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14. ABSTRACT
This grant proposal was based on the hypothesis that hypocretin (Hcrt) antagonists produce less functional impairment than benzodiazepine receptor agonists (BzRA) because BzRAs cause a general inhibition of neural activity whereas Hcrt specifically disfacilitates wake-promoting systems. During the funding period, we obtained several lines of evidence that were consistent with this hypothesis. First, we determined that the Hcrt antagonist almorexant (ALM) was most likely promoting sleep by antagonism of both Hcrt receptors 1 and 2 rather than only HcrtR2. Next, in tests of both spatial reference memory and spatial working memory, we found that rats treated with ALM performed far superior to those treated with the BzRA agonist zolpidem (ZOL) at equipotent doses in terms of sleep induction. Next, we found that this superior performance was likely due to the ability to activate wake-promoting nuclei in the presence of ALM but not ZOL. Furthermore, we explored the neural mechanisms underlying ALM-induced sleep and found that ALM, but not ZOL, requires an intact basal forebrain for maximum NREM-promoting efficacy and that ALM elicits a neurochemical profile more consistent with the transition to normal sleep than does ZOL. Furthermore, lesions of the wake-promoting noradrenergic locus coeruleus or histaminergic tuberomammillary nuclei compromised the hypnotic efficacy of ALM without affecting that of ZOL. Thus, Hcrt neurotransmission influences distinct aspects of NREM and REM sleep at different locations in the sleep-wake regulatory network. By selectively disfacilitating these subcortical wake-promoting populations, Hcrt antagonism effectively promotes sleep without negatively impacting cognitive performance and without globally blocking the capability for arousal.

15. SUBJECT TERMS
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

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FINAL REPORT

"Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance"

USAMRAA Grant W81XWH-09-2-0081

DR080789P1

9/1/09 to 8/31/15

Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component (W81XWH-09-2-0080; Thomas Neylan, M.D., Principal Investigator) was designed to establish whether ALM is indeed superior to ZOL in neurocognitive tests, the animal studies (W81XWH-09-2-0081; Thomas Kilduff, Ph.D., Principal Investigator) compared the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

2a. Assessment of Almorexant effects on spatial reference memory in rats.
Status: Data collection and analysis COMPLETED; papers published (Morairty et al., 2012; Morairty et al., 2014) (**Appendices 11 and 12**).

2b. Assessment of Almorexant effects on spatial working memory in rats:
Status: Data collection and analysis COMPLETED; paper published (Morairty et al., 2014) (**Appendix 12**).

2c. Assessment of Almorexant effects on psychomotor vigilance in rats
Status: Data collection completed; analysis ongoing (see below).

2d. Synthesis of ALM (months 1-4).
Status: COMPLETED.

Task 2a and 2b: Tasks 2a and 2b have been completed and an article entitled "The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats" was published in *Frontiers in Neuroscience* in January, 2014 (see **Appendix 12**).

Progress – Task 2c: The studies assessing the effects of ALM in the rodent psychomotor vigilance (rPVT) have been completed. Our results are described below.

Methods: The general protocol for the rPVT is as follows (**Figure 1**). Rats were motivated to perform the operant rPVT task for water reinforcements by having water

unavailable to them for 23 h prior to all operant training and testing. Rats were gradually acclimated to the water restriction schedule over several days by reducing the amount of time

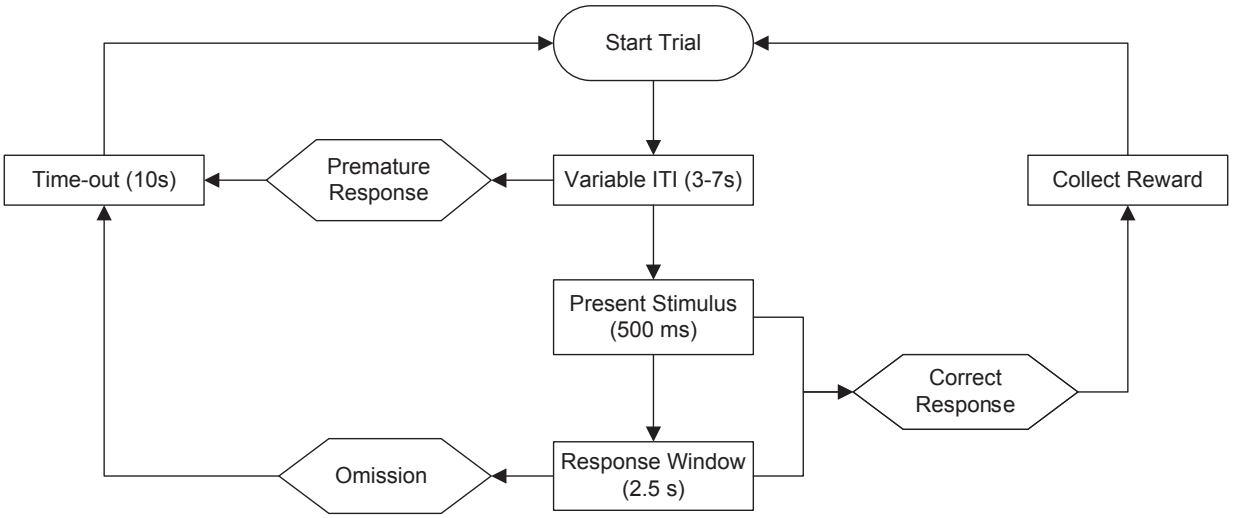

Figure 1. Flowchart of the rodent Psychomotor Vigilance Task. During the task, the inter-trial interval (ITI) varied from 3 to 7 s in 1 s increments in a quasi-random fashion (equal density of intervals throughout session). Responses during the ITI (a 'premature response') or failing to respond within 3 s of the stimulus presentation (an omission) were treated as errors and resulted in a 10 s 'time-out' (housing light extinguished and absence of trials). At the end of the 10 s time-out, the house light was re-illuminated and a new ITI started.

each day that water was available in the home cage. rPVT training took 3 mo to complete. Following this 3 mo training period, rats that did not meet criteria (> 100 correct responses per test session) were removed from the study. rPVT testing consisted of a stimulus light on for a duration of 0.5 s followed by a 3 s response period. The intertrial interval varied between 3-7 s. Errors resulted in a 10 s “time out” period during which the dim house lights were turned off. Test measures were the following:

- Correct responses (**CR**): Responding during stimulus presentation or within the response window.
- Omission (**OM**): Failure to respond within the 3 s window of opportunity.
- Premature errors (**PE**): Responding during the inter-trial interval.
- Response latencies (**RL**): Time from stimulus onset to a correct response.
- Numbers of trials: Total number of trials per session.
- Number of responses: Number of entries in the reward trough (data not shown).
- Lapses: Trials in which response latencies were >2x the average basal response latency for each rat.

Results: Seventeen rats were implanted with telemetry for devices for EEG recordings. Of these, 4 rats did not meet criteria following 3 mo of training and were removed from the study. We anticipated that up to a third of the rats might not meet criteria, so these results were expected. In addition, 2 rats had transmitter malfunctions prior to completion of the study and could not be included in our results. Therefore, 11 rats completed the rPVT study.

When the testing was about to begin, rats were acclimated to the dosing procedure by administration of 1 ml of VEH (p.o.). However, when we examined the performance following

this dose of VEH, we found a significant decline in all rPVT measure. Therefore, we reformulated the VEH solution using a base of physiological saline rather than just H_2O. This reformulation was effective at keeping the rats' performance in the rPVT above minimum criteria following dosing with VEH.

When the experiments were initiated, it became clear very early on that there were significant deficits in performance following ZOL at 100 mg/kg, p.o. Some rats had very few responses to the stimulus following ZOL. In addition, ALM-treated rats showed a noticeable deficit compared to VEH. Therefore, we added 2 additional conditions, ALM and ZOL at 30 mg/kg (p.o.). These additional concentrations of ALM and ZOL have been shown to be sleep-promoting but at more moderate levels compared to 100 mg/kg doses.

While performance in the rPVT declined following ALM and ZOL at both concentrations, the magnitude of the decline was significantly greater following ZOL (**Figure 2**). All rPVT performance measures decreased significantly following ZOL administration. Following ZOL, CR and the number of trials decreased while OM, response latencies and lapses increased. Interestingly, the number of PE decreased; following ZOL, rats were simply engaging less in the task. While ALM showed a decrease in sustained attention (decreased CR and the number of trials, increased OM and lapses), no impairment was seen in RL or PE. Further, the effects on CR, OM, the number of trials and lapses were greater following ZOL than ALM.

To investigate deeper into rPVT performance, we determined the density distributions for response latencies following all conditions (**Figure 3**). For both VEH and ALM, most responses occurred in less than 0.5 s. and the density distribution patterns of the VEH and ALM were similar. Following ZOL, however, the density distribution showed a much broader distribution across the response period. These data show that rats performed equally as well following ALM as following VEH, while responses often occurred more slowly in the presence of ZOL.

Changes in rPVT performance could not be attributed to prior sleep history. As can be seen in **Figure 4**, rats slept equivalent amounts for the hour prior to testing following ALM and ZOL. However, while the EEG power spectra during NREM sleep following VEH and ALM were indistinguishable (**Figure 5**), ZOL was followed by very large changes across the entire NREM EEG power spectrum (**Figure 6**). While the full meaning of such changes in the EEG power spectrum is yet to be understood, these data support the hypothesis that ALM produces physiological sleep while ZOL produces generalized CNS inhibition that results in a pharmacological, rather than physiological, sleep state.

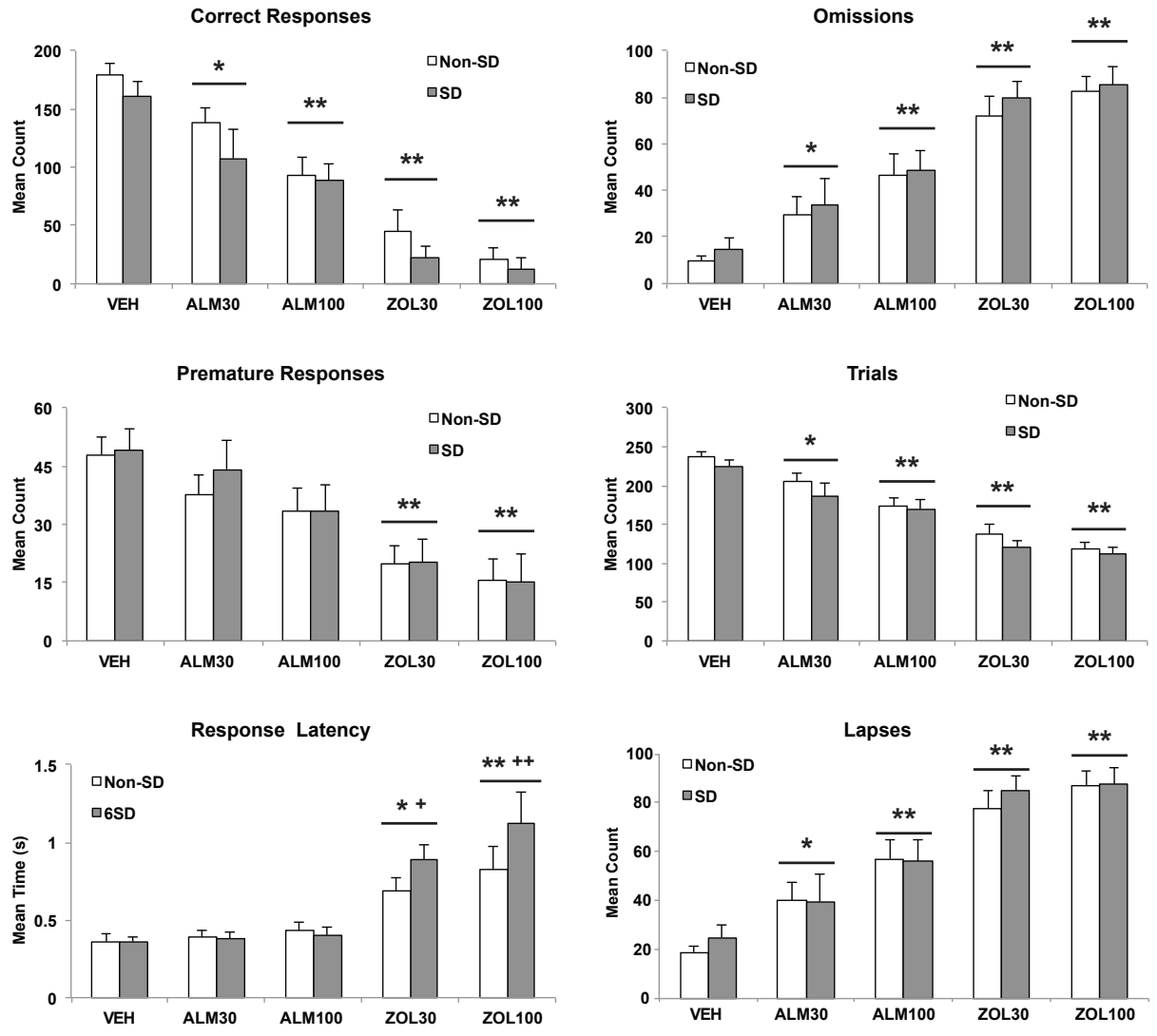

Figure 2. rPVT outcome measures. rPVT performance decreased significantly across all measures following ZOL administration. While ALM showed a decrease in sustained attention (decreased CR, increased OM and lapses), no impairment was seen in RL. Data shown as group mean ± SEM (n = 9–10). Multiple comparisons vs. control group (Bonferroni t-test): *= $p < 0.05$ significantly different from vehicle condition. ** = $p < 0.01$ significantly different from vehicle condition. + = $p < 0.05$ significantly different SD condition within drug treatment. ++ = $p < 0.01$ significantly different SD condition within drug treatment.

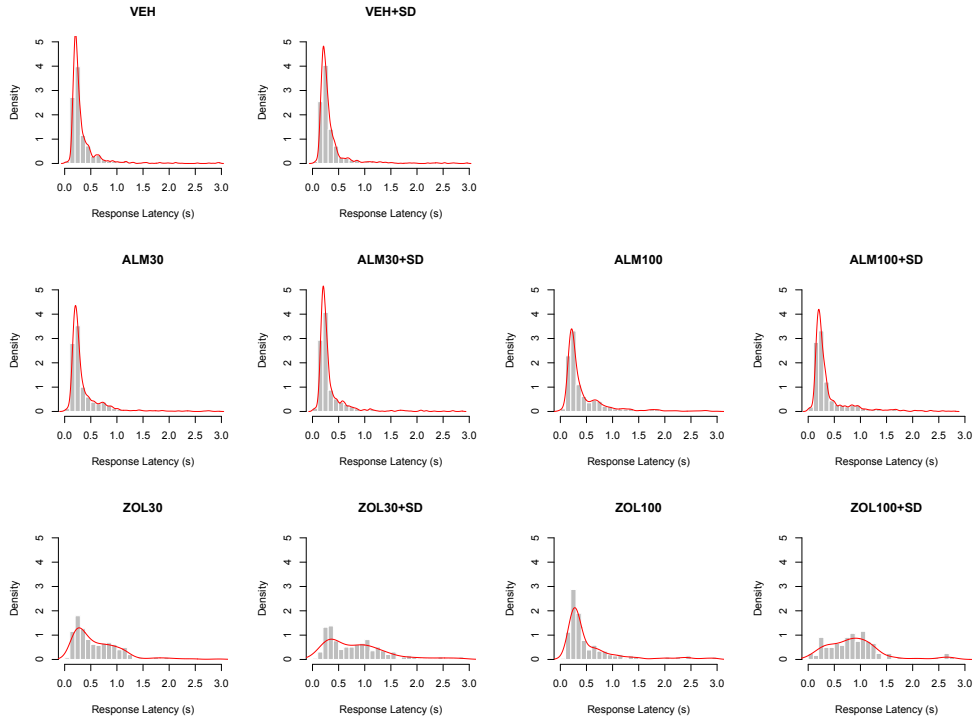

Figure 3. Density distributions for the response latencies in each test condition. RL distributions are similar for VEH and Almorexant (ALM) following either baseline or SD conditions. However, ZOL administration shifted the RL distributions following both baseline and SD conditions.

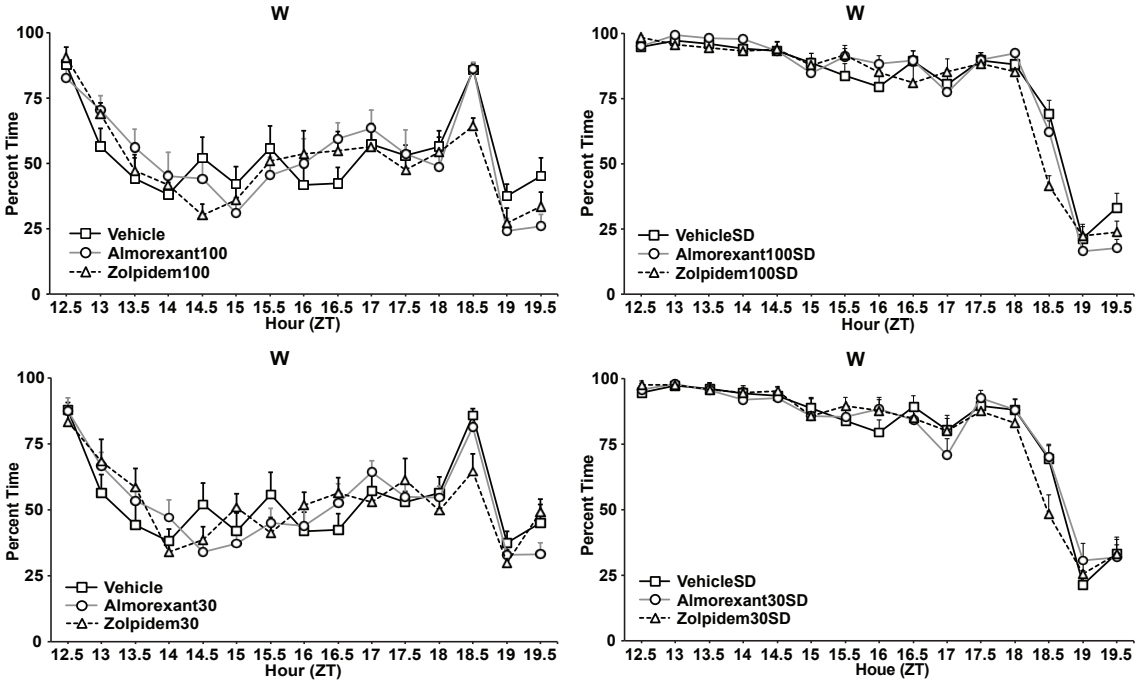

Figure 4. Effects of Almorexant and Zolpidem (each at 100 mg/kg and at 30 mg/kg po) on time spent awake under baseline conditions (left panel) or following 6 h of SD (right panel) during the active phase (lights off). Note that for the 60 min prior to rPVT test, the doses of Almorexant and Zolpidem were equally effective at inducing sleep.

