Role of orexin-A in the ventrolateral preoptic area on components of total energy expenditure

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BACKGROUND: Identifying whether components of total energy expenditure (EE) are affected by orexin receptor (OXR1 and OXR2) stimulation or antagonism with dual orexin receptor antagonists (DORAs) has relevance for obesity treatment. Orexin receptor stimulation reduces weight gain by increasing total EE and EE during spontaneous physical activity (SPA).
OBJECTIVE: The purpose of this study was to determine if a DORA (TCS-1102) in the ventrolateral preoptic area (VLPO) reduced orexin-A-induced arousal, SPA, total EE and EE during sleep, rest, wake and SPA and whether the DORA alone reduced total EE and its components. We hypothesized that: (1) a DORA would reduce orexin-A induced increases in arousal, SPA, components of total EE, reductions in sleep and the EE during sleep and (2) the DORA alone would reduce baseline (non-stimulated) SPA and total EE.
SUBJECTS/METHODS: Sleep, wakefulness, SPA and EE were determined after microinjection of the DORA (TCS-1102) and orexin-A in the VLPO of male Sprague–Dawley rats with a unilateral cannula targeted towards the VLPO. Individual components of total EE were determined based on time-stamped data.
RESULTS: The DORA reduced orexin-A-induced increases in arousal, SPA, total EE and EE during SPA, wake, rest and sleep 1 h post injection ($P < 0.05$). Orexin-A significantly reduced sleep and significantly increased EE during sleep 1 h post injection ($P < 0.05$). Furthermore, the DORA alone significantly reduced total EE, EE during sleep (NREM and REM) and resting EE 2 h post injection ($P < 0.05$).
CONCLUSIONS: These data suggest that orexin-A reduces weight gain by stimulating total EE through increases in EE during SPA, rest and sleep. Residual effects of the DORA alone include decreases in total EE and EE during sleep and rest, which may promote weight gain.

International Journal of Obesity advance online publication, 9 May 2017; doi:10.1038/ijo.2017.92

INTRODUCTION

Orexin-A also known as hypocretin-1 is an endogenous neuropeptide synthesized in lateral, dorsomedial and perifornical hypothalamic areas[1,2] that modulates the sleep–wake cycle, energy balance, reward and autonomic function.[3] Physiological effects of orexin-A are mediated by two G-protein-coupled receptors referred to as orexin 1 and 2 receptors (OXR1 and OXR2).[2] Mutations in the canine OXR2[4] and loss of orexin neurons in mice and humans[5,6] are associated with narcolepsy,[7,8] which is characterized by disorganized sleep–wake transitions. Mice lacking orexin neurons exhibit narcolepsy, obesity, hypophagia and low physical activity,[8] which illustrates the role of orexin in integrating sleep–wake states and energy balance.

Central orexin-A administration reduces sleep and enhances wakefulness,[9,10] spontaneous physical activity (SPA, for example, low-intensity physical activity excluding exercise),[9,11] energy expenditure (EE)[12] and food intake in a brain site-dependent manner.[13] Blocking both OXRs with dual orexin receptor antagonists (DORAs) promotes sleep and reduces wakefulness in several species.[14,15] DORAs also reduce baseline[14,15] and orexin-A-induced locomotor activity.[16] These data demonstrate that orexin-A impacts key behavioral processes, maintaining normal sleep–wake status and energy balance; however, the brain sites involved and the energy balance components affected are not fully defined.

The ventrolateral preoptic area (VLPO) is a brain site critical to sleep–wake regulation.[17,18] The VLPO receives innervation from orexin neurons,[19] contains both OXR subtypes[20,21] and orexin-A infusion in the VLPO increases wakefulness and decreases sleep.[22] We recently confirmed the effects of orexin-A in the VLPO on sleep–wake and also showed orexin-A increased SPA, total EE and EE during SPA (i.e. non-exercise activity thermogenesis),[23] with no effect on feeding.[24] Taken together, these data imply that the VLPO may be an important node for integration of orexin-A signals that influence sleep–wake and metabolism.

The contribution of orexin-A in the VLPO to sleep–wake status, SPA and total EE for regulating energy metabolism is evident. However, a better understanding of how orexin-A in the VLPO contributes to overall increases in total EE is warranted. Here we determined the effect of orexin-A in the VLPO on individual components of total EE; whether blocking both OXRs with a DORA abolished effects of orexin-A; and the effect of a DORA alone on sleep–wake, SPA, total EE and components of total EE. We hypothesized that (1) orexin-A in the VLPO would increase components of total EE, (2) the DORA would reduce orexin-A-stimulated increases in wakefulness, SPA, total EE, components of EE and prevent the reduction in sleep and rest and (3) a DORA alone would reduce baseline total EE and its components.

