OX2 29, Tocris Bioscience, Bristol, United Kingdom) or vehicle. ICV injections were performed between 8:40 and 8:50. Recording were performed between 09:00 and 17:00. At 17:00 mice were returned to their housing containers. Following 2–3 days rest, the testing procedure was repeated with Days 2 and 3, with the mice randomized to receive a different injection than they received previously.

2.3 Data Analysis

Ventilation was obtained by scoring normoxic breathing, excluding sniffs. Variations of pressure in the chamber are recorded as voltage swings by the transducer output, and were entered for data analyses using Lab View 7.1 (National Instruments, Austin, TX). The output was analyzed and scored using a customized program (Breath Detect using LabView programming by Innovative Computer Engineering, Cleveland, OH)(Moore et al., 2012). The program identifies an individual breath according to the crest and trough of the transducer's voltage output, but the investigator visually verifies the result. Frequency (f) is determined by the length of each breath, tidal volume (V_T) is estimated by the amplitude of the voltage change, compared to a standard (Han et al., 2001). For this analysis, the focus was on spontaneous apneas, defined as a pause for 2 respiratory cycles (Dias et al., 2009; Yamauchi et al., 2010). For studies of state, the EEG and EMG signals were recorded and

analyzed using Somnologica (Embla Systems, Amsterdam, Netherlands), and scored separately (Friedman et al., 2004) into wake, NREM and REM sleep. The files of sleep and breathing were merged using common time signals and results compiled for pauses according to state.

2.4 Pharmacologic Agents

Approximately 90% of orexin B binds to OX2R. Because modified orexin B exhibits a much higher potency than endogenous orexin B, this study used OB^{DL} and as for the OX2R antagonist TCS OX2 29 which, unlike current compounds of SB-334867 and almorexant, TCS OX2 29, specifically binds on OX2R. Based on previous work, the doses chosen were 2nMol and 4nMol of the Agonist OB^{DL}, and the same doses for the OX2R antagonist, TCS OX2 29. Drugs were diluted to their dosages using Dulbecco's solution so that the dose volume was 2µl. The vehicle volume was 2µl.

2.5 Data and Statistical Approaches

Descriptive statistics are presented as appropriate to each Part and included mean, SEM, range, etc.; tabular and graphical values are provided as the mean $\pm$ the standard error of the mean. ANOVAs were used as the main testing for results both for absolute and percent change (1-way) between test injection groups and baseline non-injection values. Significance levels were set at $p < 0.05$. If a given interaction showed statistical significance, post hoc probing was performed using t-tests of estimated marginal means (simple main effects) using a Bonferroni correction for multiple comparisons.

3. Results

3.1 Screening

Female (n=35) and male (n=28) animals of 2–3 months of age were chosen by convenience from the B6 breeding colony. Each animal was placed in a plethysmograph and recorded for 1 hour of acclimatization and 7 hours of recording during the subjective night (6am to 6pm). In females there were an average of 161.6 $\pm$ 4.6 pauses (median=99; range 2–729) or about 23 pauses/hr or about 18/hr if one outlier is excluded (this animal did not enter the study). In males there were an average of 111.6 $\pm$ 3.4 pauses (median=87; range: 2–244) or about 15/hr. The total number of apneas observed during the 7 hour screening was similar between the males and females ($p=0.08$, $F=3.56$). We excluded animals for further testing if the apnea count was outside a range of 40 to 200 ($<6/hr$ or $>30/hr$) in a 7 hour recording time. This resulted in eligibility of 57% of females and 62% of male animals.

A post hoc analysis comparing the number of apneas observed at screening, i.e. before implantation of the ICV or the EEG +ICV, and post-surgical baseline found an average 1.4 % drop/animal in the number of apneas after the procedure. For animals that completed screening and sleep/breathing measures there was a change from an average screening value of 20.6 $\pm$ 2.5/hr to 16.8 $\pm$ 2.7/hr on the baseline ($p=0.38$ $F=1.02$).