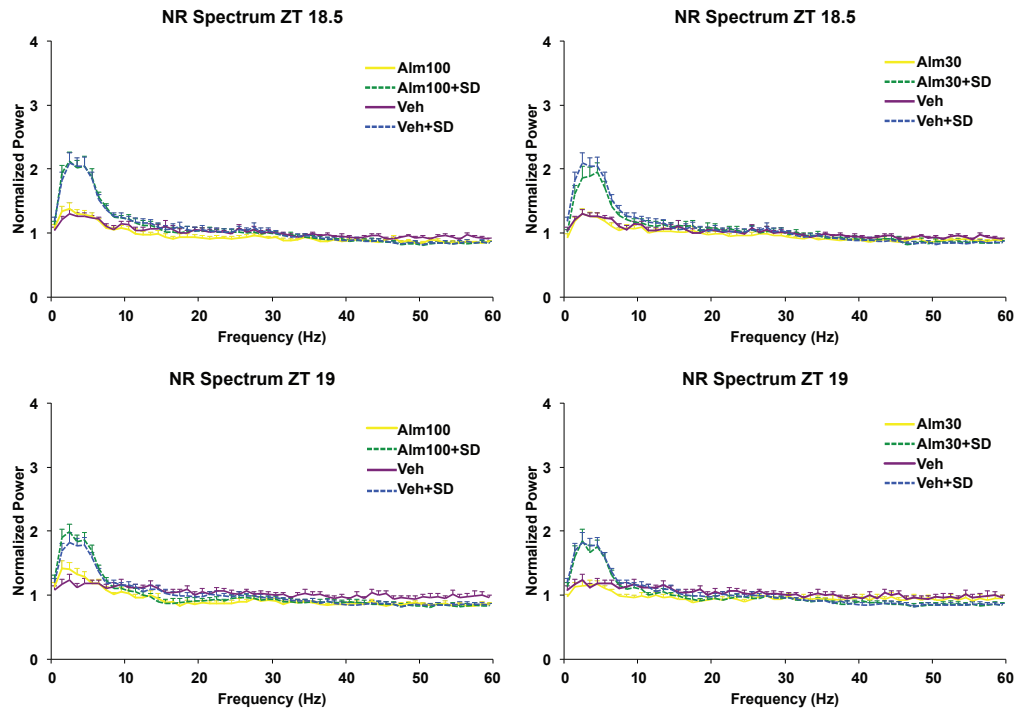

Figure 5. Rats showed no significant differences in EEG spectra during NREM sleep at 30 min (top) or 60 min (bottom) following administration of Almorexant (Alm) when compared to vehicle (Veh).

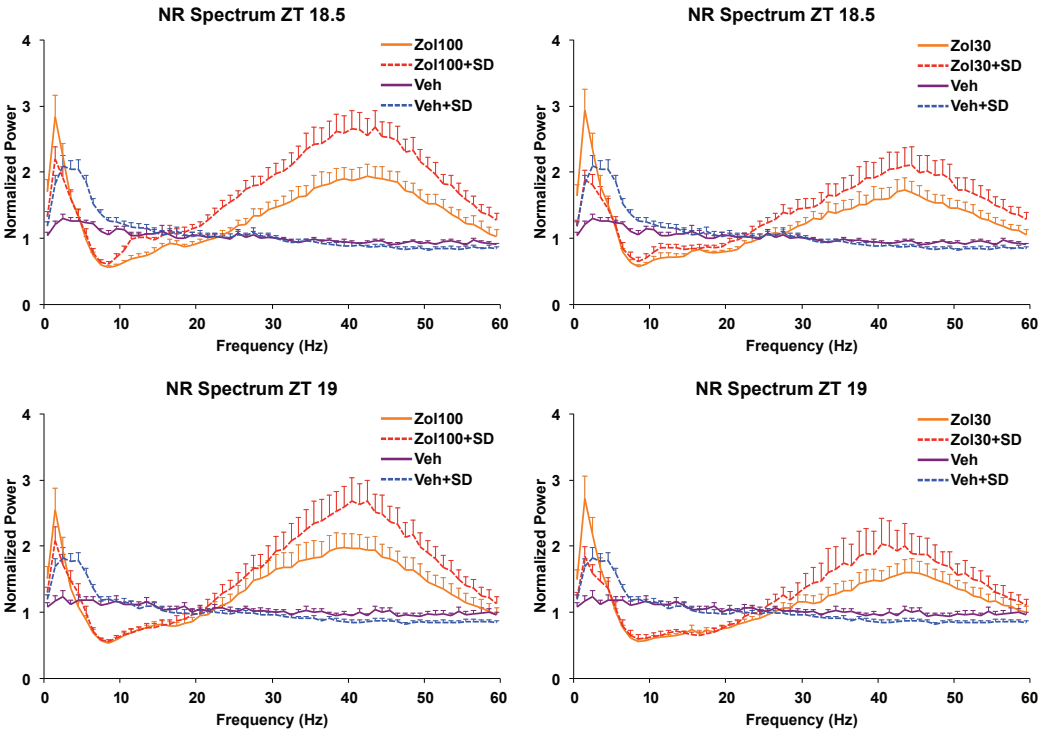

Figure 6. Rats showed significant alterations in EEG spectra during NREM sleep (0-60 Hz at 1 Hz resolution) 30 min (top) and 60 min (bottom) after administration of Zolpidem (Zol; left panel: 100 mg/kg; right panel: 30mg/kg) compared to vehicle (Veh). These changes appear to be dose-dependent and are further potentiated following 6 h of sleep deprivation (SD) during the active phase.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*

3a. Double-label immunohistochemistry with Fos and phenotypic markers.
Status: COMPLETED; Parks et al. manuscript in press in *Neuropsychopharmacology* (Parks et al., 2015) (**Appendix 15**).

3b. Assessment of hypnotic efficacy in saporin-lesioned rats.

3b.1 Status: Tuberomammillary nucleus lesion study -- COMPLETED; manuscript to be submitted in December, 2015 (**Appendix 16**).

3b.2 Status: Locus coeruleus lesion study-- data collection COMPLETED; manuscript to be submitted in December, 2015 (**Appendix 16**).

3b.3 Status: Basal forebrain lesion study COMPLETED; manuscript published in *Brain Structure and Function* (Vazquez-DeRose et al., 2014) (**Appendix 13**).

Task 3a: The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) may promote sleep through selective disfacilitation of wake-promoting systems whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through general inhibition of neural activity. Previous studies have indicated that HcrtR antagonists cause less functional impairment than BzRAs. To gain insight into the mechanisms underlying these differential profiles, we compared the effects of ALM and ZOL on functional activation of wake-promoting systems at doses equipotent for sleep induction. Sprague-Dawley rats, implanted for EEG/EMG recording, were orally administered vehicle (VEH), 100mg/kg ALM, or 100mg/kg ZOL during their active phase and either left undisturbed or kept awake for 90 min after which their brains were collected. ZOL-treated rats required more stimulation to maintain wakefulness than VEH- or ALM-treated rats. We measured Fos co-expression with markers for wake-promoting cell groups in the lateral hypothalamus (Hcrt), tuberomammillary nuclei (histamine; HA), basal forebrain (acetylcholine; ACh), dorsal raphe (serotonin; 5HT), and singly-labelled Fos^+ cells in the locus coeruleus (LC). Following SD, Fos co-expression in Hcrt, HA, and ACh neurons (but not in 5HT neurons) was consistently elevated in VEH- and ALM-treated rats whereas Fos expression in these neuronal groups was unaffected by SD in ZOL-treated rats. Surprisingly, Fos expression in the LC was elevated in ZOL- but not in VEH- or ALM-treated SD animals. These results indicate that Hcrt signaling is unnecessary for the activation of Hcrt, HA, or ACh wake-active neurons, which may underlie the milder cognitive impairment produced by HcrtR antagonists compared to ZOL. See **Appendix 15** for details (Parks et al., 2015).

Task 3b.1: This year we completed the TMN lesion study using a modified injection protocol.

Methods: Adult male rats were injected bilaterally with ~300 nL of the neurotoxin saporin conjugated to Hcrt2 (Hcrt-SAP; 228ng/μL) using calibrated pulled glass micropipettes connected to a Picospritzer at -4.35mm AP, ± 0.8mm ML from bregma, and -9.3mm from dura. Rats were instrumented for EEG at this time and, following full recovery, were administered HPMC vehicle, ALM (30/100/300 mg/kg) or ZOL (10/30/100 mg/kg) p.o. in fully-balanced order at lights-out. Sleep EEG was scored for the first 6 h following dosing.

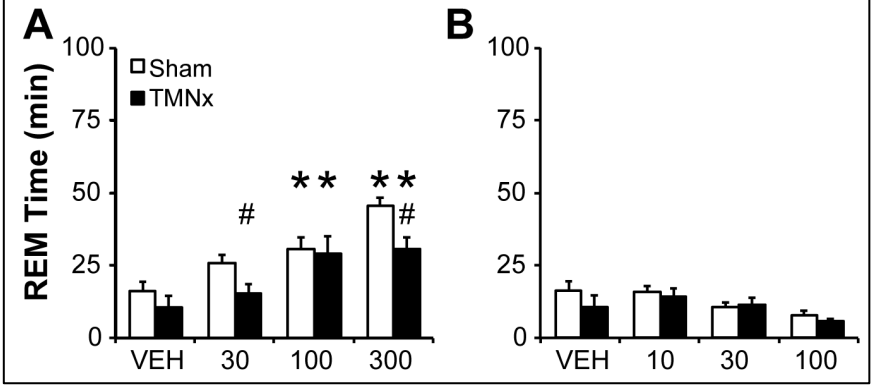

Figure 7. Cumulative REM sleep time for 6 hr after dosing with ALM (A), ZOL (B) or Veh at lights-out (ZT12). Vehicle doses are repeated in both graphs. TMNx attenuated ALM-induced increases in REM sleep time, but did not affect ZOL-induced REM sleep (*, $p < 0.05$ vs Vehicle (Bonferroni *post hoc*); #, $p < 0.05$ TMNx vs Sham (pairwise comparison *post hoc*). N=6 TMNx, N=7 Sham.

Results: Lesions of the histaminergic tuberomammillary neurons (TMNx) of the posterior hypothalamus (n=6) attenuated ALM-induced increases, but not ZOL-induced decreases, in REM sleep compared to Sham-lesioned rats (n=7; Figure 7). By contrast, both ALM and ZOL decreased wake time and increased NREM time, with no effect of lesion. Together with the results from Task 3b.2 and 3b.3, our findings lend further support to the hypothesis that Hcrt antagonism facilitates sleep by selectively inhibiting subcortical wake-promoting systems.

Task 3b.2: Following up on the last Progress Report, locus coeruleus lesions (LCx) attenuated ALM-induced but not ZOL-induced decreases in NREM sleep latency, and attenuated ALM-induced increases, but not ZOL-induced decreases, in REM sleep compared to Sham-lesioned rats. These results suggest that Hcrt inputs to the LC are a critical substrate for at least two of ALM's effects on sleep. A manuscript describing these results and results from Task 3b.1 will be submitted for publication in December, 2015 (see draft in **Appendix 16** for details).

Task 3b.3: Hypocretin/orexin (HCRT) neurons provide excitatory input to wake-promoting brain regions including the basal forebrain (BF). The dual HCRT receptor antagonist almorexant (ALM) decreases waking and increases sleep time. We hypothesized that HCRT antagonists induce sleep, in part, through disfacilitation of BF neurons; consequently, ALM should have reduced efficacy in BF-lesioned animals. To test this hypothesis, rats with bilateral BF lesions or sham-lesions were given oral ALM, the benzodiazepine agonist zolpidem (ZOL) or vehicle (VEH) at lights-out and the occurrence of sleep and wakefulness was assessed. ALM was less effective than ZOL at inducing sleep in BF-lesioned rats compared to intact animals. To investigate the underlying mechanism, BF adenosine (ADO), γ-amino-butyric acid (GABA), and glutamate (GLU) levels were determined via microdialysis from intact, freely-behaving rats following oral ALM, ZOL or VEH. ALM increased BF ADO and GABA levels during waking and mixed wake-sleep states, and mimicked sleep-associated increases in GABA under low and high sleep pressure conditions. ALM infusion into the BF also enhanced cortical ADO release, demonstrating that HCRT input is critical for ADO signaling in the BF. In contrast, ZOL had no effect on ADO and blocked sleep-associated GABA increases under low, but not high, sleep pressure. ALM elicited BF neurochemical profiles similar to those associated with normal sleep and required an intact BF for maximal efficacy, whereas ZOL elicited distinct neurochemical profiles and did not require functional contributions from the BF to induce sleep. These observations suggest that HCRT antagonism facilitates sleep by selectively inhibiting subcortical wake-promoting systems. See **Appendix 13** for details (Vazquez-DeRose et al., 2014).

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*

4a. Effects of ALM and ZOL on sleep-active brain areas.
Status: COMPLETED; manuscript published in *Neuropsychopharmacology* (Dittrich et al., 2015) (**Appendix 14**).

4b. BF adenosine (ADO) release in response to oral ALM and ZOL.
Status: COMPLETED; manuscript published in *Brain Structure and Function* (Vazquez-DeRose et al., 2014) (**Appendix 13**).

4c. Effects of BF microinjections of ALM and ZOL on sleep/wake and neurotransmitter release in the cerebral cortex.
Status: COMPLETED; manuscript published in *Brain Structure and Function* (Vazquez-DeRose et al., 2014) (**Appendix 13**).

Task 4a: Cortical interneurons, immunoreactive for neuronal nitric oxide synthase (nNOS) and the receptor NK1, express the functional activity marker Fos selectively during sleep. NREM sleep "pressure" is hypothesized to accumulate during waking and to dissipate during sleep. We reported previously that the proportion of Fos^+ cortical nNOS/NK1 neurons is correlated with established electrophysiological markers of sleep pressure. Since these markers covary with the amount of NREM sleep, it remained unclear whether cortical nNOS/NK1 neurons are activated to the same degree throughout NREM sleep or whether the extent of their activation is related to the sleep pressure that accrued during the prior waking period. To distinguish between these possibilities, we used hypnotic medications to control the amount of NREM sleep in rats while we varied prior wake duration and the resultant sleep pressure. Drug administration was preceded by 6 h of sleep deprivation ("high sleep pressure") or undisturbed conditions ("low sleep pressure"). We find that the proportion of Fos^+ cortical nNOS/NK1 neurons was minimal when sleep pressure was low, irrespective of the amount of time spent in NREM sleep. In contrast, a large proportion of cortical nNOS/NK1 neurons was Fos^+ when an equivalent amount of sleep was preceded by sleep deprivation. We conclude that, while sleep is necessary for cortical nNOS/NK1 neuron activation, the proportion of cells activated is dependent upon prior wake duration. See **Appendix 14** for details.

Tasks 4b and 4c: Hypocretin/orexin (HCRT) neurons provide excitatory input to wake-promoting brain regions including the basal forebrain (BF). The dual HCRT receptor antagonist almorexant (ALM) decreases waking and increases sleep time. We hypothesized that HCRT antagonists induce sleep, in part, through disfacilitation of BF neurons; consequently, ALM should have reduced efficacy in BF-lesioned animals. To test this hypothesis, rats with bilateral BF lesions or sham-lesions were given oral ALM, the benzodiazepine agonist zolpidem (ZOL) or vehicle (VEH) at lights-out and the occurrence of sleep and wakefulness was assessed. ALM was less effective than ZOL at inducing sleep in BF-lesioned rats compared to intact animals. To investigate the underlying mechanism, BF adenosine (ADO), γ-amino-butyric acid (GABA), and glutamate (GLU) levels were determined via microdialysis from intact, freely-behaving rats following oral ALM, ZOL or VEH. ALM increased BF ADO and GABA levels during waking and mixed wake-sleep states, and mimicked sleep-associated increases in GABA under low and high sleep pressure conditions. ALM infusion into the BF also enhanced cortical ADO release, demonstrating that HCRT input is critical for ADO signaling in the BF. In contrast, ZOL had no effect on ADO and blocked sleep-associated GABA increases under low, but not high, sleep

pressure. ALM elicited BF neurochemical profiles similar to those associated with normal sleep and required an intact BF for maximal efficacy, whereas ZOL elicited distinct neurochemical profiles and did not require functional contributions from the BF to induce sleep. These observations suggest that HCRT antagonism facilitates sleep by selectively inhibiting subcortical wake-promoting systems. See **Appendix 13** (Vazquez-DeRose et al., 2014) for details.

Task 6: *Utilize optogenetics and in vivo physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.*

6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL. COMPLETED; manuscript to be written. See text below and Heiss et al. (2015) abstract in **Appendix 10**.

6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons. Data collection ongoing (see below).

6c. Determine how ALM and ZOL affect the activity of cortical neurons.

Technology Development: We used *orexin-tTA; Tet-O ChR2(C128S)* mice that endogenously express the blue light-sensitive protein channelrhodopsin-2 (ChR2) in the Hcrt neurons. Upon repetitive stimulation, we observed a strong desensitization of the response to subsequent pulses of light. Since Hcrt is released during sustained firing of these neurons (Schone et al., 2014), we realized that this preparation would not be adequate to study the effects of ALM during Hcrt release. Therefore, we complemented our optogenetic approach with a pharmacogenetic approach using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). We used a modified form of the Gq-coupled human M3-muscarinic receptor, hM3Dq (Armbruster et al., 2007) that is activated only by the otherwise inert drug clozapine-N-oxide (CNO). After systemic administration of CNO, neurons expressing the hM3Dq receptor exhibit a concentration-dependent increase in firing rate for several hours. To transfect Hcrt neurons with DREADDs, we injected 350 nL of AAV-TetO-hM3Dq-mCherry in the lateral hypothalamus at 1mm L, 1.5mm A-P and 5mm D-V. This viral construct was obtained from Prof. Akihihro Yamanaka (Nagoya University, Japan).

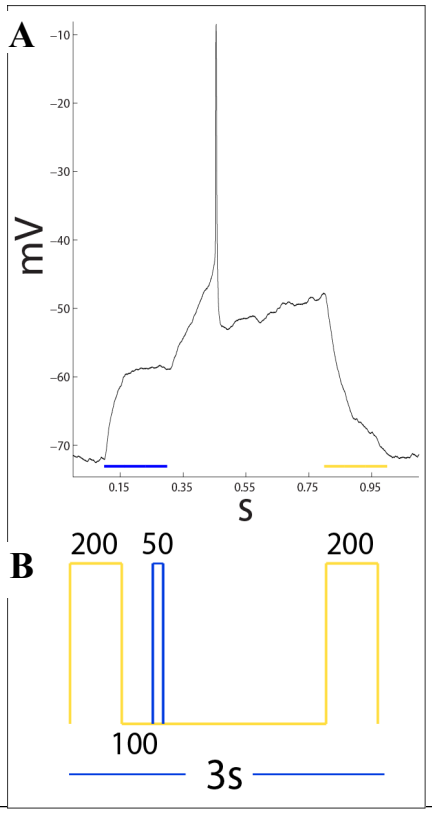

Figure 8. **A**. Depolarization of an Hcrt neuron from an *orexin-tTA; Tet-O ChR2(C128S)* mouse when illuminated by 100 ms pulse of blue and yellow light. **B**. For *in vivo* experiments, a 200 ms yellow pulse was followed by a 50 ms blue pulse and, 2650 ms later, by another 200 ms yellow pulse.