MATERIALS AND METHODS

Animals

One set of 3-month-old male Sprague–Dawley ($N = 7$) rats (Charles River Laboratories, Kingston, NY, USA) were housed individually in solid-bottom

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Received 18 July 2016; revised 20 March 2017; accepted 26 March 2017; accepted article preview online 10 April 2017

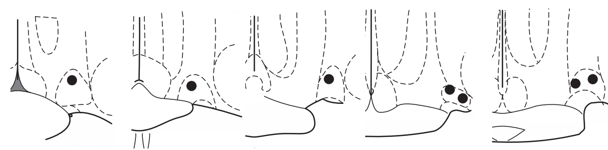

Figure 1. Histological verification map showing correct placement of injection sites into the VLPO.

cages in a temperature-controlled room (21–22 °C) with a 12 h light/12 h dark cycle (lights on at 0600 hours). Rodent chow (Harlan Teklad 8604) and water were allowed *ad libitum*. Study procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Surgery

Rats were anesthetized and surgically implanted with a 26-gauge stainless-steel cannula (Plastics One, Roanoke, VA, USA) targeted towards the VLPO and a radiotelemetric transmitter connected to electroencephalogram (EEG) and electromyogram (EMG) electrodes (F40-EET; Data Sciences International, Saint Paul, MN, USA) as described.[21,24] Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson.[25] Coordinates for the cannula and EEG electrodes, respectively, were as follows: −0.12 and 3.1 mm posterior to bregma, ±1.5 mm lateral to bregma and 0.8 mm below the skull surface. Experimental trials began 10 days after surgery.

Drugs

Orexin-A (American Peptides, Sunnyvale, CA, USA) was dissolved in artificial cerebrospinal fluid (Sigma-Aldrich, St Louis, MO, USA), which served as the vehicle control for orexin-A. The DORA (TCS-1102; Tocris Bioscience, Saint Paul, MN, USA) was dissolved in dimethyl sulfoxide/methanol HCl/sterile water, which served as the vehicle control for the DORA. All drugs were stored frozen prior to injections.

Injections

A volume of 0.5 μl was injected over 30 s with a 33-gauge injector (Plastics One) that extended 1.0 mm beyond the tip of the guide cannula.[24] Injections were performed between 0800–1000 hours (>48 h between injections). Previous studies demonstrate that repeated injections do not cause tissue damage as measured by lack of gliosis around the injection site[26] or reduce the efficacy of orexin-A to stimulate SPA,[27] suggesting maintenance of tissue integrity and behavioral responses to orexin-A with repeated injections.

Verification of cannula placement by histology

Brains were dissected out and stored in 10% formaldehyde. Cannulae were deemed incorrectly placed if >0.25 mm from the targeted site. This rationale is based on diffusion coefficients of the injection volume delivered.[28] All cannulae were correctly placed (Figure 1).

Concurrent EEG, EMG, SPA and indirect calorimetry measurements

A receiver was placed beneath the test cage to allow EEG and EMG signals to be recorded from the implanted EEG and EMG electrodes and transmitter.[21] EE and SPA were determined with an indirect calorimeter and infrared beam break sensors (for example, 1 cm spacing) that measured O_2, CO_2, water vapor and distance traveled continuously each second from each chamber simultaneously (Promethion-C; Sable Systems Inc., Las Vegas, NV, USA).[24] Analyzers were calibrated before each test with primary gas standards (100% nitrogen and 1% CO_2).[24,29] The flow rate was maintained at 2500 ml min^{-1}. Rats were acclimated to the chambers (3 h per day for three consecutive days) with food and water *ad libitum* before the test injections. Water was available *ad libitum* during testing. Data were processed with the Expedata software v.1.7.30 (Sable Systems Inc.).[24] The respiratory quotient was defined as the mean respiratory exchange ratio during the measurement period.

Determining sleep–wake behavioral states

EEG and EMG data were visualized with the Neuroscore software (version 2.0.1; Data Sciences International).[24] Consecutive 15-s epochs of EEG and EMG were manually scored to determine active wake (AW), quiet wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep.[21]

Determining components of total EE

Total EE was calculated with the Weir equation.[30] Individual components of total EE include: EE during SPA, wake (AW+quiet wake), AW, quiet wake, rest, sleep (NREM sleep+REM sleep), NREM sleep, and REM sleep. These individual components of EE will be referred to as EE during SPA, EE during wake, EE during AW, EE during quiet wake, resting EE, EE during sleep, EE during NREM sleep and EE during REM sleep. These data were derived from EEG and EMG recordings (scored as AW, quiet wake, NREM sleep and REM sleep), distance traveled indicated by infrared beam break sensors and total EE based on the timestamp.[24] Individual components of total EE were calculated in accordance with previously described methods.[31] Briefly, EE during SPA was calculated as the sum of EE when rats were awake and moving based on the infrared beam break sensors and EEG/EMG recordings. Resting EE was calculated as the calories when the rat was awake but not moving or sleeping based on infrared beam break sensors, EEG and EMG. AW and quiet wake EE was calculated as the calories when the animal was awake and either moving (i.e. AW) or not moving (i.e. quiet wake) based on EMG radiotelemetric activity counts. EE during NREM sleep and REM sleep was calculated as the calories when the rat was either in NREM sleep or REM sleep based on EEG and EMG. EE during total wake was calculated as the calories when the animal was in either active or quiet wake. Total sleep EE was calculated as the calories during both NREM sleep and REM sleep. We did not distinguish diet-induced thermogenesis from resting EE since diet-induced thermogenesis was likely minimal because food was unavailable during testing and tests were performed in the early light cycle. We have previously shown that this rat strain consumes <10% of their total 24-h caloric intake within this time interval.[32]