3.2 Determination of dose

Ten animals (5 males and 5 females) were instrumented and 7 completed the study (5 males and 2 females), with dropouts occurring either with a loose head plug (n=2) or by euthanasia because of failure to gain weight (n=1). A significant effect for drug was present across the dataset, when presented either as the absolute number of events (Table 2: $p < 0.0001$, $F = 14.8$), or the percent change from the baseline before any given intervention ($p < 8.3E-07$; $F = 10.49$; data not shown). Post-hoc analyses indicated that values for the highest agonist and antagonist doses were different from control ($p < 0.03$).

3.3 Effects on Apnea according to sex

Eight males and 8 females completed the protocol, with 2 of each sex having loose head plugs which resulted in withdrawal from the study. The average number of pauses at baseline over an eight hour period was 186.2 ± 39.0 for females and 95.7 ± 26.5 for males did not reach statistical significance ($p = 0.07$, $F = 3.56$). None of the percent changes from baseline for each condition were different between genders (highest p value of 0.85 and lowest of 0.23). Hence data from males and females were combined for analysis.

The overall effect on pauses in the eight hour recording was examined in several ways. Shown in Table 3 is the overall number of apneas for all animals; there was a significant difference among groups ($p = 0.0003$, $F = 6.81$). In an analysis comparing the percent change from baseline for all three conditions, there was an overall effect ($p = 0.01$; $f = 4.87$). In the post hoc analysis, the significant decrease occurred from baseline only with the agonist agent ($p = 0.01$).

3.4 Effects on sleep-wake time

Table 4 provides the data for the percent time for each sleep stage. There were differences among the four conditions: baseline, control, agonist, and antagonist ($p = 3.3E-10$, $F = 40.24$). These included differences in Total Sleep Time ($p = 4.99E-10$, $f = 49.02$), NREM sleep ($p = 8.07E-10$, $f = 36.97$), and wakefulness ($p = 5.72E-11$, $f = 45.04$), but there was no impact on REM sleep ($p = 0.24$, $f = 1.46$). In contrast to the effects on pause rate, there was no difference in sleep state percentages between baseline and control (vehicle) ($p = 0.53$, $F = 0.385$) (Figure 2), indicating that the injection itself was not a factor influencing subsequent sleep-wake distributions. However, with the administration of the agonist, there was an augmentation of wake and a decrease in NREM and total sleep, and the opposite effect with administration of the antagonist (wake was decreased and sleep was increased) ($F = 40.24$, $p = 3.3E-10$, and $F = 40.06$, $p = 3.51E-10$ respectively). The effects on REM were not significant with either peptide ($F = 1.75$, $p = 0.19$).

3.5 Apnea as a function of state

Combining sleep and pause data, there was a significant effect of the agonist on the expression of pauses in all states (Table 5). There was a significant reduction ($p = 0.035$; $f = 2.99$) in the number of apneas per 8 hour recording period, with a significant difference between baseline and any treatment (Figure 5). Expressed as a function of the change from baseline, there was a significant difference among groups in Wake ($p = 0.03$, $f = 3.8$), in NREM ($p = 0.003$, $f = 6.98$) and REM ($p = 0.03$, $f = 3.92$) and total sleep ($p = 0.01$, $f = 4.84$).

The agonist reduced apnea number in any given state ($p < 0.01$). Presented as event rate, while there is a reduction by vehicle in NREM and REM sleep to >5 – $10/\text{hr}$, it is the agonist that reduces the event rate to $\sim 5/\text{hr}$.

3.6 Respiratory Frequency

Baseline frequency was similar among all conditions ($p = 0.33$) across the entire recording period (data not shown). There was a significant, but small, increase the agonist on frequency in NREM and REM sleep ($p = 0.002$, $F = 5.33$ and $p = 1.13 \times 10^{-5}$, $F = 10.24$ respectively). In regard to a change from baseline, there were difference among groups in Wake ($p = 0.001$, $F = 7.79$) and in NREM ($p = 0.002$, $F = 7.28$) but not in REM ($p = 0.14$, $F = 2.04$) and the agonist was the factor significantly different, i.e. highest frequency.