Progress - Task 6a: Optogenetic excitation of Hcrt neurons. *In vitro* recordings of Hcrt neurons of *orexin-tTA; Tet-O ChR2(C128S)* mice show that a pulse of blue light depolarizes these neurons (**Figure 8**). *ChR2(C128S)* encodes a "step function opsin" (SFO) in which Na^+ channels remain open after blue light stimulation until closed by yellow light stimulation. Thus, in **Figure 8A**, V_m remains depolarized after the blue pulse until a yellow pulse closes the channels. To prevent desensitization of the SFO and to ensure that all channels were closed before delivering the pulse of blue light, we stimulated mice with a 50 ms pulse of blue light flanked by 200 ms yellow pulses (see **Figure 8B**) once every 4 min. To

control for a possible effect of illumination itself, we interleaved this stimulus pattern with

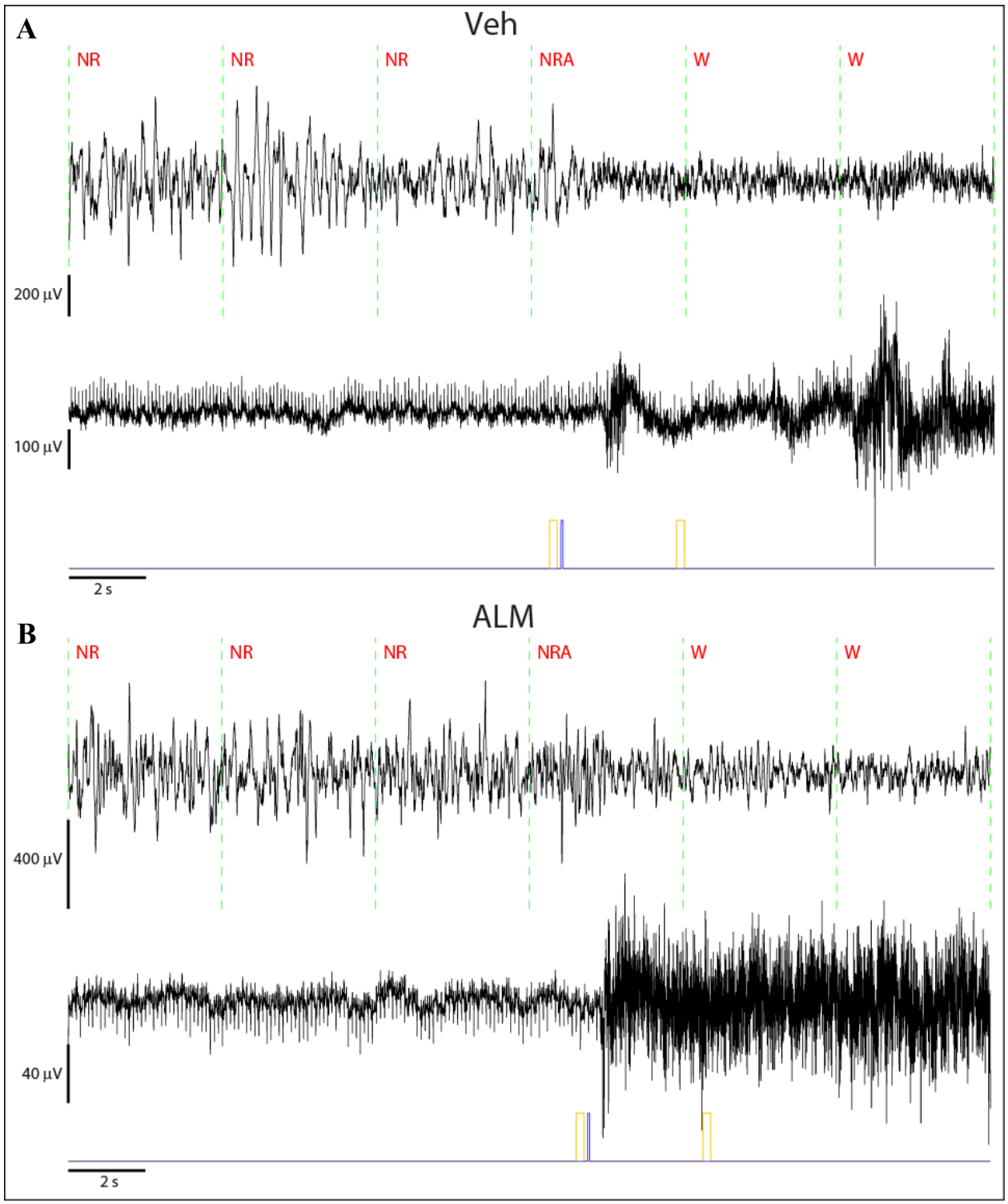

Figure 9. EEG/EMG recording of a mouse dosed with either Veh (**A**) or ALM (**B**) during optogenetic stimulation of Hcrt neurons. In each panel, the upper trace shows the EEG, middle trace is the EMG, and lower trace shows the light stimulus. As indicated in red font, 4 sec epochs were scored as either Wake (W), NREM sleep (NR) or NREM with artifact (NRA).

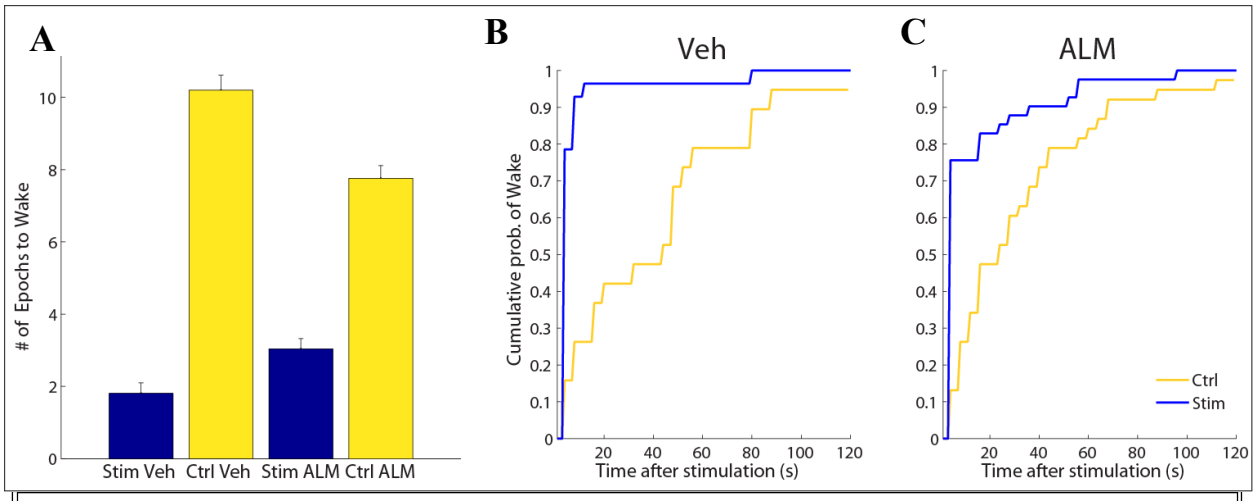

Figure 10. **A.** Number of 4s epochs elapsed after the light pulse until awakening occurred. Pulses were delivered during NREM sleep. **B, C.** Cumulative probability of wake after illumination when stimulated with blue light (Stim) or with control (Ctrl) yellow light after Veh (**B**) or ALM (**C**) treatment.

yellow stimulation in the same temporal sequence, i.e., a yellow 50 ms pulse was repeated once every 4 min. Thus, every 2 min, mice received either blue light flanked by yellow stimulation or only a yellow stimulus.

For *in vivo* studies, mice (N=4) were implanted with optical fibers above the Hcrt neuron field and with a DSI telemetry transmitter to enable EEG and EMG recording. Recordings started at ZT4 and animals were dosed with either vehicle (Veh) or 300 mg/Kg almorexant (ALM) at ZT5. The volume injected was 0.15 ml IP. One hour later, optogenetic stimulation commenced at ZT6 for 1 h and an additional hour of EEG/EMG was recorded.

Figure 9 shows the raw EEG, EMG and light stimulus in a mouse dosed with either Veh (above) or ALM (below). In both treatment conditions, mice tended to awaken after the blue pulse either briefly or for a prolonged period as depicted in **Figure 9**. Note that, in both cases, the stimulation and awakening occurred within the same 4s epoch. When the stimulus was delivered during NREM sleep, animals woke up within 4 s after the blue pulse in 79% and 76% of the cases for Veh and ALM, respectively. **Figure 10A** shows the average number of epochs that elapsed from stimulation during NREM sleep until mice (N=4) awoke. In Veh-injected mice, the latencies to awakening were 1.8±0.3 and 10.2±0.4 epochs for 50 ms blue and yellow light pulses, respectively. For ALM-treated mice, the latencies to awakening were 3±0.3 and 7.8±0.4 epochs for 50 ms blue and yellow light pulses, respectively. Blue and yellow stimulation caused a significant difference (U test, $P<0.01$) but there was no difference between drug treatments in the 4 animals tested to date.

Figure 10B and C show the cumulative probability of wake after stimulation with either blue (Stim) or yellow (Ctrl) light after Veh or ALM treatment. The fast rise of the Stim curve indicates that, in the large majority of cases, wakefulness occurred in the next epoch after blue stimulation irrespective whether the mice had been treated with either Veh or ALM. After the Ctrl stimulus, the latencies to awakening were much longer, suggesting that illumination *per se* does not induce awakening. The fast time course of the awakening and the absence of a clear difference in the presence of the Hcrt antagonist ALM suggests that the observed arousals are mediated by glutamate release from Hcrt neurons projecting to downstream wake-promoting brain areas.

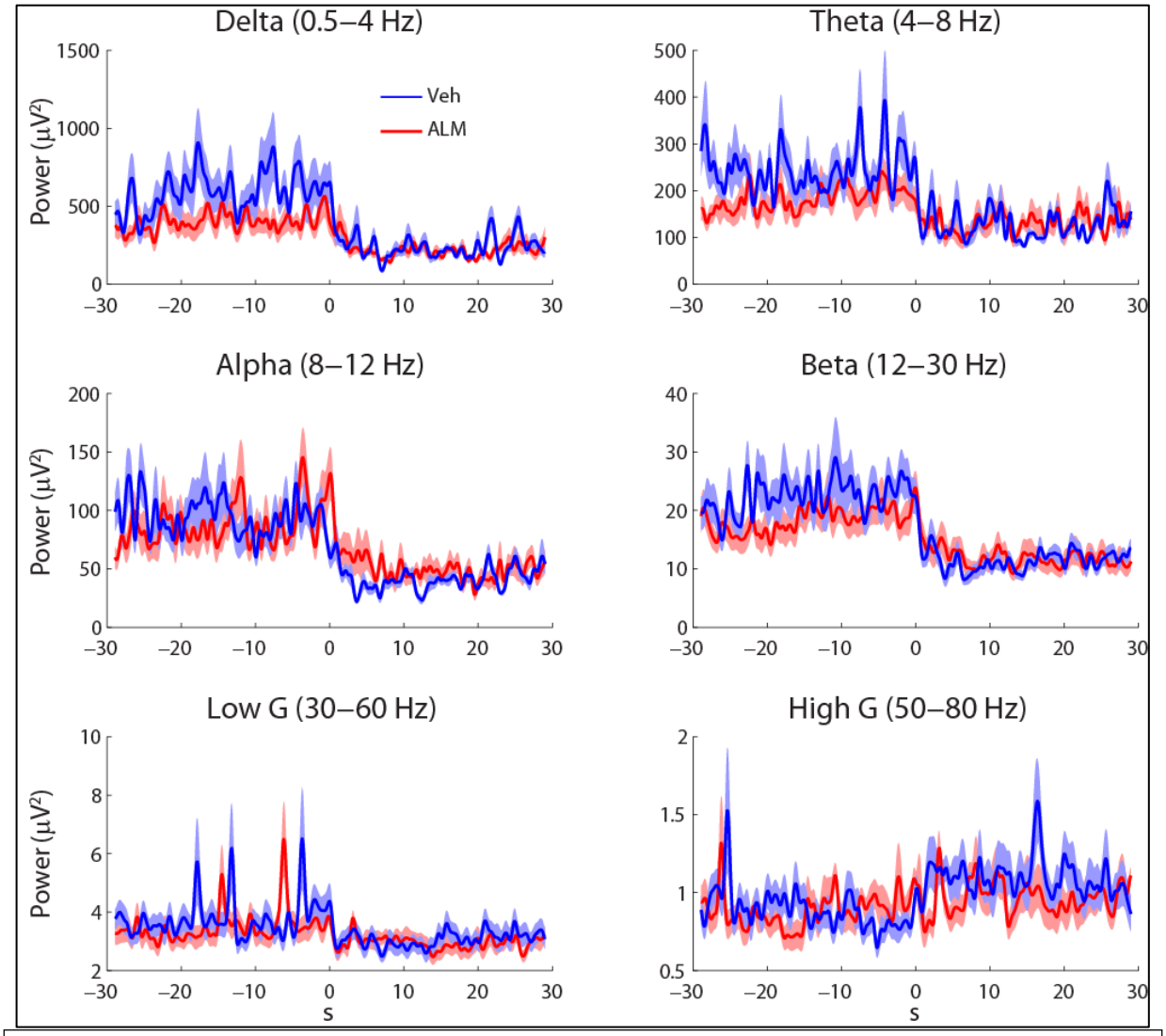

Figure 11. Time-frequency analysis for the average power in the standard EEG spectral bands. No significant difference was observed between ALM and Veh. Traces are centered on the time of blue light stimulation and the average power and SEM is depicted.

To further characterize the changes in EEG upon Hcrt activation, we performed time-frequency analysis using 2 s windows. To calculate the average power in the standard EEG frequency bands, the FFT was shifted in 100 ms steps around the time of stimulation during NREM sleep. **Figure 11** shows that, for all bands below 60 Hz, optogenetic stimulation produced the decrease in spectral power amplitude that is expected during a transition from sleep to wake. This type of plot allows visualization of the effect of Hcrt activation with greater time resolution in contrast to the coarse-grained analysis afforded by the 4s epoch classification shown in **Figure 10**. For both ALM and Veh, the change in spectral power is almost instantaneous at the time of stimulation, as reported when locus coeruleus (LC) neurons were activated by direct optogenetic excitation (Carter et al., 2010). This result suggests the hypothesis that Hcrt neuron-mediated glutamate release evoked firing of LC neurons that, in turn, resulted in a general arousal.

Pharmacogenetic excitation of Hcrt neurons. DREADD activation of Hcrt neurons during the light phase has been shown to increase the percentage of time awake during the first hour after injection from 44% to 70% (Sasaki et al., 2011). We attempted to assess the effect of the Hcrt peptide on arousal by injecting either ALM (200 mg/kg, i.p.), Veh or Zolpidem (20 mg/kg, i.p.) at ZT4 followed by either CNO (3 mg/Kg, i.p.) or saline at ZT5 in a counter-balanced manner, while EEG and EMG were recorded from ZT3 to ZT10. The protocol involved a total of 6 dosings per animal with at least 1 week of recovery between each dose. By injecting CNO, sustained firing of Hcrt neurons should be produced that should result in release of Hcrt peptides at downstream Hcrt projection sites. We are currently performing *in vitro* experiments as well as Fos immunohistochemistry to evaluate the efficacy of this preparation. **Figure 12A** shows the percentage of time awake during the 2h immediately after CNO injection (1 h after either Veh or ALM dosing). Surprisingly, we observed a much larger wake-promoting effect than what has been published by Sasaki et al. (97.5% vs 70%). In some cases, mice remained awake continously for more than 5 h! An even more striking result was the high level of wakefulness in the mice treated with ALM (79%). Despite the relatively high dose of ALM utilized, the reduction in CNO-induced arousal was non-significant (P=0.25, Mann-Whitney U test). These results suggest that most of the observed increase in arousal was not caused by Hcrt peptide release. **Figure 12B** shows that two mice remained awake almost continuously after CNO injection whether ALM was on board or not, while ALM attenuated CNO-induced arousal in the other two mice. Preliminary histological verification of DREADD transfection, shown in **Figure 12C** reveals that, although most Hcrt neurons within the range of the AAV injection are indeed

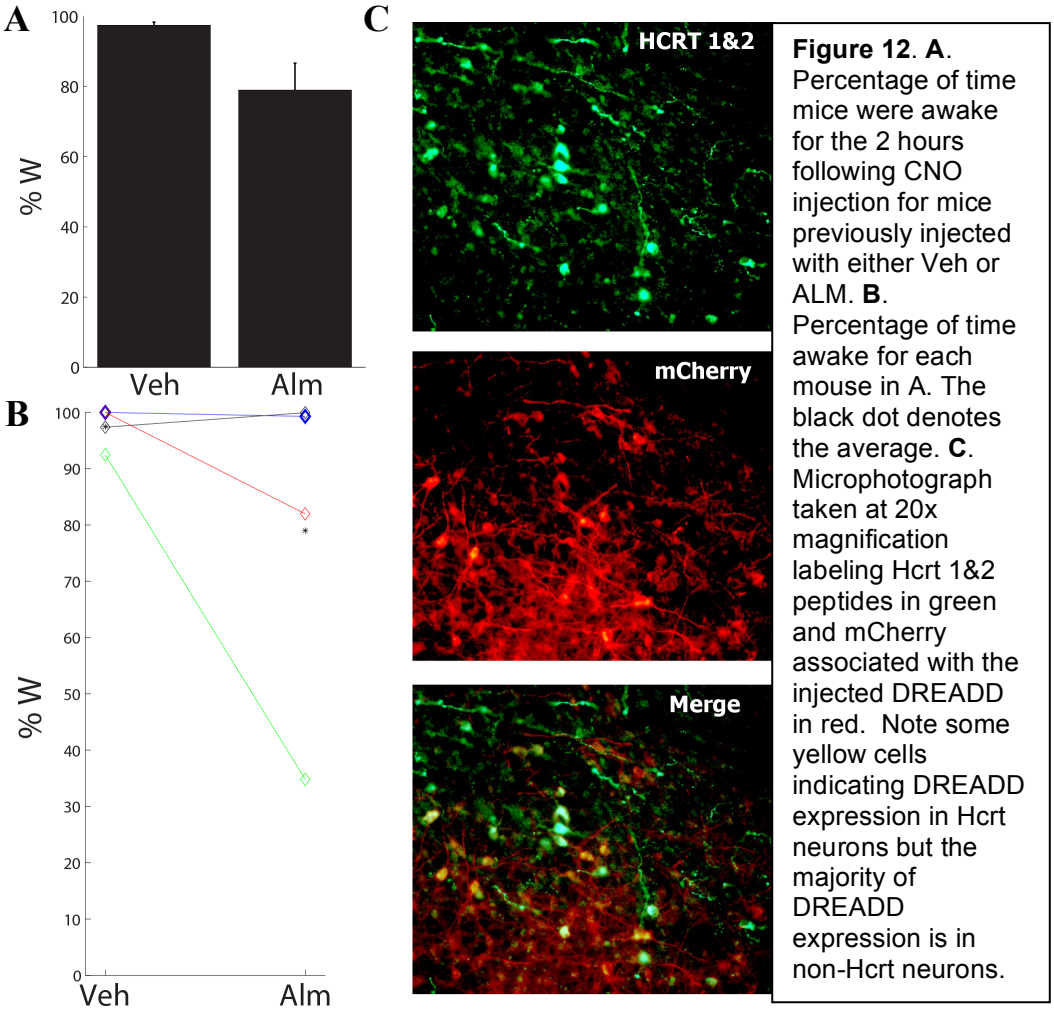

Figure 12. A. Percentage of time mice were awake for the 2 hours following CNO injection for mice previously injected with either Veh or ALM. **B.** Percentage of time awake for each mouse in A. The black dot denotes the average. **C.** Microphotograph taken at 20x magnification labeling Hcrt 1&2 peptides in green and mCherry associated with the injected DREADD in red. Note some yellow cells indicating DREADD expression in Hcrt neurons but the majority of DREADD expression is in non-Hcrt neurons.

transfected with DREADDs, there are many other nearby neurons that are also transfected, indicating that viral construct is not adequate to study Hcrt neuron-specific activation. However, these data also suggest the existence of previously unknown wake–promoting hypothalamic neurons. A detailed histological analysis of transfected neurons of the mice depicted in blue and black vs the mouse depicted in green in **Figure 12B** could shed light on which neurons are responsible for this strong wake-promoting effect.

To dissect the arousal effect of activation of only the ectopically-transfected neurons vs. the ectopic and Hcrt neurons, we implanted EEG/EMG leads in 9 mice bilaterally injected with AAV-TetO-hM3Dq-mCherry. These mice were maintained on chow containing doxycycline (DOX), which prevents the tetracycline-controlled transactivator from binding its target and thus inactivates the TetO system and results in a blockade of DREADD transcription in the Hcrt neurons. **Figure 13** shows an example of the time course of CNO injection in DOX-treated mice bilaterally injected with AAV-TetO-hM3Dq-mCherry. In this case, the mouse exhibited almost continuous wakefulness for more than 150 min as can be seen from the elevated muscle tone, increased high gamma power (70-90 Hz), and suppression of delta power (0.5-4 Hz). Since DREADD expression should have been suppressed in the Hcrt neurons due to the DOX, the wakefulness was likely mediated by ectopically-transfected neurons in the posterior hypothalamus (e.g., the mCherry-labelled cells in **Figure 12C**). Identification of the neurons responsible for this wake-promoting effect could lead to the development of novel tools to excite these neurons and to promote wakefulness. Conversely, pharmacological inactivation of these neurons may enhance sleep, which could ultimately benefit a large number of patients.