Experimental design

The DORA (62.5 nmol 0.5 μl^{-1}) or vehicle control was injected into the VLPO through the cannula 20 min before an injection of orexin-A (62.5 pmol 0.5 μl^{-1}) or vehicle control. Treatments were given in a randomly assigned latin-square unblinded design. Measurements (EEG, EMG, SPA and EE) were taken for 2.5 h post injection. Duration of measurements and doses for the DORA and orexin-A were based on previous reports.[22,24] All experimental procedures were completed once.

Statistical analysis

Data were analyzed with Prism 6.0f (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± s.e.m. Alpha was 0.05 for all statistical tests. Data were analyzed with repeated-measures analysis of variance followed by Fischer's tests to determine differences between individual treatments. All assumptions for repeated-measures analysis of variance were met. Sample size was based on power calculations from previous report.[24] Since handling involved in the injection procedure augments wakefulness and SPA for up to 20 min post injection independent of treatment, the first 20 min of data collection post injection were excluded from data analysis.[33] Therefore, data were analyzed in the 20–80, 80–140 and 20–140 min post injection time periods, which will be referred to as the 1, 1–2 and 2 h post injection. A separate analysis was completed for each time period and end point (Table 1).

The following end points were analyzed: (1) *sleep–wake status*: percent time spent in wake, AW, quiet wake, total sleep, NREM sleep, and REM sleep, number of episodes and mean duration of each sleep–wake state; total transitions between sleep–wake states; latency to sleep onset; (2) *physical activity and EE*: respiratory quotient, total EE, SPA indicated by distance traveled based on infrared sensors and EE during SPA; (3) *EE during sleep–wake states*: EE during wake, EE during AW, resting EE, EE during sleep, EE during NREM sleep and EE during REM sleep. There were no main effects on quiet wake for the aforementioned end points (data not shown). Thus, the effect of treatments on wake was due to AW rather than quiet wake since wake was defined as the sum of active plus quiet wake (Table 1).