4. Discussion

4.1 Summary

There are 3 novel features. First, in the B6 mouse apneas occur not only during NREM and REM sleep but also during wakefulness, as has been described recently in humans with central and mixed sleep apnea (Yamauchi et al., 2011). Second, while variability in expression of apnea among animals occurs at baseline, the rate/hour does not substantially change with placement of an IVC. A study design based on these data had sufficient power to examine the effect of a single peptide dose to affect apnea rate in female and male mice. Third, we demonstrate that 4nMol of OB^{DL} , an agonist peptide directed at the OX2R, will reduce pause/apnea rate across all states, compared to vehicle and to the same dose of the OX2R antagonist, TCS OX2 29.

4.2 Orexin as vigilance peptide

The effects of OX2R agonist and antagonist action on sleep-wake expression in the present study are consistent with those in the literature showing an increase in sleep with an inhibition of orexin and an augmentation of wakefulness with the agonist (Anacleot et al., 2009; Diniz Behn et al., 2010; Mieda et al., 2004). However, even at the highest dose the antagonist did not lead to drop attacks or cataplexy, features observed in the genetic knock out of preproorexin or by destruction of orexin containing neurons in the hypothalamus (Chen et al., 2009; Scammell et al., 2009; Yamauchi et al., 2010).

4.3 Orexin and respiratory drive

Orexin will stimulate respiratory drive (Kuwaki, 2008), an effect seen by the agonist in this study. The mechanisms of action inferred from the literature include changes in temperature, resetting of chemosensitivity, and direct effects on respiratory medullary neurons (Kuwaki, 2010; Shahid et al., 2012; Young et al., 2005). Leptin and orexin receptors are in the brainstem, both peptides influence metabolism, and anatomic connections occur that could influence autonomic output and respiratory drive (Haxhiu et al., 2003; Shirasaka et al., 2003). Pauses occur in the leptin receptor deficient mouse, the *ob/ob* mouse, a strain arising as a spontaneous mutation out of the B6 strain (Davis and O'Donnell, 2013). OX1R is involved in the modulation of central chemosensitivity (Corcoran et al., 2010), as is the leptin receptor (O'Donnell et al., 2000). Hence, orexin and leptin pathways might interact to

produce/modulate apneas. This study did not set out to dissect these possibilities, nor to replicate all prior studies of effects of orexin, but instead focused on whether ICV administration of agonists and antagonists affects apnea during wakefulness and sleep, i.e. respiratory rhythmogenesis.

4.4 Apnea effects

The B6 mouse is a strain where pauses are present, but there are few previous studies of state effects on apnea expression in adult animals (See discussion). In a study using leptin receptor deficient ob/ob mice compared to lean mice, the genetically obese mice exhibited a greater circadian variation in respiratory rate and diaphragmatic burst amplitude, while apnea expression in sleep was unchanged (Davis et al., 2013). In contrast to our baseline observations, in this study there was a greater expression of apneas in NREM than in REM sleep, but no examination for apneas in wakefulness. While the true cause for the difference between studies in baseline expression of apnea according to state is not known, it might be due to instrumentation or the manner of monitoring. There are no standardized/recommended methods for rodent monitoring, in contrast to those for clinical polysomnography.

The methods and apnea definition for this study are similar to those used in apnea studies in the rat, for instance those which examined mirtazapine (Carley and Radulovacki, 1999) or neonatal hydralazine (Carley et al., 1996). Reductions in spontaneous sleep apnea (>2 respiratory cycles) occurred using either pharmacologic agent. The latter study concluded that a persistent sleep-related breathing disorder occurred despite effective cardiovascular normalization by neonatal hydralazine in the phenotypically normotensive but genetically hypertensive rat (Carley et al., 2000). In the former study mirtazapine was observed to suppress apnea >2 respiratory cycles in all sleep stages in adult rats and attributed this effect to a mixed agonist/antagonist profile at serotonin receptors (Carley and Radulovacki, 1999). While the functional site and mechanism of action on apnea expression by the orexin agonist cannot be directly determined from our data, in the present study respiratory stimulation and apnea suppression were clearly evident in all states with OX2R activation. All these animal models provide preclinical tools for pharmacologic investigation of sleep-related breathing disorders across species.