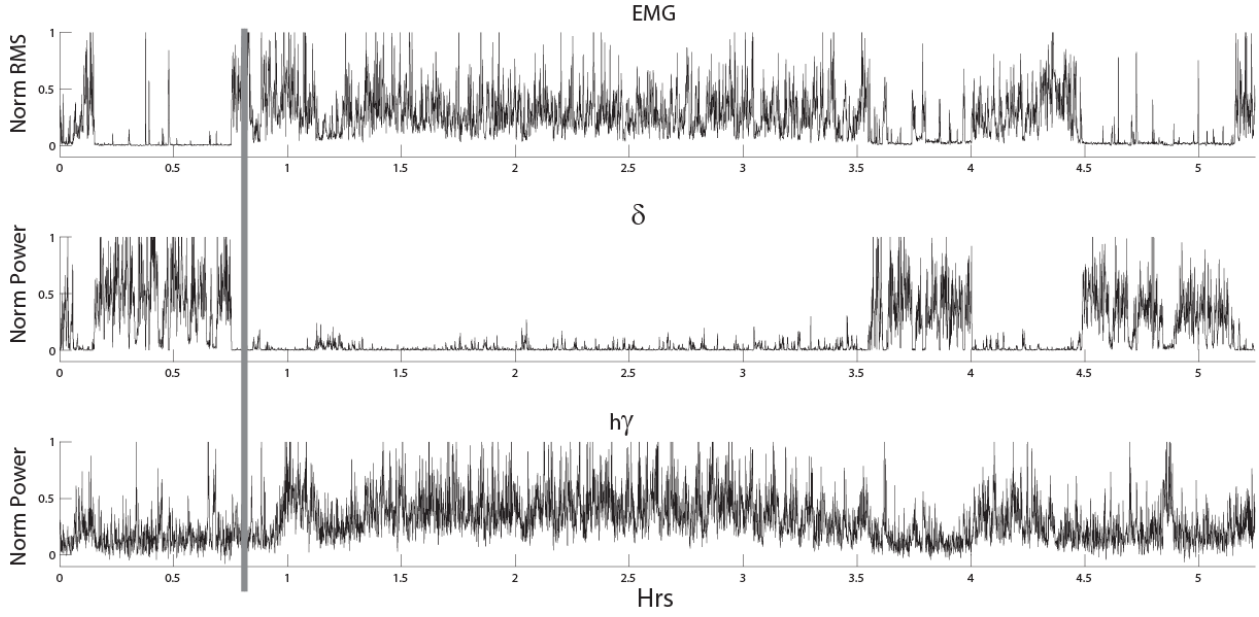

Figure 13. Time course of the changes in EMG amplitude and EEG power in the delta and high gamma bands for 5 h after CNO injection. Each data point is calculated from a 4s epoch. Grey vertical bar denotes the moment when CNO (3mg/Kg, i.p.) was injected.

KEY RESEARCH ACCOMPLISHMENTS

Year 1: 9/1/2009-8/31/2010

• Obtaining approval and commitment from SRI International to construct a new 535 s.f. laboratory suite within the Animal Facility to support the *in vivo* portion of this research program (construction initiated 19 May 2010; expected occupancy 3 Aug 2010).

• Set up of the water maze and video tracking system and initiation of data collection in a temporary location until construction of above-mentioned laboratory suite is completed

• Establishment of a 985 s.f. Analytical Neurochemistry Facility in LB212 to support this research program containing:

- 1 ESA CoulChem HPLC for analysis of dopamine and its metabolites relocated to LB212.
- 3 ESA Coul Array HPLCs for analysis of acetylcholine, norepinephrine and serotonin purchased from Roche Palo Alto on internal SRI funds; setup of machines supported by rebudgeting of current grant.
- 1 HPLC for analysis of adenosine received on 19 Jul 2010; awaiting installation.
- 1 HPLC for analysis of GABA, glutamate, glycine and other amino acids ordered on 14 Jun 2010; delivery expected this week.

• Full system reinstallation of all hardware equipment and software, and validated communication and automation capabilities for three ESA Coul Array HPLCs.

• Establishment of limits of detection for 4 of the ESA Coul Array HPLCs (Figs. 5-7).

• Determination of the effect of 3 doses of ALM vs. ZOL on sleep/wake and other physiological parameters in the Sprague-Dawley rat (Figs. 1-4)

• Upgrade of our existing Neurolucida and StereoInvestigator software from Microbrightfield, Inc. to facilitate cell counts necessary for quantification of the studies to be executed in Tasks 3 and 4a.

Year 2: 9/1/2010-8/31/2011

• Occupation of a new laboratory for behavioral performance assessment and microdialysis sampling in November, 2010.

• Full system installation of all hardware equipment and software, and validated communication and automation capabilities for two HPLCs: one to measure adenosine and the other to measure amino acids.

• Establishment of a spatial reference memory test and demonstration that ZOL impairs performance on this test whereas ALM does not (Figure 2).

• Preliminary results obtained indicating that the wake-active Hcrt neurons could be activated in the presence of ALM but not in the presence of ZOL (Figure 3).

• Determination that both ALM and ZOL activated a sleep-active cortical neuron population (Figure 4).

• Establishment of limits of detection for the two new HPLCs (Figures 5-6).

• Determination of the effect of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat (Figures 8-10).

• Submission of two abstracts to be presented at the annual Society for Neuroscience meeting to be held in Washington, D.C. in Nov 2011.

Year 3: 9/1/2011-8/31/2012
• Spatial reference memory study completed.

• Spatial working memory study under undisturbed conditions has been completed. All animals needed to complete the spatial working memory study under sleep-deprived conditions have been implanted and are currently under study.

• Equipment for the rPVT study (Task 2c) has been purchased.

• **Publication**: "Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone" in *PLoS One* by Morairty et al.

• Completed rat perfusion and tissue processing for histological Tasks 3a and 4a.
• Completed the first of three lesion studies in Aim 3b, evaluating the efficacy of ALM vs. ZOL in basal-forebrain-lesioned or sham-operated animals.

• Successfully piloted a locus coeruleus lesion protocol for the second lesion study in Aim 3b.

• Completed immunohistological analysis of neuronal activation of wake-active hypocretin neurons (Task 3a) and sleep-active cortical nNOS neurons (Task 4a).

• Presented poster entitled "The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake" at the Society for Neuroscience meeting held in Washington, D.C. in 2011 based on data collected in Tasks 2a, 3a and 4a.

• Presented poster entitled "Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat" at the Society for Neuroscience meeting held in Washington, D.C. in 2011 based on data collected in Task 4b.

• Completed the number of animals needed for Task 4b evaluating the effects of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat.

• Have preliminary findings of the effects of oral ALM vs. ZOL on animals under conditions of constant wakefulness and recovery sleep.

Year 4: 9/1/2012-8/31/2013
• Completed analysis of EEG records for Task 2a that confirmed the hypnotic efficacy of the administered doses of ALM and ZOL.

• Completed data collection and analysis of all WM data needed for Task 2b that discerned significant differences between ALM and ZOL in the performance of the spatial working memory task.

• Completed the acquisition and setup of the rPVT system. Completed the in-life portion of the rPVT experiments in Task 2c. This included all conditions initially proposed (ALM and ZOL at 100 mg/kg, p.o., and VEH following both undisturbed and SD conditions) and 4 additional conditions (ALM and ZOL and 30 mg/kg, p.o., following both undisturbed and SD conditions). Since this was a repeated measures experiment, all rats underwent all conditions in a semi counter-balanced order. Analysis has been initiated on this data set.

• Completed processing and analysis of the effects of ZOL and ALM on Fos expression patterns following sleep deprivation for most of the animals described in Task 3a.

• Identified that histamine- and hypocretin-producing neurons are inhibited by ZOL but not ALM following sleep deprivation.

• Completed the number of animals needed for Task 3b.2 evaluating the effects of LC lesions on ALM vs. ZOL-induced sleep in the Sprague-Dawley rat.

• Completed experiments and analysis to compare effects of ZOL and ALM on sleep-active cortical neurons.

• Completed experiments to compare interactions of ZOL and ALM with sleep pressure.

• Completed the number of animals needed for Task 4b evaluating the effects of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat.

• Completed the number of animals needed for Task 4b.2 evaluating the effects of oral ALM vs. ZOL on animals under conditions of extended wakefulness and recovery sleep that was established as a complementary study under Task 4b.

• Began a preliminary cohort of animals needed for the new study in Task 4c, evaluating the behavioral and neurochemical effects of central, localized microinjections of ALM vs. ZOL in the BF and cortical neurotransmission.

• Established a healthy colony of transgenic *orexin-tTA; Tet-O ChR2(C128S)* mice needed for Task 6 and showed that they can be excited by blue light pulses and that *in vivo* optogenetic stimulation of Hcrt cells can cause changes in sleep architecture.

Year 5: 9/1/2013-8/31/2014

• Aims 2a, 2b, and 4a completed and published:
Publication: "The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats." by Morairty et al. in *Front. Neurosci.*

• Manuscript describing results of Aims 3b.1 and 4b submitted and in revision.

• Data collection for Aims 2c and 3b.2 completed and data analysis ongoing.

• Data collection and analysis of Aim 3a continued.

• Data collection for Aims 3b.3, 4c and 6a initiated. In Task 6a, performed optogenetic excitation in freely behaving *orexin-tTA; Tet-O ChR2(C128S)* mice, which seem to tolerate the implant and the optogenetic stimulation without noticeable side effects.

• Initial results from Task 6a indicate that Hcrt neuron activation can cause a fast arousal that does not seem to be mediated by release of the Hcrt peptides as is not blocked in the presence of the Hcrt antagonist ALM.

• Implementation of a microdrive and a Neuronexus probe for recording multiunit activity in deep brain areas in Task 6b.

• Incorporated use of the Inscopix technology to record the activity of populations of neurons in specific brain areas.

Year 6: 9/1/2014-8/31/2015

• **Publications**: "Hypocretin/orexin antagonism enhances sleep-related adenosine and GABA neurotransmission in rat basal forebrain" by Vazquez-DeRose *et al.* in *Brain Structure and Function.* PMID:25431268.

"Homeostatic sleep pressure is the primary factor for activation of cortical nNOS/NK1 neurons" by Dittrich et al. in *Neuropsychopharmacology* 40(3):632-9. PMID:25139062.

"The dual hypocretin receptor antagonist almorexant is permissive for activation of wake-promoting systems." by Parks et al. in *Neuropsychopharmacology,* doi: 10.1038/npp.2015.256. [Epub ahead of print]. PMID: 26289145.

• **Manuscript prepared for submission**: Schwartz MD, Nguyen AT, Warrier DR, Palmerston JB, Thomas AM, Morairty SR, Neylan TC, Kilduff TS. Locus coeruleus and tuberomammillary nuclei ablations attenuate hypocretin/orexin antagonist-mediated REM sleep. (To be submitted in December 2015).

REPORTABLE OUTCOMES

Abstracts

Vazquez J., A. Nguyen, T. Kilduff. Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat. Program No. 720.09. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Dittrich L, S. Morairty, A. Wilk, D. Warrier, K. Silveira, T.-M. Chen, T. S. Kilduff. The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake. Program No. 720.10. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

L. Dittrich, D. P. Warrier, A. J. Wilk, S. R. Morairty, T. S. Kilduff (2012). Non-REM sleep is permissive but sleep pressure is required for full activation of cortical nNOS neurons. Program No. 17.07. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

S. W. Black, S. R. Morairty, S. P. Fisher, T.-M. Chen, D. R. Warrier, T. S. Kilduff (2012). Almorexant promotes sleep and exacerbates cataplexy in a murine model of narcolepsy. Program No. 486.19. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

M. D. Schwartz, L. Dittrich, S. P. Fisher, W. Lincoln, H. Liu, M. A. Miller, D. R. Warrier, A. J. Wilk, S. R. Morairty, T. S. Kilduff (2012). Effects of a dual hypocretin receptor antagonist on sleep and wakefulness in rats. Program No. 799.23. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

S.R. Morairty, A. Wilk, W. Lincoln, L. Dittrich, T.C. Neylan, T.S. Kilduff (2013). The hypocretin receptor antagonist almorexant promotes sleep in rats and does not impair performance in spatial reference memory or spatial working memory tasks. Sleep 36 Abstract Supplement: A73-A74.

J. Vazquez-DeRose, A. Nguyen, S. Gulati, T. Mathew, and T. S. Kilduff (2013). Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats. Program No. 478.11. *2013 Neuroscience Meeting Planner.* San Diego, CA: Society for Neuroscience, 2013. Online.

W. Lincoln, J Palmertson, T.C. Neylan, T.S. Kilduff, S.R. Morairty (2013). Zolpidem impairs attention/motivation in the rodent Psychomotor Vigilance Task more than almorexant. Program No. 658.24. *2013 Neuroscience Meeting Planner.* San Diego, CA: Society for Neuroscience, 2013. Online.

Parks GS, Warrier DR, Dittrich L, Wilk AJ, Schwartz MD, Neylan TC, Morairty SR, Kilduff TS. The dual hypocretin receptor antagonist almorexant is permissive for activation of wake-promoting systems. Program No. 257.11. *2014 Neuroscience Meeting Planner.* Washington, DC: Society for Neuroscience, 2014. Online.

Heiss JE, Yamanaka A, Kilduff TS (2015). Instantaneous and persistent arousal induced by bilateral optogenetic and pharmacogenetic excitation of HCRT neurons. Program No. 814.12. *2015 Neuroscience Meeting Planner.* Chicago, IL: Society for Neuroscience, 2015. Online.

Peer-reviewed Publications

Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni (2012). Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. doi: 10.1371/journal.pone.0039131. PMID:22768296.

Morairty SR, Wilk A. Lincoln W, Neylan TC, Kilduff TS. (2014). The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats. *Front. Neurosci.* 8:3. doi: 10.3389/fnins.2014.00003. PMID: 24550767.

Vazquez-DeRose J, Schwartz MD, Nguyen AT, Warrier DR, Gulati S, Mathew TK, Neylan TC, Kilduff TS (2014). Hypocretin/orexin antagonism enhances sleep-related adenosine and GABA neurotransmission in rat basal forebrain. *Brain Structure and Function*, Nov 28. [Epub ahead of print]. PMID: 25431268.

Dittrich L, Morairty SR, Warrier D, Kilduff TS (2015). Homeostatic sleep pressure is the primary factor for activation of cortical nNOS/NK1 neurons. *Neuropsychopharmacology* 40(3):632-9. PMID: 25139062.

Parks GS, Warrier DR, Dittrich L, Schwartz MD, Palmerston JB, Neylan TC, Morairty SR, Kilduff TS (2015). The dual hypocretin receptor antagonist almorexant is permissive for activation of wake-promoting systems. *Neuropsychopharmacology,* doi: 10.1038/npp.2015.256. [Epub ahead of print].

Schwartz MD, Nguyen AT, Warrier DR, Palmerston JB, Thomas AM, Morairty SR, Neylan TC, Kilduff TS. Locus coeruleus and tuberomammillary nuclei ablations attenuate hypocretin/orexin antagonist-mediated REM sleep. To be submitted (December 2015).

CONCLUSION

When submitted in October, 2008, this grant proposal was based on the hypothesis that "hypocretin (Hcrt) antagonists produce fewer functional impairments than benzodiazepine receptor agonists (BzRA) because BzRAs cause a general inhibition of neural activity whereas Hcrt specifically disfacilitates wake-promoting systems." During the funding period, several lines of evidence were obtained that were consistent with this hypothesis. First, we determined that the Hcrt antagonist almorexant (ALM) was most likely promoting sleep by antagonism of both Hcrt receptors 1 and 2 (Moriarty et al., 2012) as opposed to HcrtR2 as had been proposed by others. Next, in tests of both spatial reference memory and spatial working memory, we found that rats treated with ALM performed far superior to those treated with the BzRA agonist zolpidem (ZOL) at equipotent doses in terms of sleep induction (Moriarty et al., 2014). Next, we found that this superior performance was likely due to the ability to activate wake-promoting nuclei in the presence of ALM but not ZOL (Parks et al., 2015). Furthermore, we explored the neural mechanisms underlying ALM-induced sleep and found that ALM, but not ZOL, requires an intact basal forebrain for maximum NREM-promoting efficacy, and that ALM elicits a neurochemical release profile more consistent with the transition to normal sleep than does ZOL (Vazquez-DeRose et al., 2014). Furthermore, lesions of the wake-promoting noradrenergic locus coeruleus or histaminergic tuberomammillary nuclei compromised the hypnotic efficacy of ALM without affecting that of ZOL (Schwartz et al., to be submitted). Thus, Hcrt neurotransmission influences distinct aspects of NREM and REM sleep at different locations in the sleep-wake regulatory network. By selectively disfacilitating these subcortical wake-promoting populations, Hcrt antagonism effectively promotes sleep without negatively impacting cognitive performance and without globally blocking the capability for arousal.

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Carter ME, Yizhar O, Chikahisa S, Nguyen H, Adamantidis A, Nishino S, Deisseroth K, de Lecea L (2010) Tuning arousal with optogenetic modulation of locus coeruleus neurons. Nat Neurosci 13:1526-1533. PMID: 21037585

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Morairty SR, Wilk AJ, Lincoln WU, Neylan TC, Kilduff TS (2014) The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats. Front Neurosci 8:3. PMID: 24550767

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Vazquez-DeRose J, Schwartz MD, Nguyen AT, Warrier DR, Gulati S, Mathew TK, Neylan TC, Kilduff TS (2014) Hypocretin/orexin antagonism enhances sleep-related adenosine and GABA neurotransmission in rat basal forebrain. Brain Struct Funct. PMID: 25431268

APPENDICES:

USAMRAA GRANT DR080789P1

"EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE"

Thomas S. Kilduff, Ph.D., Principal Investigator

FINAL REPORT

Published Abstracts

1) Vazquez J., A. Nguyen, T. Kilduff. Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat. Program No. 720.09. *2011 Neuroscience Meeting Planner*. Washington, DC: Society for Neuroscience, 2011. Online

2) Dittrich L, S. Morairty, A. Wilk, D. Warrier, K. Silveira, T.-M. Chen, T. S. Kilduff. The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake. Program No. 720.10. *2011 Neuroscience Meeting Planner*. Washington, DC: Society for Neuroscience, 2011. Online.

3) L. Dittrich, D.P. Warrier, A.J. Wilk, S.R. Morairty, T.S. Kilduff (2012). Non-REM sleep is permissive but sleep pressure is required for full activation of cortical nNOS neurons. Program No. 17.07. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

4) S.W. Black, S.R. Morairty, S.P. Fisher, T.-M. Chen, D.R. Warrier, T.S. Kilduff (2012). Almorexant promotes sleep and exacerbates cataplexy in a murine model of narcolepsy. Program No. 486.19. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

5) M.D. Schwartz, L. Dittrich, S.P. Fisher, W. Lincoln, H. Liu, M.A. Miller, D.R. Warrier, A.J. Wilk, S.R. Morairty, T.S. Kilduff (2012). Effects of a dual hypocretin receptor antagonist on sleep and wakefulness in rats. Program No. 799.23. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

6) S.R. Morairty, A. Wilk, W. Lincoln, L. Dittrich, T.C. Neylan, T.S. Kilduff (2013). The hypocretin receptor antagonist almorexant promotes sleep in rats and does not impair performance in spatial reference memory or spatial working memory tasks. *Sleep* 36 Abstract Supplement: A73-A74.

7) J. Vazquez-DeRose, A. Nguyen, S. Gulati, T. Mathew, and T. S. Kilduff (2013). Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats. Program No. 478.11. *2013 Neuroscience Meeting Planner*. San Diego, CA: Society for Neuroscience, 2013. Online.

8) W. Lincoln, J Palmertson, T.C. Neylan, T.S. Kilduff, S.R. Morairty (2013). Zolpidem impairs attention/motivation in the rodent Psychomotor Vigilance Task more than almorexant. Program No. 658.24. *2013 Neuroscience Meeting Planner*. San Diego, CA: Society for Neuroscience, 2013. Online.