Table 1. Repeated-measures ANOVA for all end points

	Time period		
	0–1 h	*1–2 h*	*0–2 h*
Time spent in sleep–wake stages			
Total wake	$F_{3,18}=15.4$, $P<0.0001$	$F_{3,18}=1.2$, $P=0.3330$	$F_{3,18}=3.7$, $P=0.0296$
Active wake	$F_{3,18}=15.3$, $P<0.0001$	$F_{3,18}=1.2$, $P=0.3318$	$F_{3,18}=3.9$, $P=0.0247$
Quiet wake	$F_{3,18}=1.1$, $P=0.3642$	$F_{3,18}=0.6$, $P=0.6165$	$F_{3,18}=1.9$, $P=0.1585$
Total sleep	$F_{3,18}=15.0$, $P<0.0001$	$F_{3,18}=1.2$, $P=0.3329$	$F_{3,18}=2.8$, $P=0.0696$
NREM sleep	$F_{3,18}=13.6$, $P<0.0001$	$F_{3,18}=1.5$, $P=0.2416$	$F_{3,18}=1.9$, $P=0.1697$
REM sleep	$F_{3,18}=7.1$, $P=0.0024$	$F_{3,18}=0.5$, $P=0.6805$	$F_{3,18}=2.5$, $P=0.0873$
Sleep quality			
Episodes			
Total wake	$F_{3,18}=1.0$, $P=0.4016$	$F_{3,18}=0.6$, $P=0.6192$	$F_{3,18}=1.4$, $P=0.2823$
Active wake	$F_{3,18}=1.6$, $P=0.2270$	$F_{3,18}=0.8$, $P=0.4972$	$F_{3,18}=1.7$, $P=0.2007$
Quiet wake	$F_{3,18}=1.6$, $P=0.2326$	$F_{3,18}=0.4$, $P=0.7800$	$F_{3,18}=1.7$, $P=0.1930$
Total sleep	$F_{3,18}=4.4$, $P=0.0176$	$F_{3,18}=1.1$, $P=0.3557$	$F_{3,18}=2.7$, $P=0.0784$
NREM sleep	$F_{3,18}=3.0$, $P=0.0566$	$F_{3,18}=1.0$, $P=0.3924$	$F_{3,18}=1.9$, $P=1.609$
REM sleep	$F_{3,18}=4.4$, $P=0.0169$	$F_{3,18}=0.7$, $P=0.5794$	$F_{3,18}=2.4$, $P=0.1037$
Mean duration of episodes of			
Total wake	$F_{3,18}=0.2$, $P=0.8921$	$F_{3,18}=1.4$, $P=0.2784$	$F_{3,18}=0.1$, $P=0.9815$
Active wake	$F_{3,18}=0.2$, $P=0.9103$	$F_{3,18}=1.3$, $P=0.3150$	$F_{3,18}=0.0$, $P=0.9848$
Quiet wake	$F_{3,18}=1.5$, $P=0.2520$	$F_{3,18}=0.6$, $P=0.6189$	$F_{3,18}=1.3$, $P=0.2943$
Total sleep	$F_{3,18}=6.0$, $P=0.0051$	$F_{3,18}=0.6$, $P=0.6241$	$F_{3,18}=2.5$, $P=0.0925$
NREM sleep	$F_{3,18}=4.1$, $P=0.0213$	$F_{3,18}=1.2$, $P=0.3487$	$F_{3,18}=1.4$, $P=0.2750$
REM sleep	$F_{3,18}=4.9$, $P=0.0117$	$F_{3,18}=0.6$, $P=0.5941$	$F_{3,18}=1.9$, $P=0.1636$
Latency sleep onset	$F_{3,18}=11.3$, $P=0.0002$	$F_{3,18}=1.8$, $P=0.1822$	$F_{3,18}=2.0$, $P=0.1424$
Total transitions	$F_{3,18}=3.0$, $P=0.0555$	$F_{3,18}=1.2$, $P=0.3258$	$F_{3,18}=2.6$, $P=0.0829$
Distance traveled	$F_{3,18}=17.6$, $P<0.0001$	$F_{3,18}=1.9$, $P=0.1610$	$F_{3,18}=6.6$, $P=0.0034$
RQ	$F_{3,18}=1.3$, $P=0.2995$	$F_{3,18}=3.0$, $P=0.0568$	$F_{3,18}=2.2$, $P=0.1204$
Total EE	$F_{3,18}=21.6$, $P<0.0001$	$F_{3,18}=2.8$, $P=0.0668$	$F_{3,18}=8.5$, $P=0.0010$
Components of total EE			
EE SPA	$F_{3,18}=22.5$, $P<0.0001$	$F_{3,18}=1.6$, $P=0.2231$	$F_{3,18}=9.1$, $P=0.0007$
EE wake	$F_{3,18}=13.5$, $P<0.0001$	$F_{3,18}=1.4$, $P=0.2884$	$F_{3,18}=11.8$, $P=0.0002$
EE active wake	$F_{3,18}=15.7$, $P<0.0001$	$F_{3,18}=2.4$, $P=0.1058$	$F_{3,18}=25.0$, $P<0.0001$
EE rest	$F_{3,18}=18.2$, $P<0.0001$	$F_{3,18}=3.3$, $P=0.0474$	$F_{3,18}=17.9$, $P<0.0001$
EE sleep	$F_{3,18}=5.9$, $P=0.0056$	$F_{3,18}=2.9$, $P=0.0627$	$F_{3,18}=3.3$, $P=0.0431$
EE NREM sleep	$F_{3,18}=6.9$, $P=0.0028$	$F_{3,18}=3.2$, $P=0.0499$	$F_{3,18}=3.3$, $P=0.0453$
EE REM sleep	$F_{3,18}=3.6$, $P=0.0858$	$F_{3,18}=2.6$, $P=0.0820$	$F_{3,18}=3.3$, $P=0.0452$

Abbreviations: ANOVA, analysis of variance; EE, energy expenditure; NREM, non-rapid eye movement sleep; REM, rapid eye movement sleep; RQ, respiratory quotient; SPA, spontaneous physical activity.

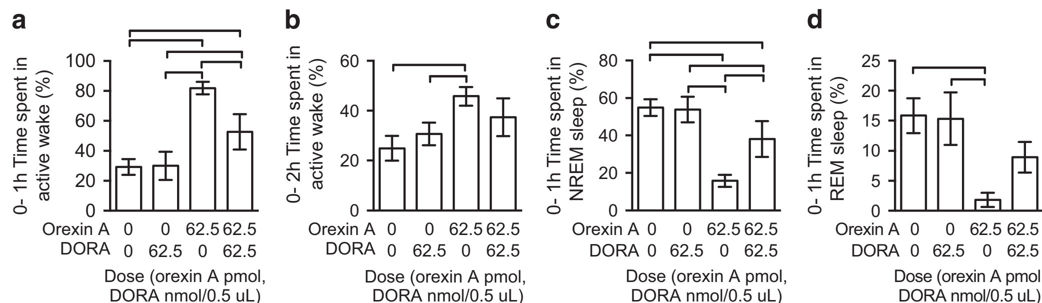

Figure 2. Orexin-A infusion in the VLPO significantly increases time spent in (**a** and **b**) AW and decreases time spent in (**c**) NREM sleep and (**d**) REM sleep compared with control. Pretreatment with the DORA (TCS-1102, DORA) in the VLPO reduces orexin-A-stimulated increases in (**a**) time spent in AW and the orexin-A-stimulated reduction in (**c**) time spent in NREM sleep. Data are expressed as mean ± s.e.m.; $N=7$. Brackets indicate bars that are significantly different from each other ($P<0.05$). Note different scaling on y axis.