4.5 The animal model

Rodent models of sleep apnea can provide information on pathways for rhythmogenesis and have been used as a platform by others to explore drug therapy (Davis and O'Donnell, 2013). Apneas in rodents in the unanesthetized state are classified as central apnea; and as we have shown, there can be apneas in not only NREM and REM sleep, but wakefulness as well. Animal models of obstructive apnea include the bulldog and elephant seal (Dempsey et al., 2010), but these are unlikely to become platforms for pharmacologic studies.

We directly addressed several issues in the development of this model for the testing of drugs. As variation in apnea expression overall among animals was substantial, we reasoned that screening will be needed to select those animals with enough pauses to show either an increase or decrease in event rate. This approach we reason mitigated a Type 2 error in the

study of [breathing x state x dose] effects. While we took these steps, a larger number of animals may be needed in future studies to study different doses and/or duration of state or pause effects. Extreme or higher doses were not examined, and might be important as orexin excess could lead to unwanted behavioral outcomes such as insomnia, hyperactivity, and/or anxiety (Feng et al., 2008; Feng et al., 2007). Future studies will use these data here to appropriately power pre-clinical studies for efficacy and safety; however, financial constraints limited a replication of results in this study with a larger set of animals, with a large dose variation, or in another species. We did not directly measure metabolism, which can be a strong modulator of respiratory drive. We know that orexinergic neurons can affect metabolism, and we suggest that future studies should either measure metabolism directly or use a surrogate marker like temperature to judge potential metabolic effects on pauses during wakefulness and sleep. None of the animals in this study suffered behaviorally or nutritionally from the acute administration of 4nmol of either OX2R agonist or antagonist, suggesting no adverse effects under the circumstances of the study. Finally, this study examined a single dose administered ICV before an eight hour observation period in the middle of the subjective night for the mouse. Future studies could observe time-of-day effects and multiple doses.

4.6 Relevance to human disease

It is our contention that the modification of central apneas in rodent models is appropriate for testing of drugs for potential use in human sleep apnea. We and others emphasize that the onset of central and obstructive apnea occurs with a reduction in respiratory drive either manifested by a slowing of breath rate or a lengthening of expiration (Carley et al., 1989; Onal et al., 1986). In a human upper airway predisposed to collapse, this reduction in drive causes obstructive apneas and hypopneas, and mixed apneas. In those with mechanically stable upper airways, a fall in drive results in central apneas or central hypopneas (Onal et al., 1986; Wellman et al., 2011). In some cases, continuous positive airway pressure converts obstructive apneas to central apneas, supporting the conclusion that unstable central patterning contributes directly to the pathogenesis of obstructive as well as central sleep apnea syndrome (Dempsey et al., 2010). Such central mechanisms are fundamental causes for apnea of any type.

4.7 Conclusions

The effect of the orexin agonist is to reduce apnea, but this is but one small step in the development of pharmacologic approaches to the treatment of apnea. The ideal drug for the treatment of sleep apnea would be one that not only improves the number of events but also the accompanying vigilance deficits found in some patients with long-standing disease. Orexin agonists might play that role.

Acknowledgements

This work is supported by an STTR award (R41 HL107037) from the NIH NHLBI to Neogene Biosciences LLC (formerly Biofunc Laboratory Sciences). The laboratory is also supported in part by the VA Research Service through a Merit Award.