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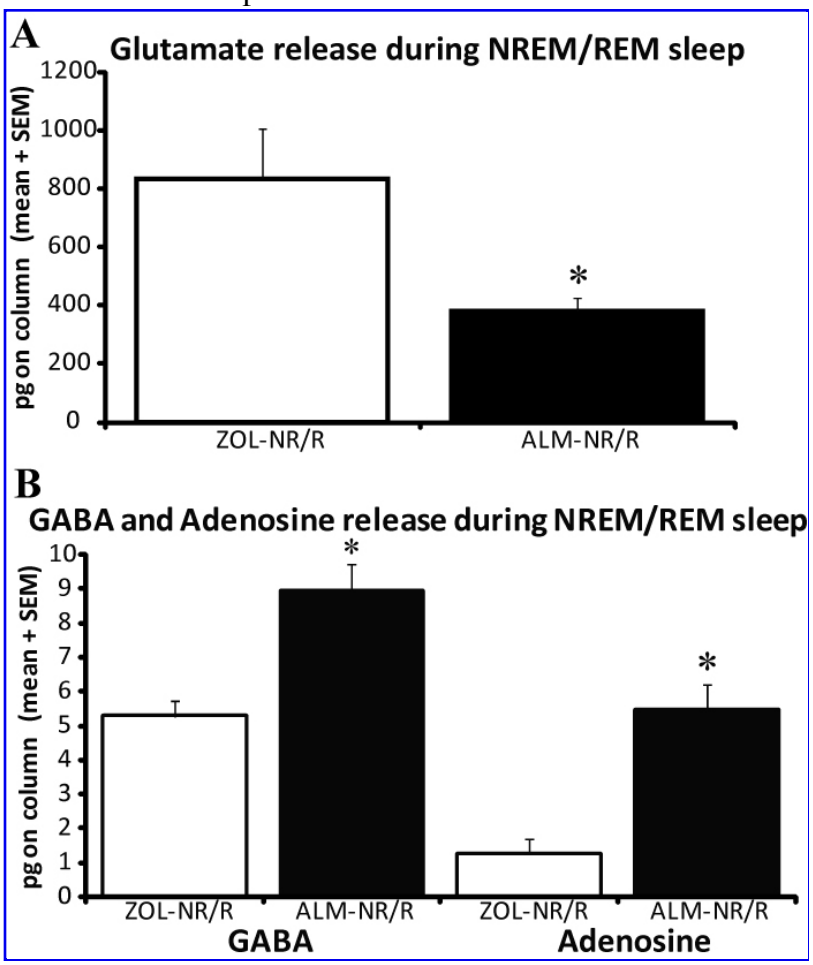
Print this Page

Presentation Abstract

Program#/Poster#:	720.09/XX21
Presentation Title:	Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat.
Location:	Hall A-C
Presentation time:	Tuesday, Nov 15, 2011, 1:00 PM - 2:00 PM
Authors:	***J. VAZQUEZ**, A. NGUYEN, T. KILDUFF; Ctr. for Neuroscience, Biosci. Div., SRI Intl., MENLO PARK, CA
Abstract:	Hypocretins (orexins) modulate diverse physiological processes such as cognitive function and alertness. Hypocretin-1 and hypocretin-2 (Hcrt) peptides regulate sleep and alertness (Kilduff and Peyron 2000) and Hcrt neurons project to several brain regions including the basal forebrain (BF; Peyron et al. 1998), a brain region critical for promoting wakefulness (Jones 2004). The BF contains cholinergic, GABAergic, and putative glutamatergic neurons important for cortical activation (Manns et al. 2003). Zolpidem (ZOL), a benzodiazepine receptor agonist, affects a Cl^- channel on the $GABA_A$ receptor, resulting in hyperpolarization and somnolence (Dang et al. 2010). In contrast, almorexant (ALM) is a dual Hcrt receptor antagonist that reversibly blocks signaling of both Hcrt peptides. Oral delivery of ALM elicits somnolence without cataplexy and, in rat, decreases active wake and increases the time spent in non-rapid eye movement (NREM) and (REM) sleep (Brisbare-Roch et al. 2007). We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects $GABA_A$ receptors that are widely distributed in the CNS, ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used in vivo microdialysis and HPLC analyses to examine BF glutamate, GABA, and adenosine efflux following oral ZOL (10mg/kg), ALM (100mg/kg), or placebo (VEH) combined with behavioral analyses. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters.

Post-hoc comparisons showed that ALM (n=4 rats; p<0.05) caused a significant decrease in BF glutamate (**A**) during NREM/REM cycling and the corresponding collection timeframes compared to ZOL (n=4) or VEH (n=3; data not shown). Oral ALM concurrently increased BF GABA and adenosine (**B**; p<0.05) during NREM/REM compared to ZOL or VEH. These results provide novel evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.



Disclosures: **J. Vazquez:** None. **A. Nguyen:** None. **T. Kilduff:** None.

Keyword(s): MICRODIALYSIS

GABA

HYPOCRETIN

Support: USAMRMC W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

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Presentation Abstract

Program#/Poster#:	720.10/XX22
Presentation Title:	The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake
Location:	Hall A-C
Presentation time:	Tuesday, Nov 15, 2011, 2:00 PM - 3:00 PM
Authors:	***L. DITTRICH**, S. MORAIRTY, A. WILK, D. WARRIER, K. SILVEIRA, T.-M. CHEN, T. S. KILDUFF; Biosci., SRI Intl., Menlo Park, CA
Abstract:	Most commonly prescribed hypnotics, such as benzodiazepine receptor agonists, cause general inhibition of neural activity. As a result, these hypnotics are less than optimal to aid sleep if there is a risk of being awakened with the need to perform without impairment, e.g., healthcare workers or emergency response personnel. A more specific mechanism of action is exerted by almorexant (ALM), a dual antagonist for hypocretin/orexin (Hcrt) receptors. We hypothesized that challenged rats would be able to stay awake more easily and function with less impairment after a sleep-promoting dose of ALM than after a dose of the benzodiazepine receptor agonist zolpidem (ZOL). To test this hypothesis, we trained 24 rats to remember the location of a platform in a spatial reference memory task (Morris Water Maze). Next day, they were dosed with either ALM (100 mg/kg i.p.), ZOL (30 mg/kg i.p.), or vehicle. Although both drugs induced sleep, the performance of rats dosed with ALM was indistinguishable from the rats dosed with vehicle whereas the group dosed with ZOL displayed weaker preference for the learned location of the platform. To assess the influence of the two compounds on the activity of sleep/wake regulatory neurons, we performed an immunohistological study using c-Fos as a marker of neuronal activity. The same rats were administered the drugs as described above but half of the animals were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling. In agreement with the behavioral results, we found that the percentage of

Fos-positive neurons in the wake-active Hcrt neurons in the lateral hypothalamus was higher for sleep deprived animals than for non-sleep deprived animals in the ALM and vehicle groups, whereas there was no such difference for the ZOL group. The sleep-active cortical neurons immunoreactive for neuronal nitric oxide synthase expressed more Fos in animals that were allowed to sleep than in those kept awake, independent from the compound administered. Taken together, our results indicate that ALM effectively induces sleep but unlike ZOL allows the rats to activate wake-promoting neurons and perform normally when needed.

Disclosures: **L. Dittrich:** None. **S. Morairty:** None. **A. Wilk:** None. **D. warrier:** None. **K. Silveira:** None. **T. chen:** None. **T.S. Kilduff:** None.

Keyword(s): WATER MAZE

C-FOS

SLEEP DEPRIVATION

Support: USAMRMC Grant W81XWH-09-2-0081

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 17.07

Presentation Title: Non-REM sleep is permissive but sleep pressure is required for full activation of cortical nNOS neurons

Location: 388

Presentation time: Saturday, Oct 13, 2012, 2:30 PM - 2:45 PM

Authors: ***L. DITTRICH**, D. P. WARRIER, A. J. WILK, S. R. MORAIRTY, T. S. KILDUFF;
Biosci., SRI Intl., Menlo Park, CA

Abstract: Cortical neurons immunoreactive for neuronal nitric oxide synthase (nNOS) are selectively activated during sleep. We have proposed that cortical nNOS neurons are inhibited by wake-promoting brain regions and activated by sleep-promoting factors that accumulate during prolonged wakefulness. To test this hypothesis, we compared nNOS neuron activation in animals that slept comparable amounts but under conditions of either high or low sleep pressure. To this end, we administered the GABA-A agonist zolpidem (ZOL, 100 mg/kg p.o., n=5) or vehicle (VEH, n=5) to Sprague Dawley rats at ZT12, when sleep pressure is naturally lowest. As a comparison, sleep pressure was increased by 6h of sleep deprivation before administering ZOL (n=6) or VEH (n=5) at ZT12. All 4 groups of rats were perfused 90-120 min after dosing (ZT13.5-14). To test whether ZOL was permissive for nNOS neuron activation in the absence of sleep, additional rats were administered ZOL (n=6) or VEH (n=7) at ZT18 and sleep deprived until perfusion 90-120 min later. EEG/EMG recordings for sleep/wake determination were performed for 8h prior to perfusion and a preceding 24h baseline period. The percentage of cortical nNOS neurons immunoreactive for c-FOS was determined in histological sections.
The ZOL-treated groups that were allowed to sleep did not differ in the time spent asleep in the 1.5h before perfusion. However, the ZOL-treated group with low sleep pressure showed a significantly lower proportion of c-FOS/nNOS neurons (19.9%±0.05 SEM) than either the ZOL-treated or the

VEH-treated groups with high sleep pressure (58.1%±0.05, 44.2%±0.06, respectively), indicating the importance of sleep pressure for activation of cortical nNOS neurons. The proportion of c-FOS/nNOS neurons in the rats treated with ZOL at ZT18 and not allowed to sleep did not differ from VEH control (6.1%±1.4 vs. 3.3%±0.8, respectively), indicating that neither sleep pressure nor GABA-A agonism in the absence of sleep is sufficient for activation of cortical nNOS neurons.
Thus, in agreement with our hypothesis, the occurrence of sleep removes wake-related inhibition of cortical nNOS neurons but prior accumulation of sleep pressure is required for full activation of this neuronal population. These results support the concept of a role for nNOS neurons in sleep homeostasis, the physiological adaptation of increased sleep intensity or duration in response to elevated sleep pressure.

Disclosures: **L. Dittrich:** None. **D.P. Warrier:** None. **A.J. Wilk:** None. **S.R. Morairty:** None. **T.S. Kilduff:** None.

Keyword(s): SLEEP DEPRIVATION

SLEEP HOMEOSTASIS

NITRIC OXIDE SYNTHASE

Support: USAMRAA Award Number W81XWH-09-2-0081

DFG fellowship DI 1718/1-1

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 486.19/VV7

Presentation Title: Almorexant promotes sleep and exacerbates cataplexy in a murine model of narcolepsy

Location: Hall F-J

Presentation time: Monday, Oct 15, 2012, 3:00 PM - 4:00 PM

Authors: ***S. W. BLACK**, S. R. MORAIRTY, S. P. FISHER, T.-M. CHEN, D. R. WARRIER, T. S. KILDUFF;
Biosci. Div., SRI Intl., MENLO PARK, CA

Abstract: Disruption of the hypocretin (Hcrt, also known as orexin) neuropeptide signaling system results in a narcoleptic phenotype characterized by excessive sleepiness, fragmented sleep, abnormally timed Rapid-Eye-Movement (REM) sleep, and cataplexy_an emotionally triggered, sudden loss of muscle tone. Hcrt neurodegeneration underlies human narcolepsy and is recapitulated in the orexin/ataxin-3 transgenic (TG) mouse model. Acute antagonism of Hcrt receptors has recently been investigated as a novel mechanism of sleep promotion, however, the use of Hcrt antagonists in narcoleptics has not yet been evaluated. Here, we determined the hypnotic and cataplexy-inducing efficacy of Hcrt receptor antagonism by almorexant in the orexin/ataxin-3 mouse model of chronic Hcrt deficiency for comparison with wild type (WT) controls. We also examined the effects of disrupted Hcrt signalling on body temperature (Tb) during sleep. During the 12-h dark period immediately following dosing, almorexant exacerbated cataplexy, decreased wakefulness, and increased nonREM sleep with heightened sleep/wake fragmentation. The antagonist showed greater hypnotic potency in WT than in TG mice. The 100 mg/kg dose of ALM conferred maximal promotion of cataplexy in TG mice and maximal promotion of REM sleep in WT mice. In TG mice, the 30 mg/kg dose of ALM paradoxically induced a transient increase in alertness with elevated motor activity. Tb decreased after acute Hcrt receptor blockade, but the reduction in Tb that normally accompanies the wake-to-sleep transition was blunted in TG mice. These complex dose- and genotype-dependent

interactions underscore the importance of effector mechanisms downstream from Hcrt receptors that regulate arousal state.

Disclosures: **S.W. Black:** None. **S.R. Morairty:** None. **S.P. Fisher:** None. **T. Chen:** None. **D.R. Warrier:** None. **T.S. Kilduff:** None.

Keyword(s): HYPOCRETIN

OREXIN

SLEEP

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[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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NEUROSCIENCE 2012

Presentation Abstract

Program#/Poster#: 799.23/BBB24

Presentation Title: Effects of a dual hypocretin receptor antagonist on sleep and wakefulness in rats.

Location: Hall F-J

Presentation time: Wednesday, Oct 17, 2012, 10:00 AM -11:00 AM

Authors: ***M. D. SCHWARTZ**, L. DITTRICH, S. P. FISHER, W. LINCOLN, H. LIU, M. A. MILLER, D. R. WARRIER, A. J. WILK, S. R. MORAIRTY, T. S. KILDUFF;
SRI Intl., Menlo Park, CA

Abstract: Benzodiazepine receptor agonists promote sleep by activating $GABA_A$ receptors, leading to generalized reduction in cortical activity. They are widely used as hypnotic medications, but have side effects including risk for tolerance and/or dependence, as well as cognitive impairment while under their influence. The excitatory hypocretin (HCRT) neuropeptides promote wakefulness by activating multiple subcortical wake-promoting neurotransmitter systems which, in turn, project to and regulate cortical activity. Blocking HCRT signaling should therefore promote sleep by acting specifically on subcortical brain areas regulating sleep and wake without adversely impacting cortical function. Here, we assessed the ability of the dual HCRT receptor antagonist almorexant (ALM) to promote sleep in rats following ablation of a major sleep-wake regulatory region, the cholinergic basal forebrain (BF). We predicted that ALM would be less effective at inducing sleep in BF-lesioned rats compared to neurologically-intact rats, whereas benzodiazepine-based compounds should be equally as effective in lesioned and intact rats. Male rats received bilateral stereotaxic injections of saline or the selective cholinergic neurotoxin192-IgG-saporin (SAP) directed at the BF and were implanted with telemetry for recording sleep EEG. Following recovery, animals were given increasing doses of ALM, the GABA-A receptor agonist zolpidem (ZOL), or vehicle. Spontaneous sleep/wake regulation and homeostatic recovery from sleep deprivation was

also assessed. At baseline, NREM sleep in the dark (active) phase was reduced in SAP rats compared to intact rats; SAP rats also exhibited decreased NREM recovery sleep following 6 h sleep deprivation in the dark phase. Sleep in the light (rest) phase was unaffected by SAP. Analysis of ALM and ZOL administration in these animals is currently in progress.

Disclosures: **M.D. Schwartz:** None. **L. Dittrich:** None. **S.P. Fisher:** None. **W. Lincoln:** None. **H. Liu:** None. **M.A. Miller:** None. **D.R. Warrier:** None. **A.J. Wilk:** None. **S.R. Morairty:** None. **T.S. Kilduff:** None.

Keyword(s): SLEEP DEPRIVATION

ADENOSINE

CHOLINERGIC

Support: USAMRAA Grant W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

0196

THE HYPOCRETIN RECEPTOR ANTAGONIST ALMOREXANT PROMOTES SLEEP IN RATS BUT DOES NOT IMPAIR PERFORMANCE IN SPATIAL REFERENCE MEMORY OR SPATIAL WORKING MEMORY TASKS

Morairty S[1], Wilk A[1], Lincoln W[2], Dittrich L[1], Neylan TC[2], Kilduff TS[1]
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Introduction: The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but little is known about performance in the presence of ALM. Since Hcrt antagonists produce sleep by disfacilitation of wake-promoting systems whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through general inhibition of neural activity, we tested whether ALM produced less functional impairment than ZOL when rats were tested in the presence of the drug.

Methods: 96 rats were implanted with telemetry devices for recording EEG and EMG. Rats were tested in water maze (WM) spatial reference memory (SRM) or spatial working memory (SWM) tasks. The effects of ALM and ZOL on both memory tasks were assessed following undisturbed and sleep deprivation (SD) conditions. SRM: On the day following acquisition, rats were either left undisturbed and dosed with ALM (100 mpk, po), ZOL (100 mpk, po) or vehicle (VEH) 6 h into their active period (ZT18) or kept awake for the first 6 h of the dark and then dosed. 90 min later, a retention probe trial was performed. Parameters measured were distance traveled, latency to enter, time within, and number of entries into, the target quadrant. SWM: This test consisted of 6 pairs of trials starting 60 min after dose administration. In the first trial, rats were released into the WM with a cued platform and were allowed 120 s to locate the platform. After 1, 5, or 10 min, the rat was placed back into the WM and given 60 s to locate the platform without cues. Another pair of trials with a novel platform location occurred 10 min later. Test measures were velocity, time and distance traveled to locate the platform.

Results: SRM: During the WM probe trial following undisturbed conditions, rats administered ZOL showed impairments in all parameters measured compared to VEH or ALM whereas ALM-treated rats were indistinguishable from VEH for all measures. ALM-treated rats were also indistinguishable from VEH for all measures during the WM probe trial following 6 h SD. SWM: During the WM test trials in both undis-

turbed and SD conditions, rats administered ZOL showed impairments in almost all parameters measured compared to VEH or ALM. ALM rats were indistinguishable from VEH for all measures. ZOL-treated rats failed to find the platform much more often than ALM- or VEH-treated rats.

Conclusion: These results are consistent with the hypothesis that less functional impairment results from HcrtR antagonism than from BzRA-induced inhibition.

Support (If Any): Supported by USAMRMC grant W81XWH-09-2-008.

Presentation Abstract

Program#/Poster#:	478.11/JJJ25
Presentation Title:	Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats
Location:	Halls B-H
Presentation time:	Monday, Nov 11, 2013, 3:00 PM - 4:00 PM
Topic:	++E.08.c. Sleep: Molecular, cellular, and pharmacology
Authors:	***J. VAZQUEZ-DEROSE**[1], A. NGUYEN[1], S. GULATI[1], T. MATHEW[1], T. C. NEYLAN[2], T. S. KILDUFF[1]; [1]Ctr. for Neuroscience, Biosci. Div., SRI Intl., MENLO PARK, CA; [2]VA Med. Center/NCIRE, UCSF San Francisco, San Francisco, CA
Abstract:	Hypocretin (Hcrt-1 and Hcrt-2) peptides are well-known to regulate sleep and alertness and send projections to the basal forebrain (BF), an area critical for promoting wakefulness. The BF contains a heterogeneous mix of neurons that send diverse projections important for cortical arousal. Almorexant (ALM) is a dual Hcrt receptor antagonist that reversibly blocks signaling of both Hcrt receptors, whereas Zolpidem (ZOL) is a benzodiazepine receptor agonist affecting Cl- channels. Previous studies have shown that oral delivery of ALM elicits somnolence without cataplexy and, in rat, decreases active wake and increases the time spent in non-rapid eye movement (NREM) and (REM) sleep with differential effects on various neurotransmitter systems. To date, no studies have reported the effects of central microinjections of ALM or ZOL and its effect on behavior or transmitter levels in brain. We used in vivo microdialysis and HPLC analysis to examine cortical adenosine (ADO) levels following BF microinjections of ZOL (0.6 μg/0.2 μl), ALM (1.0 μg/0.2 μl), or VEH (aCSF) combined with behavioral analyses. Preliminary analyses revealed a significant main effect of drug on ADO levels. Post-hoc comparisons showed that ALM microinjected in to the BF (n=3 rats; * $p<0.05$) caused a significant increase in cortical ADO that lasted up to 6 h post microinjection compared to VEH control (n=3). Conversely, administration of ZOL (n=3) to the BF significantly decreased cortical ADO levels (# $p<0.05$) compared to VEH and ALM. These results provide novel evidence for dynamic

neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

Disclosures: **J. Vazquez-Derose:** None. **A. Nguyen:** None. **S. Gulati:** None. **T.C. Neylan:** None. **T. Mathew:** None. **T.S. Kilduff:** None.