RESULTS

The DORA prevents the effects of orexin-A in the VLPO on sleep–wake states

We hypothesized that the DORA in the VLPO would reduce orexin-A-stimulated increases in arousal and reductions in sleep. The DORA reduced the effect of orexin-A on sleep–wake states and improved sleep quality (Figure 2 and Table 1). Orexin-A significantly increased wake and AW 1 and 2 h post injection compared with control (Figures 2a and b, $P<0.05$ for all comparisons). Orexin-A significantly reduced sleep (total, NREM sleep and REM sleep) 1 h post injection relative to control (Figures 2c and d, $P<0.05$ for all comparisons). The DORA reduced the orexin-A-stimulated increase in wake and AW (Figures 2a, $P<0.05$ for all comparisons). Similarly, the DORA

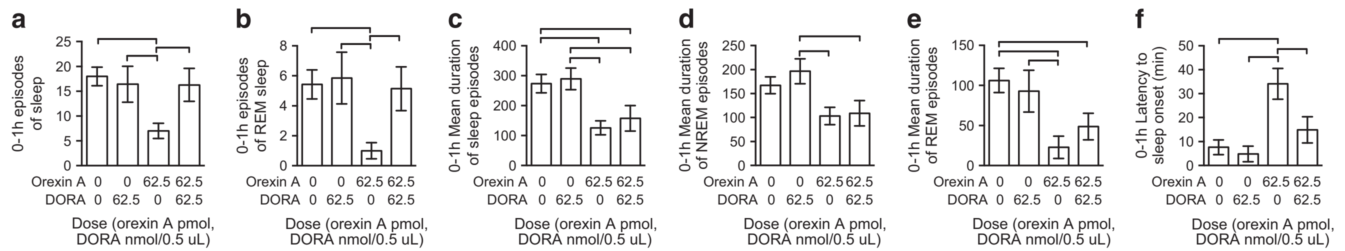

Figure 3. Orexin-A in the VLPO significantly (**f**) prolongs the latency to sleep onset and reduces (**a**) episodes of sleep, (**b**) episodes of REM sleep, (**c**) mean duration of sleep episodes and (**e**) mean duration of REM sleep episodes but not (**d**) mean duration of NREM sleep episodes ($P=0.058$) compared with control. Pretreatment with the DORA (TCS-1102, DORA) in the VLPO significantly reversed the orexin-A-stimulated increase in the (**a**) episodes of sleep and (**b**) episodes of REM sleep and the latency to sleep onset. Data are expressed as mean ± s.e.m.; $N=7$. Brackets indicate bars that are significantly different from each other ($P<0.05$). Note different scaling on y axis.

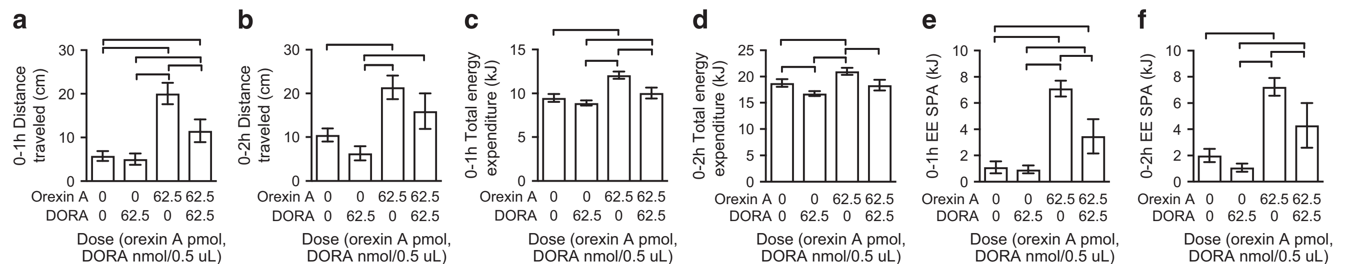

Figure 4. Pretreatment with the DORA (TCS − 1102, DORA) in the VLPO significantly reduced orexin-A stimulated increases in (**a** and **b**) distance traveled and (**c** and **d**) total EE and (**e** and **f**) EE during SPA (**d**). The DORA alone significantly reduced (**d**) total EE compared with control 2 h post injection. Data are expressed as mean ± s.e.m.; $N=7$. Brackets indicate bars that are significantly different from each other ($P<0.05$). Note different scaling on y axis.