References

- Anaclet C, Parmentier R, Ouk K, Guidon G, Buda C, Sastre JP, Akaoka H, Sergeeva OA, Yanagisawa M, Ohtsu H, Franco P, Haas HL, Lin JS. Orexin/hypocretin and histamine: distinct roles in the control of wakefulness demonstrated using knock-out mouse models. *J Neurosci*. 2009; 29:14423–14438. [PubMed: 19923277]
- Carley DW, Berecek K, Videnovic A, Radulovacki M. Sleep-disordered respiration in phenotypically normotensive, genetically hypertensive rats. *Am J Respir Crit Care Med*. 2000; 162:1474–1479. [PubMed: 11029364]
- Carley DW, Onal E, Aronson R, Lopata M. Breath-by-breath interactions between inspiratory and expiratory duration in occlusive sleep apnea. *J Appl Physiol*. 1989; 66:2312–2319. [PubMed: 2745295]
- Carley DW, Radulovacki M. Mirtazapine, a mixed-profile serotonin agonist/antagonist, suppresses sleep apnea in the rat. *Am J Respir Crit Care Med*. 1999; 160:1824–1829. [PubMed: 10588592]
- Carley DW, Trbovic SM, Radulovacki M. Hydralazine reduces elevated sleep apnea index in spontaneously hypertensive (SHR) rats to equivalence with normotensive Wistar-Kyoto rats. *Sleep*. 1996; 19:363–366. [PubMed: 8843525]
- Chen L, Brown RE, McKenna JT, McCarley RW. Animal models of narcolepsy. *CNS Neurol Disord Drug Targets*. 2009; 8:296–308. [PubMed: 19689311]
- Conduit R, Sasse A, Hodgson W, Trinder J, Veasey S, Tucker A. A neurotoxinological approach to the treatment of obstructive sleep apnoea. *Sleep Med Rev*. 2007; 11:361–375. [PubMed: 17646118]
- Corcoran A, Richerson G, Harris M. Modulation of respiratory activity by hypocretin-1 (orexin A) in situ and in vitro. *Adv Exp Med Biol*. 2010; 669:109–113. [PubMed: 20217331]
- Davis EM, Locke LW, McDowell AL, Strollo PJ, O'Donnell CP. Obesity accentuates circadian variability in breathing during sleep in mice but does not predispose to apnea. *J Appl Physiol*. 2013; 115:474–482. (1985). [PubMed: 23722707]
- Davis EM, O'Donnell CP. Rodent models of sleep apnea. *Respir Physiol Neurobiol*. 2013; 188:355–361. [PubMed: 23722067]
- Dempsey JA, Veasey SC, Morgan BJ, O'Donnell CP. Pathophysiology of sleep apnea. *Physiol Rev*. 2010; 90:47–112. [PubMed: 20086074]
- Dias MB, Li A, Nattie EE. Antagonism of orexin receptor-1 in the retrotrapezoid nucleus inhibits the ventilatory response to hypercapnia predominantly in wakefulness. *J Physiol*. 2009; 587:2059–2067. [PubMed: 19273574]
- Diniz Behn CG, Klerman EB, Mochizuki T, Lin SC, Scammell TE. Abnormal sleep/wake dynamics in orexin knockout mice. *Sleep*. 2010; 33:297–306. [PubMed: 20337187]
- Dutschmann M, Kron M, Morschel M, Gestreau C. Activation of Orexin B receptors in the pontine Kolliker-Fuse nucleus modulates pre-inspiratory hypoglossal motor activity in rat. *Respir Physiol Neurobiol*. 2007; 159:232–235. [PubMed: 17652033]
- Feng P, Vurbic D, Wu Z, Hu Y, Strohl KP. Changes in brain orexin levels in a rat model of depression induced by neonatal administration of clomipramine. *J Psychopharmacol*. 2008; 22:784–791. [PubMed: 18753273]
- Feng P, Vurbic D, Wu Z, Strohl KP. Brain orexins and wake regulation in rats exposed to maternal deprivation. *Brain Res*. 2007; 1154:163–172. [PubMed: 17466285]
- Friedman L, Haines A, Klann K, Gallagher L, Salibra L, Han F, Strohl KP. Ventilatory behavior during sleep among A/J and C57BL/6J mouse strains. *J Appl Physiol*. 2004; 97:1787–1795. [PubMed: 15475556]
- Fung SJ, Yamuy J, Sampogna S, Morales FR, Chase MH. Hypocretin (orexin) input to trigeminal and hypoglossal motoneurons in the cat: a double-labeling immunohistochemical study. *Brain Res*. 2001; 903:257–262. [PubMed: 11382413]
- Gestreau C, Bevengut M, Dutschmann M. The dual role of the orexin/hypocretin system in modulating wakefulness and respiratory drive. *Curr Opin Pulm Med*. 2008; 14:512–518. [PubMed: 18812827]
- Han F, Mignot E, Wei YC, Dong SX, Li J, Lin L, An P, Wang LH, Wang JS, He MZ, Gao HY, Li M, Gao ZC, Strohl KP. Ventilatory chemoresponsiveness, narcolepsy-cataplexy and human leukocyte antigen DQB1*0602 status. *Eur Respir J*. 2010; 36:577–583. [PubMed: 20110394]