Keyword(s): HYPOCRETIN

MICRODIALYSIS

ADENOSINE

Support: W81XWH-09-2-0081

Presentation Abstract

Program#/Poster#:	658.24/BBB16
Presentation Title:	Zolpidem impairs attention/motivation in the rodent psychomotor vigilance task more than almorexant
Location:	Halls B-H
Presentation time:	Tuesday, Nov 12, 2013, 4:00 PM - 5:00 PM
Topic:	++E.08.e Sleep: Systems and behavior
Authors:	**W. LINCOLN**[1], J. PALMERSTON[1], T. NEYLAN[2], T. KILDUFF[1], *S. R. MORAIRTY[1]; [1]Ctr. for Neurosci., SRI Int'l, MENLO PARK, CA; [2]UCSF/SFVAMC, San Francisco, CA
Abstract:	The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but less is known about its effects on performance. Since Hcrt antagonists produce sleep by disfacilitation of wake-promoting systems whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through a generalized inhibition of neural activity, we hypothesized that ALM would produce less functional impairment than ZOL. We have previously shown that rats tested in spatial reference memory or spatial working memory tasks in a water maze show no impairment following ALM whereas significant impairment was evident following ZOL. Here, we tested the effects of ALM and ZOL on the Rodent Psychomotor Vigilance Task (rPVT), a sensitive test of attention and motivation. 10 rats were implanted with telemetry devices for recording EEG and EMG. The effects of ALM and ZOL on attention/motivation administered in the middle of the active period were assessed at 2 sleep-promoting concentrations (30 & 100 mpk, po) following undisturbed and sleep deprived (SD, 6 h prior to dosing) conditions. 90 min following dosing, trained, water-restricted rats were placed in operant chambers with infrared detection beams in front of the water dispenser. rPVT testing consisted of a stimulus light (duration of 0.5 s) followed by a 3 s response period. The inter-trial interval varied between 3-7 s. Errors resulted in a cued 10 s “time out” period. Performance measures were 1) response latencies (RL), 2) correct responses (CR), 3) omissions (OM), and 4) premature errors (PE).

Impaired performance is indicated by increases in RL, OM and PE and decreases in CR.
SD had a relatively small but significant effect on performance following VEH: RL decreased (96.2%), CR decreased (95.5%) and OM increased (146.1%) while PE decreased (95.1%) following SD+90 min recovery compared to baseline. Following ALM at 30 mpk compared to VEH, OM and RL decreased (51.7 & 96.2%; indicative of increased performance) while CR decreased and PE increased (indicative of impaired performance). ZOL at 30 mpk decreased performance markedly: RL increased (131.3%), CR decreased (30.4%) and OM increased (724.1%) while PE decreased (36.9%) compared to VEH. However, performance decreased significantly following both drugs at 100 mpk, particularly with ZOL. Following ALM at 100 mpk, RL and OM increased (150.6 & 556.3%) and CR and PE decreased (42.8 & 58.0%). Following ZOL at 100 mpk, RL and OM increased (126.6 & 855.6%) and CR and PE decreased (9.2 & 26.0%).
These results are consistent with the hypothesis that less functional impairment results from HcrtR antagonism than from BzRA-induced inhibition.

Disclosures: **W. Lincoln:** None. **S.R. Morairty:** None. **J. Palmerston:** None. **T. Kilduff:** None. **T. Neylan:** None.

Keyword(s): ATTENTION

SLEEP DEPRIVATION

MOTIVATION

Support: USAMRMC grant W81XWH-09-2-0081

Internal SRI funds

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NEUROSCIENCE 2014

Presentation Abstract

Program#/Poster#:	257.11/NN24
Presentation Title:	The dual hypocretin receptor antagonist almorexant is permissive for the activation of wake-promoting systems
Location:	WCC Hall A-C
Presentation time:	Sunday, Nov 16, 2014, 1:00 PM - 5:00 PM
Presenter at Poster:	Sun, Nov. 16, 2014, 3:00 PM - 4:00 PM
Topic:	++E.08.c. Sleep: Molecular, cellular, and pharmacology
Authors:	***G. S. PARKS**[1], D. R. WARRIER[1], L. DITTRICH[1], A. J. WILK[1], M. D. SCHWARTZ[1], T. C. NEYLAN[2], S. R. MORAIRTY[1], T. S. KILDUFF[1]; [1]Ctr. for Neurosci., SRI Intl., Menlo Park, CA; [2]VA Med. Center/NCIRE, UCSF, San Francisco, CA
Abstract:	The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions and is thought to promote sleep by selective disfacilitation of wake-promoting systems whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through general inhibition of neural activity. Consequently, HcrtR antagonists are predicted to cause less functional impairment than BzRAs. Recent behavioral studies have supported this hypothesis as ALM causes less impairment in spatial memory tasks in rats awoken from hypnotic-induced sleep than ZOL does. Other dual HcrtR antagonists also promote sleep at doses that do not disrupt locomotor activity or cognition. In order to gain insight into the neural mechanisms underlying the differential functional impairment of these drugs, we compared the effects of ALM and ZOL on functional activation of

the currently known wake-promoting systems. Sprague Dawley rats, implanted for EEG/EMG recording, were orally administered vehicle (VEH), 100mg/kg ALM, or 100mg/kg ZOL during their active phase and were either left undisturbed or kept awake (i.e., sleep-deprived; SD) for 90 min after which their brains were collected. We measured Fos coexpression with markers for wake-promoting cell groups in the basal forebrain (BF; ChAT), tuberomammillary nuclei (ADA), lateral hypothalamus (Hcrt), and dorsal raphe (DR; 5HT). In the locus coeruleus (LC), we counted singly-labelled Fos+ cells because the density of DBH staining obscured Fos immunoreactivity in double-labeled sections. Following sustained wakefulness, Fos coexpression in histamine and Hcrt neurons was higher in VEH and ALM-treated rats than in ZOL-treated rats; moreover, the level of co-expression was indistinguishable between the VEH- and ALM-treated groups. In these neuronal populations, Fos levels were consistently elevated in ALM-treated SD rats compared to undisturbed animals whereas Fos levels were unchanged by SD in ZOL-treated animals. In contrast, no significant differences were found between groups regardless of treatment in the BF and DR. Interestingly, there were no differences in Fos expression between VEH and ALM-treated animals in the LC following SD, but ZOL-treated rats exhibited elevated Fos compared to vehicle. These results indicate that, in contrast to ZOL, ALM does not inhibit activation of the histamine and Hcrt systems and is thus unlikely to prevent activation of wake-promoting systems in response to situational demands. These results may also relate to the lower levels of cognitive impairment produced by dual HcrtR antagonists compared to ZOL.

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Keyword (s): HYPOCRETIN

SLEEP DEPRIVATION

HISTAMINE

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Neuroscience 2015

Presentation Abstract

Program#/Poster#: 814.12/Y28

Presentation Title: Instantaneous and persistent arousal induced by bilateral optogenetic and pharmacogenetic excitation of HCRT neurons

Location: Hall A

Presentation time: Wednesday, Oct 21, 2015, 4:00 PM - 5:00 PM

Topic: ++E.08.e Sleep: Systems

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Abstract: HCRT (Orexin) neurons located in the lateral hypothalamus are a key regulator of arousal; loss of this neuronal group leads to the sleep disorder narcolepsy. HCRT cells provide excitatory input to noradrenergic, histaminergic and serotonergic neurons, thereby regulating their activity and influencing the global state of the brain. In addition to the Hcrt1 and Hcrt2 peptides, HCRT neurons can release glutamate and dynorphin and perhaps other unknown neurotransmitters. In vitro studies have shown that HCRT neurons can induce brief glutamate-mediated excitation and persistent HCRT-mediated depolarization of histaminergic neurons. In vivo optogenetic studies have shown that unilateral stimulation of HCRT neurons during REM and NREM sleep induces an increase in the probability of awakening that is mediated by HCRT peptide release. To test whether brief stimulation of a larger population of HCRT neurons has a more robust effect on arousal than previously reported, we performed single pulse, bilateral optogenetic excitation of HCRT neurons in Orexin/tTA;TetO Chr2 (C128S) mice which constitutively express a "step function" opsin in HCRT neurons that is activated by a single pulse of blue light (475 nm) and inactivated by yellow light (575 nm). All manipulations described below were performed in the middle of the day (around ZT 6). In a pilot study (N=4 mice), a single 50 ms pulse of blue light flanked by a 200 ms yellow light pulse 50 ms before and 30 s later was delivered 1 mm above

both HCRT fields. Blue light flanked by yellow pulses was alternated with yellow light flanked by yellow pulses (control) every 2 min for 30 min. Blue light stimulation induced awakening within 4 s after stimulation in over 75% of the trials. In contrast, the probability of a NREM-Wake transition was 20% for control trials. To determine whether prolonged stimulation of larger populations of HCRT neurons affects arousal, we injected Orexin/tTA mice with AAV-TetO-hM3Dq-mCherry bilaterally. Persistent pharmacogenetic stimulation of HCRT neurons by CNO injection (0.5-3 mg/Kg, IP) induced prolonged wakefulness that lasted 2-4 h (N=4). In the presence of the dual orexin receptor antagonist almorexant (200 mg/Kg, IP), preliminary data shows that the effect of optogenetic excitation of HCRT cells was unaffected (N=3) whereas the effects of persistent excitation mediated by hM3Dq DREADD was attenuated (N=3). Our results suggest that direct excitation of large populations of HCRT neurons is sufficient to induce wakefulness with a very short latency and that persistent, but not brief, arousal is mediated at least partially by HCRT peptides.

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Dual Hypocretin Receptor Antagonism Is More Effective for Sleep Promotion than Antagonism of Either Receptor Alone

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Abstract

The hypocretin (orexin) system is involved in sleep/wake regulation, and antagonists of both hypocretin receptor type 1 (HCRTR1) and/or HCRTR2 are considered to be potential hypnotic medications. It is currently unclear whether blockade of either or both receptors is more effective for promoting sleep with minimal side effects. Accordingly, we compared the properties of selective HCRTR1 (SB-408124 and SB-334867) and HCRTR2 (EMPA) antagonists with that of the dual HCRTR1/R2 antagonist almorexant in the rat. All 4 antagonists bound to their respective receptors with high affinity and selectivity *in vitro*. Since *in vivo* pharmacokinetic experiments revealed poor brain penetration for SB-408124, SB-334867 was selected for subsequent *in vivo* studies. When injected in the mid-active phase, SB-334867 produced small increases in rapid-eye-movement (REM) and non-REM (NR) sleep. EMPA produced a significant increase in NR only at the highest dose studied. In contrast, almorexant decreased NR latency and increased both NR and REM proportionally throughout the subsequent 6 h without rebound wakefulness. The increased NR was due to a greater number of NR bouts; NR bout duration was unchanged. At the highest dose tested (100 mg/kg), almorexant fragmented sleep architecture by increasing the number of waking and REM bouts. No evidence of cataplexy was observed. HCRTR1 occupancy by almorexant declined 4–6 h post-administration while HCRTR2 occupancy was still elevated after 12 h, revealing a complex relationship between occupancy of HCRT receptors and sleep promotion. We conclude that dual HCRTR1/R2 blockade is more effective in promoting sleep than blockade of either HCRTR alone. In contrast to GABA receptor agonists which induce sleep by generalized inhibition, HCRTR antagonists seem to facilitate sleep by reducing waking "drive".

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Competing Interests: The authors have the following conflicts: Dr. Revel, Dr. Malherbe, Dr. Moreau, Dr. Wettstein, and Dr. Borroni are employed by Roche. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Determination of the functions of neurotransmitters, neuromodulators and their receptors has classically been aided by use of small molecule receptor-specific antagonists. In recent years, forward and reverse genetics have provided insights into the functions of neurotransmitter/neuromodulatory systems before receptor-specific antagonists were developed. Such was the case for hypocretin (orexin), whose cell bodies in the perifornical and lateral hypothalamus synthesize a pair of neuropeptides alternatively called hypocretin-1 (HCRT1) or orexin-A and hypocretin-2 (HCRT2) or orexin-B [1,2]. Identification of a mutation in the gene encoding HCRT receptor 2 (HCRTR2 or OX2R) as the cause of canine narcolepsy [3] and demonstration that HCRT ligand-deficient mice exhibited periods of behavioral arrest that resembled both human and canine narcolepsy [4] implicated the HCRT system in sleep/wake control well before the first small molecule HCRT receptor antagonists [5,6,7] were described. An extensive literature has since led to the conclusion that the HCRT system is wake-promoting [8,9,10,11] and involved in energy homeostasis [12,13]. Other studies have suggested roles for the HCRT system in neuroendocrine, cardiovascular, water balance, and gastrointestinal control [14], nociception and hyperalgesia [15,16,17], stress and stress-induced analgesia [18,19], reward and addiction [20,21,22,23], and panic anxiety [24].

It is currently unclear whether targeting the HCRTR2 alone or both HCRT receptors is the best strategy for the development of sleep-promoting compounds. Several dual HCRTR1/R2 antagonists show significant sleep-promoting properties [25,26,27,28,29,30,31,32]. However, some reports indicate that HCRTR2 blockade alone was sufficient to produce the hypnotic actions of HCRTR antagonism [32,33]. One study compared the efficacy of the selective HCRTR1 antagonist SB-408124 [34], the selective HCRTR2 antagonist JNJ-10397049 [35], and the dual

antagonist almorexant [27] and concluded that HCRTR1 antagonism attenuates the hypnotic actions of HCRTR2 blockade [32]. While data on the affinity and selectivity of these compounds have been published, the absence of information on their pharmacokinetic properties is problematic for interpretation of their *in vivo* effects.

In the present study, we characterize the hypnotic activity of HCRTR antagonists in rats to determine whether selective or dual HCRTR antagonists are more effective for promoting sleep. To ensure a meaningful *in vivo* comparison, we determined the pharmacological and pharmacokinetic profiles in rats of two selective HCRTR1 antagonists, SB-408124 and SB-334867 [36], the selective HCRTR2 antagonist EMPA [37], and the dual HCRTR1/R2 antagonist almorexant. After showing that SB-408124 displays insufficient brain penetration, we used SB-334867 as the HCRTR1 antagonist for all *in vivo* experiments. Lastly, we determined the time course of HCRTR occupancy by almorexant and correlated this with hypnotic efficacy.

Materials and Methods

Drugs

Almorexant (ACT-078573, (2*R*)-2-[(1*S*)-6,7-Dimethoxy-1-[2-(4-trifluoromethyl-phenyl)-ethyl]-3,4-dihydro-1H-isoquinolin-2-yl]-*N*-methyl-2-phenyl-acetamide) [27], EMPA *N*-(Ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-*N*-pyridin-3-yl-methyl-acetamide) [37], SB-674042 (1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((S)-2-(5-phenyl-(1,3,4)oxadiazol-2-yl-methyl)-pyrrolidin-1-yl)-methanone) [34], and Cp-5 ((*S*)-1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-3,3-dimethyl-2-[(pyridin-4-ylmethyl)-amino]-butan-1-one) [7] were synthesized at F. Hoffmann-La Roche Ltd. (Basel, Switzerland) or SRI International (Menlo Park, CA USA) according to the patent literature [38]. SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride), zolpidem (N,N,6-Trimehtyl-2-(methylphenyl)-imidazol[1,2-a]pyridine-3-acetamide) and SB-408124 (1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea) were purchased from Tocris Bioscience (Ellisville, MO). Chemical structures are provided in Figure S1. [^{3}H]almorexant (specific activity: 42.7 Ci/mmol), [^{3}H]SB-674042 (specific activity: 24.4 Ci/mmol) and [^{3}H]EMPA (specific activity: 94.3 Ci/mmol) were synthesized at Roche.

Animals

Animal experiments performed at F. Hoffmann-La Roche were conducted in strict adherence to the Swiss federal regulations on animal protection and to the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and with the explicit approval of the local Cantonal Veterinary Office/Authority Basel City. Animal experiments performed at SRI International were approved by SRI's Institutional Animal Care and Use Committee and were in accordance with U.S. National Institute of Health guidelines. Male Wistar rats (240±20 g) used for spontaneous locomotion studies and pharmacokinetic studies at F. Hoffmann-La Roche were obtained from RCC Ltd. (Fullinsdorf, Switzerland). Male Sprague-Dawley rats (300±25 g) used for receptor occupancy studies at F. Hoffmann-La Roche were from Iffa Credo (Lyon, France). Animals were housed in separate rooms under a 12 h light/12 h dark cycle (light onset: 06:00, except where noted below; Zeitgeber time 0, ZT0) at 22±2°C, with *ad libitum* access to food and water. Male Sprague-Dawley rats (300±25 g) used for sleep studies at SRI were from Charles River (Wilmington, MA) and were housed in a temperature-controlled recording room under a 12 h light/12 h dark cycle (lights on at 05:00) with food and water available *ad libitum*. Room temperature (24±2°C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines.

Pharmacological Studies

[^{3}H]almorexant binding to rat HCRTR1 and HCRTR2. The rat cDNAs encoding HCRTR1 (Accession No. P56718) and HCRTR2 (Accession No. P56719) were subcloned into pCI-Neo expression vectors (Promega, Madison, WI) and used to transfect HEK293 cells (acquired commercially from ATCC-LGC, Molsheim, France) as previously described [37]. Membrane preparations, saturation and inhibition experiments, and determination of the association and dissociation kinetic parameters of [^{3}H]almorexant to rHCRTR2-HEK293 cell membranes were performed at F. Hoffmann-La Roche as previously described [37] and reported in the Materials and Methods S1.

Pharmacokinetic Studies

Pharmacokinetic analyses were performed at F. Hoffmann-La Roche as described in supporting Maqterials and Methods S1.

SB-334867 selectivity screen. SB-334867 was evaluated in a selectivity screen performed at CEREP (Paris, France). The screen consisted of binding assays on a panel of 79 target receptors. The specific binding (SB) of a radioligand to each target receptor was defined as the difference between the total binding and the nonspecific binding determined in the presence of a cold competitor in excess. The results are expressed as a percent of control SB obtained in the presence of SB-334867 used at 10 μM. Details on the CEREP screen are available from www.cerep.fr.

Effect of Almorexant and SB-334867 on Spontaneous Locomotor Activity in Rats

Locomotor activity (LMA) was evaluated at F. Hoffmann-La Roche as described previously [39]. Male Wistar rats were placed for 2 weeks in a 12 h light/12 h dark cycle with light onset at 22:00 (ZT0). Three h after the onset of the dark period (i.e., ZT15), rats were injected ip with either vehicle or HCRT receptor antagonist (almorexant or SB-334867 at 3, 10, 30 mg/kg in NaCl 0.9%, 0.3% Tween-80) (n = 8 per group), and returned to the recording chambers. Spontaneous LMA was recorded for a period of 30 min. At the end of the experiment, the brain and plasma were collected for determination of the drug exposure and brain/plasma concentration ratio.

Electroencephalogram, Core Body Temperature and Locomotor Activity Studies

Surgical procedures and recordings. All rodent electroencephalograph (EEG) studies were performed at SRI International. Three groups of eight male Sprague-Dawley rats (300±25 g; Charles River, Wilmington, MA) were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of EEG, electromyograph (EMG), core body temperature (T_{core}), and LMA via telemetry as previously described [40]. Data recording and scoring were performed as previously reported [40] (see also Supplemental Material and Methods). The EEG and EMG data were scored in 10 sec epochs for waking (W), rapid eye movement sleep (REM), and non-REM sleep (NR). T_{core} and LMA (counts per minute) were analyzed as hourly means. Data from the EEG studies are

reported in hourly means such that the hourly time ZT1 refers to the hour between time points ZT0 and ZT1.