Table 2. Components of total energy expenditure ($kJ\ h^{-1}$) for each treatment during the 0–1, 1–2 and 0–2 h post injection time period

Components of total EE	*Treatment*			
	Control/control	*DORA/control*	*Control/orexin-A*	*DORA/orexin-A*
EE active wake				
0–1 h	10.04 ± 0.67	9.24 ± 0.53	12.47 ± 0.40^{a,b}	10.82 ± 0.52^{b,c}
1–2 h	9.54 ± 0.50	7.85 ± 0.19	8.85 ± 0.42	8.87 ± 0.66
0–2 h	9.87 ± 0.44	8.66 ± 0.33^{a}	12.17 ± 0.40^{a,b}	10.23 ± 0.51^{b,c}
EE rest				
0–1 h	9.72 ± 0.62	8.98 ± 0.45	12.23 ± 0.36^{a,b}	10.20 ± 0.61^{b,c}
1–2 h	9.44 ± 0.49	7.82 ± 0.20^{a}	8.84 ± 0.42	8.50 ± 0.42
0–2 h	9.41 ± 0.43	8.41 ± 0.26^{a}	11.38 ± 0.27^{a,b}	9.54 ± 0.50^{b,c}
EE NREM sleep				
0–1 h	9.34 ± 0.32	8.89 ± 0.25	11.25 ± 0.59^{a,b}	9.62 ± 0.56^{c}
1–2 h	9.23 ± 0.52	7.91 ± 0.23^{a}	8.98 ± 0.33^{b}	8.13 ± 0.33^{a}
0–2 h	9.28 ± 0.35	8.39 ± 0.20^{a}	9.36 ± 0.32^{b}	8.53 ± 0.30^{c}
EE REM sleep				
0–1 h	8.95 ± 0.32	8.12 ± 0.15	9.69 ± 0.47	8.38 ± 0.24
1–2 h	9.01 ± 0.53	7.83 ± 0.22	8.81 ± 0.34	8.06 ± 0.27
0–2 h	8.99 ± 0.36	7.97 ± 0.19^{a}	8.84 ± 0.34^{b}	8.22 ± 0.25

Abbreviations: DORA, dual orexin receptor antagonist; EE, energy expenditure; NREM, non-rapid eye movement sleep; REM, rapid eye movement sleep. Data expressed as mean ± s.e.m.; $N=7$. $^{a}P<0.05$ as compared with control/control. $^{b}P<0.05$ as compared with DORA/control. $^{c}P<0.05$ as compared with control/orexin-A.

reversed the orexin-A-stimulated reduction in sleep and NREM sleep but not REM sleep 1 h post injection (Figures 2c–d, $P<0.05$ for all comparisons except REM sleep: $P=0.06$). At 2 h post injection, the DORA failed to significantly reduce orexin-A-stimulated increase in wake and AW (Figures 2b, $P>0.05$ for all comparisons, data not shown).

The DORA reduced the effect of orexin-A on sleep quality (Figure 3 and Table 1). Pretreatment with the DORA reversed orexin-A-stimulated reductions in the number of total and REM sleep episodes ($P<0.05$ for all comparisons, Figures 3a and b). The DORA failed to significantly reduce the mean duration of sleep episodes 1 h post injection ($P>0.05$, Figure 3c). Orexin-A significantly prolonged the latency to sleep onset, while the DORA blocked the orexin-A-stimulated increase in the latency to sleep onset 1 h post injection ($P<0.05$ for all comparisons; Figure 3f).

The DORA prevents the effects of orexin-A in the VLPO on SPA and total EE

We hypothesized that blocking OXRs would reduce baseline and orexin-A-stimulated increase in SPA and total EE. The DORA reduced the effects of orexin-A on SPA and total EE (Figure 4 and Table 1). Orexin-A significantly increased SPA and total EE 1 and 2 h post injection (Figures 4a–d, $P < 0.05$ for all comparisons). The DORA significantly blocked orexin-A-stimulated increases in total EE (Figures 4c and d) and reduced SPA stimulated by orexin-A 1 but not 2 h post injection (Figures 4a and b). The DORA alone significantly reduced baseline total EE 2 h post injection relative to control (Figure 4d).

The DORA prevents the effects of orexin-A on components of total EE

We hypothesized that the DORA would block orexin-A-stimulated effects on EE during SPA, wake, rest and sleep as well as reduce baseline levels of total EE and its components. Orexin-A significantly increased EE during SPA, AW, rest and during NREM sleep compared with control 1 h post injection (Figure 4e and Table 2; $P < 0.05$ for all comparisons). At 2 h post injection, orexin-A significantly increased EE during SPA, AW and rest (Figure 4f and Table 2; $P < 0.05$ for all comparisons).