- Han F, Subramanian S, Dick TE, Dreshaj IA, Strohl KP. Ventilatory behavior after hypoxia in C57BL/6J and A/J mice. *J Appl Physiol*. 2001; 91:1962–1970. [PubMed: 11641331]
- Haxhiu MA, Mack SO, Wilson CG, Feng P, Strohl KP. Sleep networks and the anatomic and physiologic connections with respiratory control. *Front Biosci*. 2003; 8:D946–D962. [PubMed: 12700084]
- Kuwaki T. Orexinergic modulation of breathing across vigilance states. *Respir Physiol Neurobiol*. 2008; 164:204–212. [PubMed: 18455970]
- Kuwaki T. Hypothalamic modulation of breathing. *Adv Exp Med Biol*. 2010; 669:243–247. [PubMed: 20217358]
- Li A, Nattie E. Antagonism of rat orexin receptors by almorexant attenuates central chemoreception in wakefulness in the active period of the diurnal cycle. *J Physiol*. 2010; 588:2935–2944. [PubMed: 20547681]
- Mieda M, Willie JT, Hara J, Sinton CM, Sakurai T, Yanagisawa M. Orexin peptides prevent cataplexy and improve wakefulness in an orexin neuron-ablated model of narcolepsy in mice. *Proc Natl Acad Sci U S A*. 2004; 101:4649–4654. [PubMed: 15070772]
- Moore MW, Chai S, Gillombardo CB, Carlo A, Donovan LM, Netzer N, Strohl KP. Two weeks of buspirone protects against posthypoxic ventilatory pauses in the C57BL/6J mouse strain. *Respir Physiol Neurobiol*. 2012; 183:35–40. [PubMed: 22595368]
- Nattie E, Li A. Respiration and autonomic regulation and orexin. *Prog Brain Res*. 2012; 198:25–46. [PubMed: 22813968]
- O'Donnell CP, Tankersley CG, Polotsky VP, Schwartz AR, Smith PL. Leptin, obesity, and respiratory function. *Respir Physiol*. 2000; 119:163–170. [PubMed: 10722859]
- Onal E, Burrows DL, Hart RH, Lopata M. Induction of periodic breathing during sleep causes upper airway obstruction in humans. *J Appl Physiol*. 1986; 61:1438–1443. [PubMed: 3781958]
- Peever JH, Lai YY, Siegel JM. Excitatory effects of hypocretin-1 (orexin-A) in the trigeminal motor nucleus are reversed by NMDA antagonism. *J Neurophysiol*. 2003; 89:2591–2600. [PubMed: 12611960]
- Scammell TE, Willie JT, Guilleminault C, Siegel JM. A consensus definition of cataplexy in mouse models of narcolepsy. *Sleep*. 2009; 32:111–116. [PubMed: 19189786]
- Shahid IZ, Rahman AA, Pilowsky PM. Orexin and central regulation of cardiorespiratory system. *Vitam Horm*. 2012; 89:159–184. [PubMed: 22640613]
- Shirasaka T, Takasaki M, Kannan H. Cardiovascular effects of leptin and orexins. *Am J Physiol Regul Integr Comp Physiol*. 2003; 284:R639–R651. [PubMed: 12571072]
- Veasey S. Treatment of obstructive sleep apnoea. *Indian J Med Res*. 2010; 131:236–244. [PubMed: 20308749]
- Wellman A, Eckert DJ, Jordan AS, Edwards BA, Passaglia CL, Jackson AC, Gautam S, Owens RL, Malhotra A, White DP. A method for measuring and modeling the physiological traits causing obstructive sleep apnea. *J Appl Physiol*. 2011; 110:1627–1637. [PubMed: 21436459]
- Yamauchi M, Dostal J, Kimura H, Strohl KP. Effects of buspirone on posthypoxic ventilatory behavior in the C57BL/6J and A/J mouse strains. *J Appl Physiol*. 2008a; 105:518–526. [PubMed: 18511527]
- Yamauchi M, Dostal J, Strohl KP. Acetazolamide protects against posthypoxic unstable breathing in the C57BL/6J mouse. *J Appl Physiol*. 2007; 103:1263–1268. [PubMed: 17673555]
- Yamauchi M, Kimura H, Strohl KP. Mouse models of apnea: strain differences in apnea expression and its pharmacologic and genetic modification. *Adv Exp Med Biol*. 2010; 669:303–307. [PubMed: 20217371]
- Yamauchi M, Ocak H, Dostal J, Jacono FJ, Loparo KA, Strohl KP. Post-sigh breathing behavior and spontaneous pauses in the C57BL/6J (B6) mouse. *Respir Physiol Neurobiol*. 2008b; 162:117–125. [PubMed: 18565803]
- Yamauchi M, Tamaki S, Yoshikawa M, Ohnishi Y, Nakano H, Jacono FJ, Loparo KA, Strohl KP, Kimura H. Differences in breathing patterning during wakefulness in patients with mixed apnea-dominant vs obstructive-dominant sleep apnea. *Chest*. 2011; 140:54–61. [PubMed: 21393396]
- Young JK, Wu M, Manaye KF, Kc P, Allard JS, Mack SO, Haxhiu MA. Orexin stimulates breathing via medullary and spinal pathways. *J Appl Physiol*. 2005; 98:1387–1395. [PubMed: 15557013]