Experimental design. For each of the three separate studies, a repeated measures counter-balanced design was employed in which each rat received five separate dosings. The dosing conditions for study 1 included SB-334867 at three concentrations (3, 10 and 30 mg/kg), zolpidem (ZOL, 7.5 mg/kg) and a vehicle control (saline 95%/ethanol 5%). The dosing conditions for study 2 included EMPA at three concentrations (10, 30 and100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). The dosing conditions for study 3 included almorexant at three concentrations (10, 30 and 100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). All dosings were administered ip in a volume of 2 ml/kg. A minimum of 3 d elapsed between doses. Dosing occurred during the middle of the rats' normal active period at the start of ZT19 and was typically completed within 10 min. Animals were continuously recorded for 6 h prior to dosing and for 18 h following dosing.

Determination of HCRTR1 and HCRTR2 occupancy by almorexant. This study was performed at F. Hoffmann-La Roche. Sixty-five male Sprague-Dawley rats, housed 5 per cage (light onset: 12:00), were injected intraperitoneally (ip) with either vehicle (1.25% hydroxypropyl methylcellulose (HPMC), 0.1% docusate sodium) or almorexant (30 mg/kg in 1.25% HPMC, 0.1% docusate sodium) at the mid-dark phase (ZT18; i.e., 6 h after lights-off), and returned to their home cage. Groups (n = 5 per group) of vehicle- or almorexant-treated animals were then sacrificed by decapitation 0.5, 2, 4, 8 or 12 h after the injection. An extra group of non-injected rats (n = 5) was also sampled at ZT18. Plasma was collected and stored at −80°C until assayed. Brains were rapidly dissected, frozen on dry ice, and stored at −80°C. Series of coronal brain sections (14 µm) were cut in a cryostat through the posterior hypothalamus (tuberomammillary nucleus level: 3.8 to 4.2 mm posterior to bregma) and the brain stem (dorsal raphe nucleus level: 7.3 to 8 mm posterior to bregma; locus coeruleus level: −9.3 to −10 mm posterior to bregma), thaw-mounted (6 sections per slide) and stored at −20°C. After sectioning, the remaining pieces of brain were kept at −80°C for later determination of almorexant brain concentration. The brain and plasma concentrations of almorexant were determined by quantitative liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

Receptor occupancy (RO) was determined as published previously [41]. For each Hcrt receptor subtype, two series of slides were thawed and incubated at room temperature with the relevant radioligand in assay buffer for 15 min (HCRTR1) or 1 h (HCRTR2). For HCRTR1, assay buffer (2 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 100 µL per section) contained either 5 nM [^{3}H]SB-674042 (for determination of total binding, TB) or 5 nM [^{3}H]SB-674042 plus 10 µM SB-408124 (for determination of non-specific binding, NSB). For HCRTR2, assay buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 120 µL per section) contained either 1 nM [^{3}H]EMPA (for determination of TB) or 1 nM [^{3}H]EMPA plus 10 µM Cp-5 (for determination of NSB). The liquid was drained, the brain sections were rinsed with ice-cold assay buffer (2 brief washes followed by 3×2 min soaking) and distilled water (3 brief dips) and air dried at 4°C for 12 h. The slides were exposed together with [^{3}H] microscales against tritium-sensitive imaging plates (BAS-TR2025) for 5 days. The plates were scanned with a high resolution phosphor imager device (Fujifilm BAS-5000) and calibrated measurements of radioactivity (fmol/mg protein) were made. All analyses were performed blind to treatment.

For each selected region, the mean signal density (TB) was measured and averaged from three consecutive sections from the same slide. The specific binding (SB) signal was then determined for each animal by subtracting the NSB signal from the TB signal. NSB was measured from adjacent brains sections incubated with the radiotracer and an excess of cold competitor. The SB signal was averaged for each experimental group and the percent RO was calculated at each time-point according to the equation $RO = (1-(SB_{almorexant}/SB_{vehicle})) \times 100$, where $SB_{almorexant}$ is the average SB for the animal group injected with almorexant and $SB_{vehicle}$ is the average SB for the animal group injected with vehicle.

Statistical Analyses

Results are shown as mean±SEM. LMA and RO data were analyzed with one-way ANOVA followed by Dunnett's analysis. EEG data were analyzed with repeated measures (rm)-ANOVA, followed by paired two-tailed *t*-tests. REM:NR ratios, sleep latencies (NR and REM) and cumulative data were analyzed with one-way rm-ANOVA and all other data with two-way rm-ANOVA. Light period and dark period data were analyzed separately as well as pre- and post-drug administration data. Statistical significance was set at $P<0.05$.

Results

Pharmacological Studies

Binding characteristics of [^{3}H]almorexant to rHCRTR1- and rHCRTR2-expressing cell membranes. To characterize the *in vitro* binding of [^{3}H]almorexant to rat HCRT receptors, saturation binding analyses were performed at binding equilibrium on membranes isolated from HEK293 cells transiently transfected with rHCRTR1 and rHCRTR2. As shown in Fig. 1A and B, [^{3}H]almorexant bound with high affinity to a single saturable site on recombinant rHCRTR1 (K_d of 3.4±0.3 nM and B_{max} of 27.2±0.7 pmol/mg prot, at 23°C) and rHCRTR2 (K_d of 0.5±0.0 nM and B_{max} of 53.0±1.4 pmol/mg prot, measured at 37°C). Binding kinetics of [^{3}H]almorexant to membrane preparations from HEK293 cells transiently expressing rHCRTR2 are shown in Fig. 1C and D and the kinetic parameters in Table 1. The association binding of [^{3}H]almorexant to the rHCRTR2 had a half-maximal binding at 10 min and reached equilibrium within 50 min. The data were fit to a one-phase exponential model with the association rate constant of 0.073±0.015 $nM^{-1}min^{-1}$. The dissociation rate for [^{3}H]almorexant binding to the rHCRTR2 was determined by the addition of an excess amount of almorexant (5 µM) after equilibrium was reached. The rate of [^{3}H]almorexant dissociation from rHCRTR2 membrane was slow; the reversal of binding was incomplete and did not reach baseline even after 2 h (Fig. 1D & Table 1).

The potencies of almorexant and of the selective HCRTR1 antagonists SB-334867 [6] and SB-408124 [34] in inhibiting [^{3}H]almorexant binding to HEK293-rHCRTR1 and HEK293-rHCRTR2 cell membranes are given in Table 2. Almorexant was able to displace [^{3}H]almorexant binding from rHCRTR1 and rHCRTR2 membranes with high affinity (Table 2). In contrast, SB-334867 and SB-408124 displaced [^{3}H]almorexant binding from rHCRTR1, but not from rHCRTR2, with high affinity (Table 2).

Pharmacokinetic Studies

Pharmacokinetic properties of SB-334867, SB-408124, EMPA and almorexant in rats. The oral bioavailability and pharmacokinetic properties of almorexant, SB-334867 and SB-408124 were evaluated in Wistar rats. The mean pharmacokinetic parameters after single iv or oral (po) bolus administration in rat

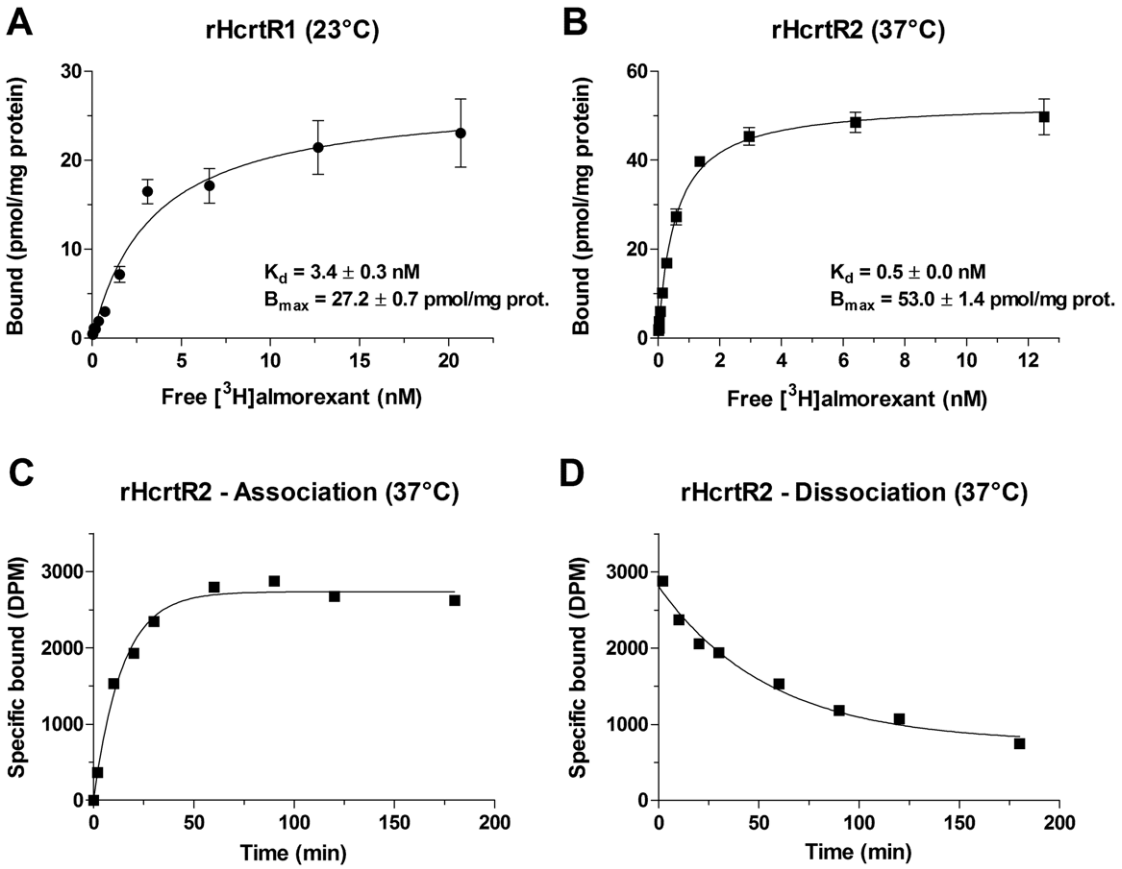

Figure 1. Binding characteristics of [^{3}H]almorexant to rHCRTR1 and rHCRTR2 cell membranes. (**A,B**) Saturation binding curves of [^{3}H]almorexant binding to membranes from HEK293 cells transiently transfected with rHCRTR1 (**A**) or rHCRTR2 (**B**). Each data point represents the mean±SEM of three independent experiments performed in triplicate. The data were analyzed by nonlinear regression analysis using GraphPad Prism 4.0 software and a single-site binding model. (**C,D**) Time course for the association (**C**) and dissociation (**D**) of [^{3}H]almorexant binding to rHCRTR2 membranes.
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are given in Table S1. Almorexant displayed a high systemic plasma clearance, high volume of distribution at steady state (Vss) and low oral bioavailability in rat. In addition, almorexant was highly bound to plasma proteins (<3.7%, and <8.7% free fraction in human and rat plasma, respectively), and its stability measured for 2 h in human and rat plasma was 90.0% and 95.0%, respectively. The mean brain/plasma concentration ratio of almorexant was 0.12 in rat.

SB-334867 exhibited a low systemic plasma clearance, medium Vss and oral bioavailability in rat. SB-334867 is highly bound to plasma proteins (1.3%, and 0.8% free fraction in human and rat plasma, respectively), and its stability measured for 1 h/4 h in human and rat plasma was 95%/93% and 104%/110%, respectively. The mean brain/plasma concentration ratio of SB-334867 (at a dose of 8.8 mg/kg, po) was 0.53 in rat.

SB-408124 had a low systemic plasma clearance, low Vss and medium oral bioavailability in rat. SB-408124 had very low free fraction in human and rat (0.3% and <0.1%, respectively) and its stability (1 h/4 h) in human and rat plasma was 94%/88% and 101%/107%, respectively. The mean brain/plasma concentration ratio of SB-408124 (at dose of 18 mg/kg, po) was 0.03 in rat. Such unfavorable pharmacokinetic properties of SB-408124, most importantly its extremely low brain penetration, prompted us to use SB-334867 for further *in vivo* studies in the rat.

The pharmacokinetic profiles of EMPA have been reported previously [37].

Table 1. Kinetic parameters for the association and dissociation of [^{3}H]almorexant in rHCRTR2-HEK293 cell membranes at 37°C.

Compound	Association kinetic	Dissociation kinetic	Apparent	
	K_{on} ($nM^{-1}min^{-1}$)	K_{off} (min^{-1})	$t_{1/2}$ (min)	K_d (nM)
[^{3}H]almorexant	0.073±0.015	0.021±0.004	36.3±5.7	0.33±0.9

The K_{on} (calculated on rate), K_{off} (observed off rate), $t_{1/2}$ (half-maximal binding) and K_d (apparent dissociation constant) values are ± SEM, calculated from three independent experiments (each performed in quadruplicate) as described under ''Materials and Methods''.
doi:10.1371/journal.pone.0039131.t001

Table 2. Potencies of almorexant, SB-408124 and SB-334867 antagonists in inhibition of [^{3}H]almorexant binding to the membrane preparations from HEK293 cells transiently expressing rHCRTR1 and rHCRTR2.

Compound	rHCRTR1	rHCRTR2
	[^{3}H]almorexant (23°C)	[^{3}H]almorexant (37°C)
	K_i (nM)	K_i (nM)
almorexant	7.1±0.7	2.0±0.0
SB-408124	45.7±4.1	5370.0±2200.0
SB-334867	58.4±2.9	2390.0±81.0

[^{3}H]almorexant was used at a concentration equal to its K_d values of 3.4 nM and 0.5 nM at rHcrtR1 and rHcrtR2, respectively, in these competition binding experiments. K_i values for [^{3}H]almorexant binding inhibition by various antagonists were calculated as described under "Materials and Methods". Values are ± SEM of the K_i calculated from three independent experiments, each performed in duplicate.

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Selectivity profile of SB-334867. The specificity of SB-334867 at the HCRTR1 was confirmed by assessment in radioligand binding assays in a broad CEREP screen (Paris, France; www.cerep.fr) (Table S2). Among the 79 receptors tested, 30 were peptide receptors. SB-334867 was inactive (<40% activity at 10 μM) at all targets tested with the exception of the A_{2A} (adenosine), A_3, MT3 (melatonin), P_{2Y} (purinergic 2Y) and $5HT_{2C}$ (serotonin 2C) receptors, where it caused 89%, 63%, 102%, 64% and 70% displacement of specific binding at 10 μM, respectively. The selectivity profiles of almorexant [27] and EMPA [37] have been reported previously.

Effect of Almorexant and SB-334867 on Spontaneous Locomotor Activity in Rats

The ability of almorexant and SB-334867 to antagonize *in vivo* the biological action of endogenous hypocretins was assessed by measuring spontaneous LMA during the active phase. Almorexant dose-dependently reduced LMA, although only the 30 mg/kg dose reached significance when compared to vehicle (Figure 2A; $F = 4.28$, $p<0.05$). Similarly, SB-334867 dose-dependently reduced spontaneous LMA, with both the 10 and 30 mg/kg doses being statistically different from vehicle (Figure 2B; vehicle: 6097±536; 10 mg/kg: 3509±383; 30 mg/kg: 2626±341; $F = 12.80$, $p<0.01$ and $p<0.001$, respectively).

The plasma and brain exposure of SB-334867 were measured at the end of the LMA experiment. When determined 35 min after ip administration, SB-334867 doses of 3, 10 and 30 mg/kg produced plasma levels of 220, 718 and 738 ng/mL vs. brain levels of 48, 171, and 142 ng/mL (ratios: 0.21, 0.23, 0.19, respectively). These results confirmed the ability of SB-334867 to enter the rat brain at the doses used in this report.

Rodent EEG Studies

The effects of almorexant, SB-334867 and EMPA administered in the middle of the dark (active) period were evaluated during the latter half of the active period and subsequent light (inactive) period to determine both efficacy for sleep promotion and whether "hangover" or rebound effects occurred. Of these three compounds, only almorexant reduced NR and REM sleep latency (Figure 3). Almorexant at 30 and 100 mg/kg reduced NR latency while only the 30 mg/kg concentration decreased latency to REM sleep. ZOL produced a decrease in NR latency in all three experiments.

In contrast, all three compounds increased NR sleep (Figure 4). SB-334867 at 3 and 30 mg/kg increased cumulative NR for the first 4 and 6 h periods following administration ($F = 10.808$, $p<0.0001$ and $F = 10.752$, $p<0.0001$, respectively). EMPA at 100 mg/kg also increased cumulative NR for the first 4 and 6 h periods post administration ($F = 17.655$, $p<0.0001$ and $F = 12.816$, $p<0.0001$, respectively). Almorexant had the strongest effect: both 30 and 100 mg/kg increased cumulative NR for 2, 4 and 6 h following administration ($F = 13.010$, $p<0.0001$; $F = 17.771$, $p<0.0001$; and $F = 16.179$, $p<0.0001$, respectively). Cumulative REM also increased for the first 2 h following almorexant at 30 mg/kg ($F = 5.418$, $p = 0.0023$) and for the 6 h period following the 100 mg/kg dose (Figure 4; $F = 8.535$, $p<0.0001$). ZOL increased cumulative NR and decreased cumulative REM in all three experiments. Whereas ZOL suppressed the REM:NR ratio in all 3 studies, none of the 3 test compounds did (Table 3). Although ZOL had significant effects on EEG delta power during NR, this parameter was little affected by any of the three test compounds compared to vehicle control (Figure S2).

There were few effects on sleep/wake amounts during the light period subsequent to administration of EMPA, SB-334867 or almorexant (Figure 5). REM was not significantly affected during this period following any of the three HCRT antagonists. NR decreased during the third hour of the light period (ZT3) following SB-334867 at 10 and 30 mg/kg while NR increased during ZT1 and ZT6 following almorexant at 30 mg/kg compared to vehicle. No significant effects on NR were found following EMPA during the light period.

Significant results occurred in measures of sleep-wake consolidation (Tables S3, S4, S5 and Figures S3, S4, S5). The strongest effects were found following almorexant at 100 mg/kg, which produced increased numbers of W and NR bouts during ZT19, ZT20, and ZT22-ZT24 ($F = 2.069$, $p = 0.0077$ and $F = 2.413$, $P = 0.0015$, respectively). The number of REM bouts was increased by almorexant at 100 mg/kg during ZT22-ZT24 ($F = 2.963$, $p = 0.002$). W bout duration was decreased following almorexant at 100 mg/kg during ZT22 compared to vehicle ($F = 2.320$, $p = 0.0023$). All three concentrations of EMPA increased the number of W bouts ($F = 4.243$, $p = 0.0065$). SB-334867 increased NR bout duration during ZT21 following 30 mg/kg and during ZT24 following 3 mg/kg ($F = 4.574$, $p<0.0001$).

Both LMA and T_{core} underwent dose-dependent decreases after drug treatment (Figure 6). ANOVA revealed condition effects for both almorexant and EMPA in which LMA was decreased across the 6 h period following administration of both compounds at 100 mg/kg compared to vehicle ($F = 7.316$, $p<0.00015$ and $F = 7.442$, $p = 0.00018$ respectively). No differences in LMA during the subsequent light period were found. Condition effects for T_{core} were found in all three studies. The high concentrations tested for all three HCRT receptor antagonists decreased T_{core} across the 6 h period following administration ($F = 7.629$, $p = 0.00027$ for SB-334867; $F = 7.442$, $p = 0.00018$ for EMPA; $F = 7.315$, $p = 0.00036$ for almorexant). ZOL administration resulted in the largest declines in T_{core} in all three studies, which was followed by a sustained rebound increase in T_{core} during the subsequent light period.