The DORA significantly reduced orexin-A stimulated increases in EE during SPA, AW, rest and NREM sleep 1 and 2 h post injection (Figures 4e and f and Tables 1 and 2; $P < 0.05$ for all comparisons). The DORA alone significantly reduced EE during AW, REM sleep, rest and NREM sleep relative to control (Tables 2; $P < 0.05$ for all comparisons).

DISCUSSION

Previously, we showed that orexin-A in the VLPO decreased sleep and enhanced wakefulness, SPA, total EE and EE during SPA, with no effect on feeding.[24] Our data here verify and extend those results.[22,24] The current study demonstrates that orexin-A in the VLPO affects individual components of baseline total EE, while blockade of both OXRs reduces stimulation of EE (total and individual components) by orexin-A. In addition, baseline total EE and its components were lower after OXR blockade. The stimulatory effect of orexin-A on total EE is due to enhancement of several individual components of total EE (i.e. resting EE and EE during SPA, wake and NREM sleep; Figure 4 and Table 2), and antagonizing both OXRs prevents these effects. Finally, we demonstrate that DORA blockade of endogenous OXR stimulation in the VLPO reduces total EE by decreasing resting EE and EE during wake, AW, NREM sleep and REM sleep. These data are novel and show that the stimulatory effect of orexin-A in the VLPO on total EE involves an increase in several components of EE. In addition, blocking OXRs lowers total EE through reductions in non-SPA-related EE independent of the time spent asleep. Given the role of orexins in obesity resistance,[34] narcolepsy[7] and insomnia,[35] these data have implications for developing effective treatments to combat obesity and sleep disorders.

Orexin-A in the VLPO significantly increased time in AW and decreased sleep (Figure 2). These data parallel results from other studies of orexin-A administration in the VLPO[22,24] and other brain nuclei central to sleep–wake regulation.[9,36] Prolongation of wakefulness by orexin-A was due to a reduction in the duration and number of sleep episodes[22] (Figure 3). We have previously shown[24] a positive effect of orexin-A in the VLPO on SPA, total EE and EE during SPA. The results here (Figure 4) are concordant with those data[24] and agree with others.[9,11,12,37,38]

Based on our work[24] and others, which shows that orexin-A increases resting metabolism,[12] body temperature,[39] brown adipose tissue thermogenesis[40] and autonomic function,[41] we hypothesized that orexin-A in the VLPO would increase other components of total EE. Our data show that the stimulatory effect of orexin-A in the VLPO on total EE is due to other EE components in addition to the EE during SPA, which is a novel finding. Moreover, the metabolic and sleep–wake effects of orexin-A can be dissociated since orexin-A decreased time spent in NREM sleep and REM sleep but only increased EE during NREM sleep. Finally, the latter suggests that orexin-A in this brain site may have an independent effect on EE during NREM sleep versus REM sleep. Taken together, our data suggest that orexin-A contributes to negative energy balance by increasing multiple components of total EE in addition to SPA.[42]

Next, we tested whether blocking both OXRs reduced the aforementioned effects of orexin-A. The DORA reduced orexin-A-stimulated increases in AW, SPA, total EE, resting EE and EE during SPA, AW and NREM sleep. Moreover, the DORA reduced orexin-A-induced reductions in NREM sleep and sleep quality. The reduction of orexin-A-induced AW by the DORA agrees with our prior work with this specific antagonist[24] and others who have reported that another DORA, Almorexant, reversed SPA stimulated by orexin-A.[16] Antagonism of OXR1 also reverses orexin-A-stimulated SPA and total EE.[37] Taken together, these data highlight the importance of OXR stimulation for increasing arousal and total EE. Future studies are needed to determine whether either receptor has a more prominent role in sleep–wake regulation or EE, and whether each receptor contributes equally to these processes.

Based on the efficacy of DORAs to increase sleep[14,43] and suppress SPA,[15] we hypothesized that preventing endogenous OXR stimulation with the DORA alone would reduce total EE mainly through effects on SPA. As expected, blocking endogenous OXRs reduced total EE and several EE components. Most interestingly, the DORA alone failed to significantly alter sleep time or reduce SPA and its resultant EE, a result that contrasts with previously published data.[14–16] This discrepancy may be related to study design since the type and dose of the DORA used, animal species tested, route of administration and the measurement duration differed between our study and these previous reports.[14–16] We also show that the DORA alone did not reduce baseline SPA or its corresponding EE, which agrees with studies that showed DORAs (Merck DORA-12 and Almorexant) had no effect on rotarod activity,[44] but contrasts with another that reported that a DORA (Merck DORA-1) reduced baseline dark cycle locomotion.[15] The fact that the DORA failed to reduce baseline SPA here may be due to a ‘floor effect’ for physical activity, as the injections were performed in the early light cycle when SPA is inherently low. It is plausible that the DORA injections in the early dark cycle may reduce SPA, as shown previously,[15] and thus its resultant EE. Interestingly, baseline total EE was significantly lower in rats treated with the DORA alone 2 h post injection (Figure 4d), which suggests that factors aside from SPA contribute to the DORA-induced decrease in baseline total EE.