Highlights

The orexinergic system acts on respiratory control as well as vigilance. An orexin 2 receptor agonist reduces apnea in sleep and in wakefulness, but one small step in the development of pharmacologic approaches to the treatment of apnea.

Wakefulness

NREM to REM transition

Figure 1. Shown are examples of the recording of sleep and breathing

In Figure 1A the top panel shows a continuous ~2 minute recording with 4 channels representing EEG1, nuccal EMG1, nuccal EMG2, and EEG2. EMG activity is high, and variable, with a lower amplitude fast EEG. Along the EMG1 channel 3 short bars- A, B, and C- represent times where there are shown representative tracings from the plethysmography. A= wakefulness examples of 4 regular breaths, 8 sniffs, and a pause of ~ 3 respiratory cycles. B= Irregular breathing but without any pauses. C= Breathing, sniffs, and a post-sigh apnea, an event which is not counted.

In Figure 1B, the format is the same as Figure 1A; however, the first minute is in NREM and the second minute REM sleep. In contrast to NREM sleep, in REM sleep EMG activity is low, the amplitude lower, and frequency faster. In this instance, the events during sleep include: A= a post-sigh pause (again not counted), B= irregular breathing with 3 pauses >2 respiratory cycles and one <2 cycles, and C = 2 significant pauses >2 cycles and 1 <2 cycles.

Figure 2.

Shown are state values expressed as a percent of total recording time as the mean and SEM of values, according to baseline, vehicle (all C), Agonist (All T ago-4nmol) and antagonist (All T ant4nmol). Wake%= dark grey, NREM sleep (white), REM%(PS% in black). Please see Results for explanation of significant differences.

Figure 3.

Shown in bars are the mean and SEM of values in regard to the pauses/hr in the recording of baseline for each of the states including wakefulness (Wake= dark grey), NREM sleep (white), and REM (black), according to Baseline (no injection), vehicle (CONT), agonist (AGO), and antagonist (ANT). Please see Results for details on statistical differences.

Table 1

Study Design

	Screening (without instrumentation)	Drug Dose Study	Core Study: State effects on Apnea
Observation Time	7 hours	8 hours	8 hours
Measures	Breathing	Breathing and Drug	Breathing, Drug and State
Intervention		Vehicle	Vehicle
Intervention		Agonist 2nMol	
Intervention		Agonist 4nMol	Agonist 4nMol
Intervention		Antagonist 2nMol	
Intervention		Antagonist 4nMol	Antagonist 4nMol
Number of Animals	n= 28 male, 35 female	n=5 male, 5 female	n=10 male, 10 female

Table 2

Descriptive data on Drug Effects on Pause Expression over a 7-Hour Recording. For statistical significance, see text.

	Baseline	Control	Ago 2nmol	Ago 4nmol	Ant 2nmol	Ant 4nmol
Total # of apnea	140.9±15.2	77.4±25.6	25.5±7.8	24.5±8.4	67.5±22.7	119.7±28.1
Apnea rate (#/hr)	20.1±2.2	11.1±3.7	3.6±1.1	3.5±1.2	9.6±3.3	17.1±4.0

Table 3

Average ($\pm$ SEM) number of apneas over a 7-hour period. For statistical significance, see text.

Condition	Number of Pauses	Number/Hr
Baseline	140.99 $\pm$ 15.19	17.62 $\pm$ 1.90
Control	77.41 $\pm$ 25.60	9.68 $\pm$ 3.20
Agonist 4nmol	24.55 $\pm$ 8.39	3.07 $\pm$ 1.05
Antagonist 4nmol	119.68 $\pm$ 28.11	14.96 $\pm$ 3.51

Table 4

State Changes. Percent Change from Baseline ($M \pm SEM$; graphically displayed in Figure 4). For statistical significance, see text.

	Wake%	NREM%	REM%	Total sleep
All Baseline	33.04% $\pm$.82%	59.87% $\pm$ 0.70%	7.09% $\pm$ 0.31%	66.96% $\pm$.82%
All Control	33.91% $\pm$ 1.13%	63.40% $\pm$ 1.36%	2.69% $\pm$ 0.59%	66.09% $\pm$ 1.13%
All T ago4nmol	45.54% $\pm$ 1.98%	50.27% $\pm$ 1.58%	4.18% $\pm$ 0.60%	54.46% $\pm$ 1.98%
All T ant4nmol	23.02% $\pm$ 1.48%	72.46% $\pm$ 1.47%	4.52% $\pm$ 1.11%	76.98% $\pm$ 1.48%

Table 5

Effect of States on the Expression of Pauses. For statistical significance, see text.

		Baseline	apneas per hr	# of apneas	VEH	apneas per hr
	# of apneas	Hours in State		# of apneas	Hours in State	
Wake	59.13±7.47	2.64±0.09	22.40±2.6	55.42±15.53	2.71±0.09	20.45±5.7
NREM	88.79±16.05	4.79±0.08	18.54±3.7	41.71±15.78	5.07±0.11	8.23±3.1
REM	5.45±0.74	0.57±0.03	9.57±1.4	1.52±0.52	0.22±0.05	6.91±2.6
Total	151.11±22.92		29.7±4.7	98.67±30.30		17.84±5.6
		AGO			ANT	
	# of apneas	Hours in State	apneas per hr	# of apneas	Hours in State	apneas per hr
Wake	19±7.15	3.64±0.15	5.22±1.7	44.7±13.70	1.84±0.12	24.29±7.8
NREM	10.5±3.34	4.021±0.13	2.61±0.8	88.1±19.25	5.8±0.12	15.19±3.2
REM	0.5±0.24	0.33±0.05	1.52±0.8	3.2±0.80	0.36±0.09	8.89±2.1
Total	30±9.31		7.3±2.4	136±30.84		22.48±4.9