Blocking endogenous OXR stimulation in the VLPO with the DORA alone significantly reduced EE during AW, rest, NREM sleep and REM sleep. This demonstrates that blocking endogenous OXR stimulation may reduce baseline total EE independent of SPA, as the DORA alone had no effect on SPA or its corresponding EE. More than 50% of total daily EE is due to resting EE.[45] Thus, the DORA-induced reductions in resting EE and EE during sleep 2 h post injection are significant factors contributing to the overall reduction in total EE. Moreover, that the DORA alone had no effect on time in AW, REM sleep or EE during sleep, yet reduced their associated EE suggests that rats simply expended less energy in response to this specific DORA regardless of whether they were awake or asleep. The latter is significant since individuals with insomnia would be prescribed a DORA before bedtime to promote sleep. Hence, blocking endogenous OXR stimulation with the DORA alone may reduce EE during sleep in these individuals. The fact that baseline total EE and several components (EE during AW, NREM sleep, REM sleep and rest) were significantly lower after

administration of the DORA alone underscores the importance of quantifying components of total EE, and the potential effect of OXR antagonism on energy balance. We tested a low dose of the DORA; thus, it is plausible that higher doses would further reduce baseline total EE. The latter and our data therefore have implications for insomnia therapies as it is unclear whether long-term antagonism of OXRs would promote positive energy balance through reductions in total EE and favor weight gain. The effects of chronic OXR antagonism on food intake, total EE and weight gain remain to be tested.

The mechanism(s) underlying the physiological effects of orexin-A in the VLPO remain unclear. Orexin-A increases firing rates of arousal-promoting neurons, including the locus coeruleus noradrenergic cells,[9] the tuberomammilary nucleus histaminergic cells,[46] cholinergic neurons in the pedunculopontine and laterodorsal tegmental nuclei,[47] and the dorsal raphe serotonergic neurons.[48] Orexin-A also stimulates release of noradrenaline, histamine, acetylcholine and serotonin.[36,49–51] The arousal centers promote wakefulness to some extent by inhibiting γ-aminobutyric acid and galanin neurons in the VLPO.[52–55] Based on these data and OXRs in the VLPO, a hypothetical mechanism by which orexin-A in the VLPO may promote wakefulness is by enhancing the activity of arousal-promoting nuclei through binding to OXRs on the VLPO neurons directly, or to the terminals from arousal-promoting neurons. Orexin-A given in some brain areas (for example, after ventricular injection) has also been shown to influence autonomic outflow,[41,56] through increases in heart rate, mean arterial blood pressure and temperature in conscious[39,41] and anesthetized[39,57] rodents, and could be one mechanism underlying increases in resting EE by orexin-A. Thus, blocking both OXRs with a DORA alone would be expected to reduce resting EE. Yet, the DORA (Almorexant) had no effect on body temperature,[14] heart rate or mean arterial pressure[58] in normal rats independent of time of administration.

It is plausible that diffusion of orexin-A into the supraoptic nucleus contributed to our results as this brain site contains OXR1 protein,[59] orexin fibers[56] and expresses c-fos after ventricular orexin-A infusion.[56] However, no studies have discriminated between orexin-A action in the supraoptic nucleus versus VLPO on sleep–wake states or other end points reported here. Moreover, while supraoptic nucleus might be involved in circadian processes,[60] its precise role in sleep–wake remains undefined. Future studies should distinguish the metabolic and behavioral effects of orexin-A injections in the supraoptic nucleus and VLPO.

In conclusion, we show that blocking OXRs with a DORA reduces the effects of orexin-A in the VLPO on total EE and several of its components. We also show that blocking endogenous OXR stimulation by the DORA alone reduces baseline total EE primarily by reducing EE during rest and sleep. This is the first demonstration that stimulation and antagonism of OXRs has disparate effects on the components of total EE. Our results suggest that OXR stimulation may contribute to negative energy balance through increases in EE during SPA, rest and NREM sleep if orexin-A was given in the active period, whereas OXR blockade may contribute to positive energy balance by decreasing non-SPA related EE. These results imply that current therapies for insomnia may have unintended and likely unwanted effects on body weight.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Funding for this research and publication was supported by a Career Development Award-level 2 (F7212W to JAT) and Merit Award (5I01RX000441-04 to CMK and CJB) from the United States Department of Veterans Affairs Rehabilitation Research and Development Service, the National Institutes of Health-NIDDK (1R01DK100281-01A1 to CMK and CJB and 5P30DK05045619 to CJB), the United States Department of Agriculture (ARZT-1360220-H23-150 and ARZT-1372540-R23-131 to JAT), the University of Arizona Department of Nutritional Sciences DeBell Research Enhancement Award, the National Needs Fellowship (2014-38420-21799) and the University of Arizona College of Agriculture and Life Science Dean's Research Advisory Committee Research Enhancement Award.

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