Time Course of HCRT Receptor Occupancy (RO) by Almorexant

To determine the time-course of HCRTR1 and HCRTR2 RO by almorexant, a single dose of almorexant at the smallest concentration shown to promote sleep (30 mg/kg, ip; Figure 4) was administered in the mid-dark phase (ZT18) and rats were

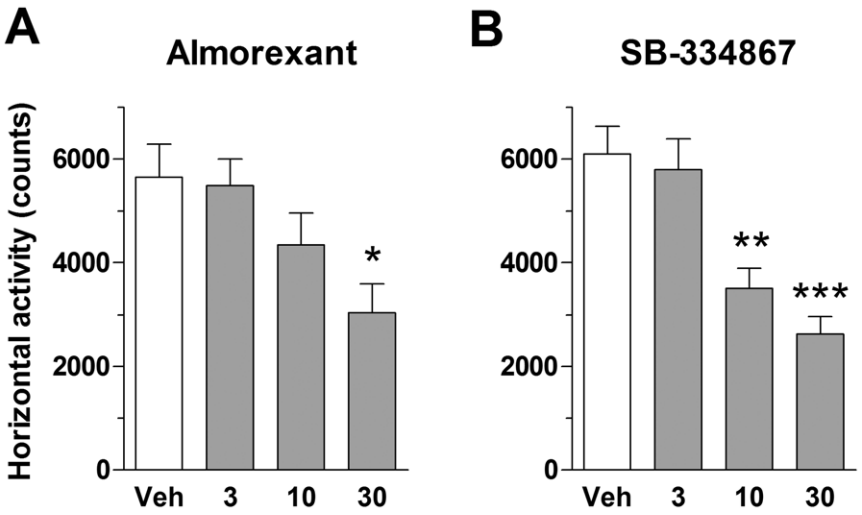

Figure 2. Effects of almorexant and SB-334867 on spontaneous locomotor activity of rats during the active phase. Both almorexant **(A)** and SB-334867 **(B)** reduced locomotor activity compared to vehicle (Veh) when administered 3 h after the onset of the dark period. Horizontal locomotor activity was recorded for a period of 30 min. Numbers on the X-axes represent intraperitoneal doses in mg/kg.***$p<0.001$, **$p<0.01$, *$p<0.05$ vs. Veh (one-way ANOVA followed by Dunnett's analysis). All data are mean±SEM (n = 8 per group).
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sacrificed after incubation periods of 0, 0.5, 2, 4, 8 or 12 h. For both HCRTR1 and HCRTR2, the NSB was minimal and represented 6.2% and 3%, respectively, of the average TB signal measured in control animals. The signal localization was in good agreement with the distribution of *HCRTR1*- and *HCRTR2*-expressing neurons [42,43], as confirmed by *in situ* hybridization on separate sections (data not shown). Figure 7A shows representative autoradiograms of HCRTR1 binding sites in the locus coeruleus (*LC)*. This signal localization is in good agreement with the distribution of *Hcrtr1*-expressing neurons [42,43], as confirmed by *in situ* hybridization (data not shown). The rats injected with vehicle displayed maximal HCRTR1 radiotracer binding at all time points (Figure 7A), whereas the animals injected with almorexant showed reduced binding 2 h after the injection. Binding of the HCRTR1 radiotracer returned to levels similar to control 8–12 h post almorexant injection.

Figure 7B shows representative autoradiograms of the HCRTR2 binding sites examined at 2 different rostro-caudal levels. At the level of the posterior hypothalamus, signal was observed in various brain regions, including the tuberomammillary nuclei (*TMN*), cerebral cortex (*CC*), retrosplenial cortex (*RSC*), and field CA3 of the hippocampus (*CA3*). The signal attributed to the *TMN* was verified by *in situ* hybridization for histidine decarboxylase mRNA on separate sections (data not shown). At the level of the anterior pons, the dorsal raphe nuclei (*DRN*), pontine nuclei (*Pn*) and parabigeminal nuclei (*PBG*) displayed specific labeling. This pattern corresponds to that already reported by Malherbe et al. [37] and was in good agreement with the distribution of *Hcrtr2*-expressing neurons previously described [42,43]. The rats injected with vehicle displayed constant HCRTR2 binding at all time points. In contrast, the animals that received almorexant exhibited a very strong reduction of HCRTR2 radiotracer binding and, 2 h after almorexant injection, no signal could be detected (Figure 7B). Reduction of TB signal was still evident for all brain regions 12 h after almorexant administration.

SB was quantified in the *LC* for HCRTR1 and in 6 brain areas (*TMN*, *CC*, *CA3*, *RSC*, *DRN* and *Pn*) for HCRTR2, and the RO by almorexant was determined for 12 h post-injection (Figure 7C and Figure S7). HCRTR1 RO reached 50–60% from 30 min to 4 h post-injection (maximum: 59% after 2 h) and then returned to basal levels after 6 h. This RO profile paralleled that of almorexant concentration in the plasma (Figure 7D) and brain (Figure S6). For both compartments, drug concentration rose rapidly and reached a peak around 30 min, with plasma levels of 1966.4±349.2 ng/mL and brain levels of 565.8±112.4 ng/g (mean brain/plasma concentration ratio: 0.28). The half-maximal concentrations were achieved between 4 and 6 h.

For HCRTR2, all 6 structures displayed a comparable RO profile (Figure 7C for *DRN* and *TMN*, and Figure S7 for *CC, RSC, Pn* and *CA3*): it was close to 100% within 30 min after dosing, remained at maximal levels at 2 h and 4 h, and started to slowly decline between 4 and 6 h. After 12 h, although the brain and plasma levels of almorexant were strongly reduced (Figure 7C and Figure S6), HCRTR2 occupancy was still elevated with levels between 49 and 67%, depending on brain structure (Figure 7C and Figure S7; *TMN*: 49.2±13.2%; *CC*: 66.1±11.6%; *CA3*:58.4±11.5%; *RSC*: 64.6±10.7%; *DRN*: 57.7±10.5%; *Pn*: 67.2±13.9%).

Discussion

This study was undertaken to determine whether blockade of either or both HCRT receptors is more effective in promoting sleep. Multiple dual HCRTR1/R2 antagonists employing different molecular scaffolds have been found to have significant significant sleep-promoting properties [25,26,27,28,29,30,31] Anatomical localization of HCRTRs suggests that both receptors are involved in the promotion of wakefulness [39,43]. High levels of HCRTR1 are found in *LC* while only HCRTR2 is abundant in the *TMN*. Both receptors are expressed at moderately high levels in the dorsal and medial raphe and in the cholinergic regions of the basal forebrain. In the laterodorsal tegmentum and the pedunculopontine nucleus (brain stem cholinergic regions), the HCRTR1 is predominant. However, some recent reports support the hypothesis that only blockade of the HCRTR2 underlies the hypnotic actions of HCRTR antagonism [30,31]. Further, one study suggests that antagonism

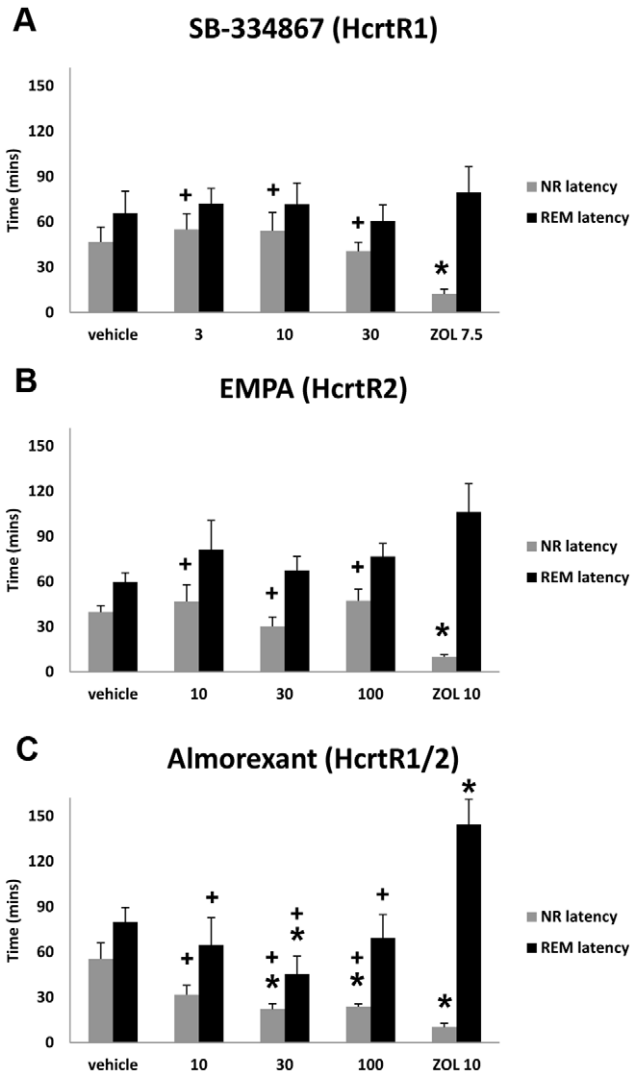

Figure 3. Latency to the onset of NR and REM sleep following administration of SB-334867. (A), EMPA **(B)**, and almorexant **(C)** as compared to zolpidem (ZOL). *=significantly different from vehicle ($p<0.05$); +=significantly different from ZOL ($p<0.05$) (One-way repeated measures ANOVA followed by paired two-tail *t* tests; n=8 per group). Data represent the mean±SEM.
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of HCRTR1 attenuates the hypnotic actions of HCRTR2 blockade [32]. Therefore, to help clarify the hypnotic effects of HCRTR blockade, we characterized the pharmacological and pharmacokinetic properties of selective and dual HCRTR antagonists in rat before evaluating their relative efficacy on sleep and wakefulness.

Pharmacokinetic Considerations

The affinities of almorexant, SB-408124 and SB-334867 at the rat HCRTR1 and HCRTR2 receptors are very similar to those reported for human HCRT receptors (for almorexant, K_i values of 4.7 nM and 0.9 nM at hHCRTR1 and hHCRTR2, 37°C, respectively [42]; for SB-334867, K_i value of 38.7 nM at rHCRTR1 [34]; for SB-408124, K_i value of 26.9 nM at rHCRTR1 [34]). Almorexant had high affinity for both HCRTRs and displayed a slow rate of dissociation from rHCRTR2 membranes *in vitro*, which translated into a long-lasting occupancy of the HCRTR2 *in vivo*. This property likely underlies some of the pharmacological effects described here. Among the three antagonists tested, almorexant had the highest systemic plasma clearance, highest Vss but lowest oral bioavailability; both SB-334867 and SB-408124 had low clearances and medium to low bioavailability. Importantly, SB-408124 had a very low free fraction and was found to penetrate the brain poorly, especially when compared to the other compounds. This prompted us to use SB-334867 for evaluating the effects of selective HCRTR1 blockade on sleep.

Effects of Selective HCRTR1 and HCRTR2 Antagonists on Sleep/wake

Selective blockade of HCRTR2 clearly results in sleep promotion. The HCRTR2 antagonist JNJ-10397049 reduced NR latency during both the light and dark phases, increased NR duration in the light phase, and increased both NR and REM duration during the dark phase [30,31]. Here, although EMPA had no effect on either NR or REM latency when administered in the mid-dark phase, it increased cumulative NR for the first 4 and 6 h. Conversely, icv infusion of an HCRTR2 agonist, [Ala^{11}]orexin-B, during the light period dose-dependently increased wake duration and decreased the amounts of both NR and REM sleep [44]. The effects of HCRT1 (orexin-A) on wakefulness and NREM sleep were reduced more in $OX2R^{-/-}$ mice than in $OX1R^{-/-}$ mice, implying that HCRTR2 has a greater influence than HCRTR1 on these parameters, at least in mice [45].

The selective HCRTR1 antagonist SB-334867 dose-dependently reduced LMA and, at 3 and 30 mg/kg i.p., increased cumulative NR for the first 4 and 6 h. These results differ from those of Dugovic *et al.* [32] who reported that selective blockade of HCRTR1 using SB-408124 had no effect on sleep, although it reduced LMA. However, the time of drug administration differed between these studies (middle vs. start of the active phase). By the middle of the active phase, both endogenous HCRT tone [46,47] and sleep pressure are increased, so HCRTR antagonists are more likely to be effective at this time of day than at dark onset.

A previous study showed that SB-334867 blocked HCRT1-induced effects on REM sleep but did not alter any sleep parameters when administered alone [36]. However, only the first hour after treatment was examined whereas, here, effects of SB-334867 on sleep were only apparent after 2 h. Importantly, we showed that SB-408124 exhibits poor pharmacokinetic properties, with notably low free fraction and little brain penetration, which likely limits its *in vivo* efficacy. The brain-to-plasma ratio for SB-408124 is 0.03, which is in the range of blood contamination levels obtained with the residual blood carried over in the brain homogenate (in the absence of compound in the brain). Although Dugovic *et al.* [32] did not specifically report brain-to-plasma ratios, they did report both brain and plasma concentrations following administration of SB-408124 at 30 mg/kg. Using these numbers, a brain-to-plasma ratio for SB-408124 is calculated to be 0.012 (using C_{max} values given in text: brain-to-plasma ratio = 1.09/84.29 = 0.012), which is in good agreement with our findings. This observation most likely explains why Dugovic *et al.* [32] did not detect effect on sleep. There are numerous examples of compounds lacking central efficacy due to insufficient brain exposure. For example, the reduced ability of second-generation H1 anti-histaminic drugs to cross the blood-brain barrier (BBB) as compared to the first generation of drugs, prevents them from causing centrally-mediated side effects such as sedation [48,49,50]. Similarly, the antidiarrheal medication loperamide is a potent agonist of the μ opiate receptor that is devoid of opioid central effects at usual doses in patients [51]. This directly results from the

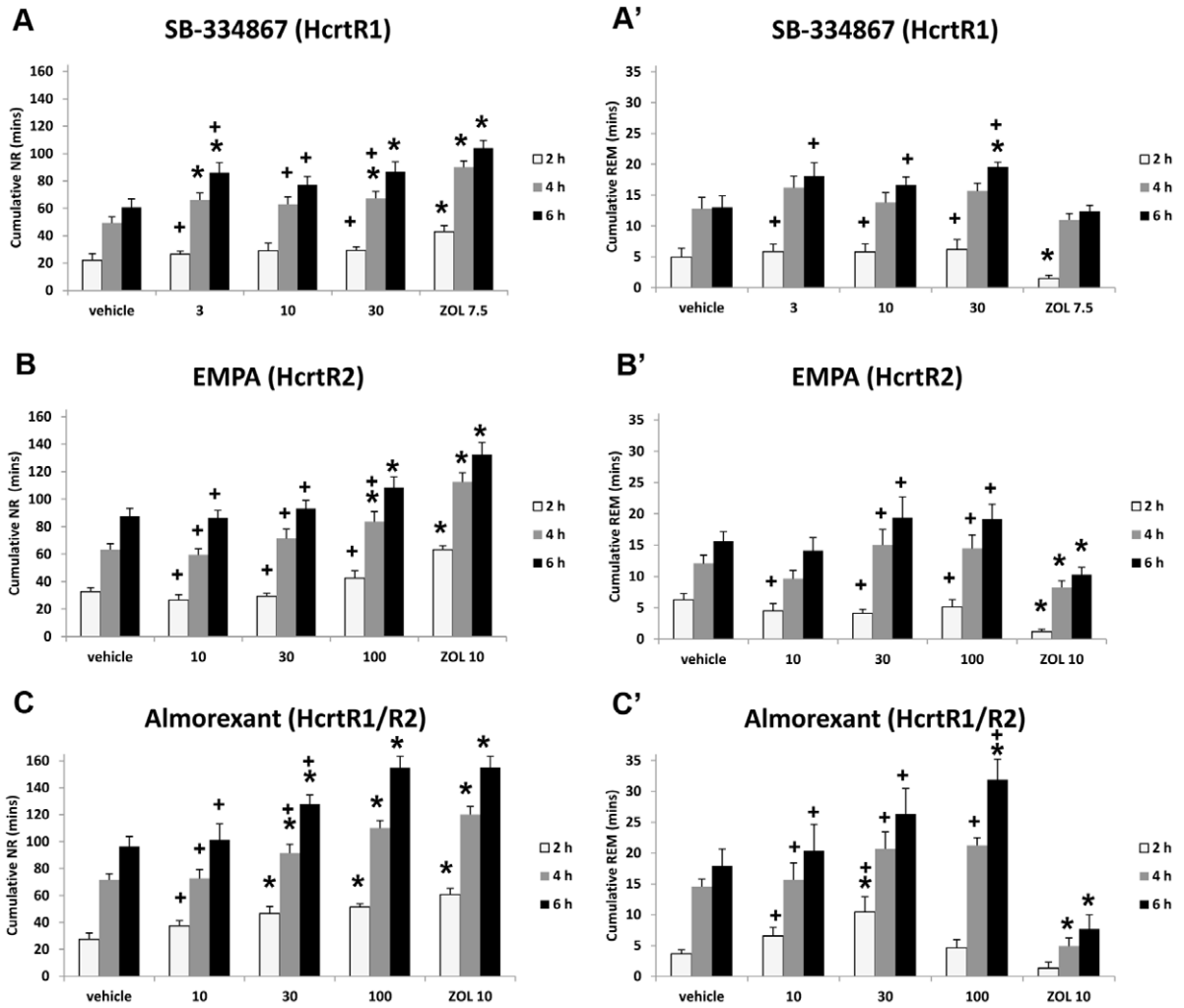

Figure 4. Cumulative time in NR and REM sleep over the first 2, 4 and 6 h following drug administration. (A–C) Cumulative time spent in NR sleep following SB-334867 (**A**), EMPA (**B**) and almorexant **(C)** compared to zolpidem (ZOL). **(A'–C')** Cumulative time spent in REM sleep for the same drug treatments. (One-way repeated measures ANOVA followed by paired two-tail *t* tests; n = 8 per group). Data represent the mean±SEM. *, significantly different from vehicle; **+**, significantly different from ZOL.
doi:10.1371/journal.pone.0039131.g004

Table 3. REM:NR ratios for the 6 h period following the administration of SB-334867, EMPA and almorexant.

Vehicle	**SB-334867**	**SB-334867**	**SB-334867**	**ZOL**
	3 mg/kg	**10 mg/kg**	**30 mg/kg**	**7.5 mg/kg**
0.22±0.039	0.21±0.016^{+}	0.22±0.023^{+}	0.23±0.016^{+}	0.12±0.009*
Vehicle	**EMPA**	**EMPA**	**EMPA**	**ZOL**
	10 mg/kg	**30 mg/kg**	**100 mg/kg**	**10 mg/kg**
0.18±0.018	0.16±0.019^{+}	0.21±0.032^{+}	0.18±0.027^{+}	0.08±0.008*
vehicle	**Almorexant**	**Almorexant**	**Almorexant**	**ZOL**
	10 mg/kg	**30 mg/kg**	**100 mg/kg**	**10 mg/kg**
0.18±0.020	0.19±0.026^{+}	0.20±0.022^{+}	0.21±0.021^{+}	0.05±0.013*

* = significantly different from vehicle ($p<0.05$), $^{+}$ = significantly different from ZOL ($p<0.05$).
doi:10.1371/journal.pone.0039131.t003

low brain exposure caused by the P-glycoprotein (P-gp) transporter at the BBB [51]. Administration of the drug to P-gp-deficient mice or co-administration with a P-gp blocker both increase brain levels and trigger central effects typically observed with brain penetrant opioids, such as analgesia [52,53] or respiratory depression [54]. Our observation made with SB-408124 underscores that verification of brain penetration is a prerequisite for the conception and use of centrally-acting drugs [55,56].

On the other hand, it is difficult to reconcile the poor brain penetration of SB-408124, both documented here and also evident in the study of Dugovic *et al.* (estimation: 0.012), with some indications of central localization following subcutaneous administration of 30 mg/kg, i.e. the 90% HCRTR1 occupancy observed in the *tenia tecta* and the SB-408124-mediated elevation of extracellular dopamine levels in the prefrontal cortex [32]. A heterogeneous distribution of the drug is unlikely, and further experiments will be necessary to delineate more precisely the free concentration of the compound, such as microdialysis studies and measures of binding to brain tissue homogenates.

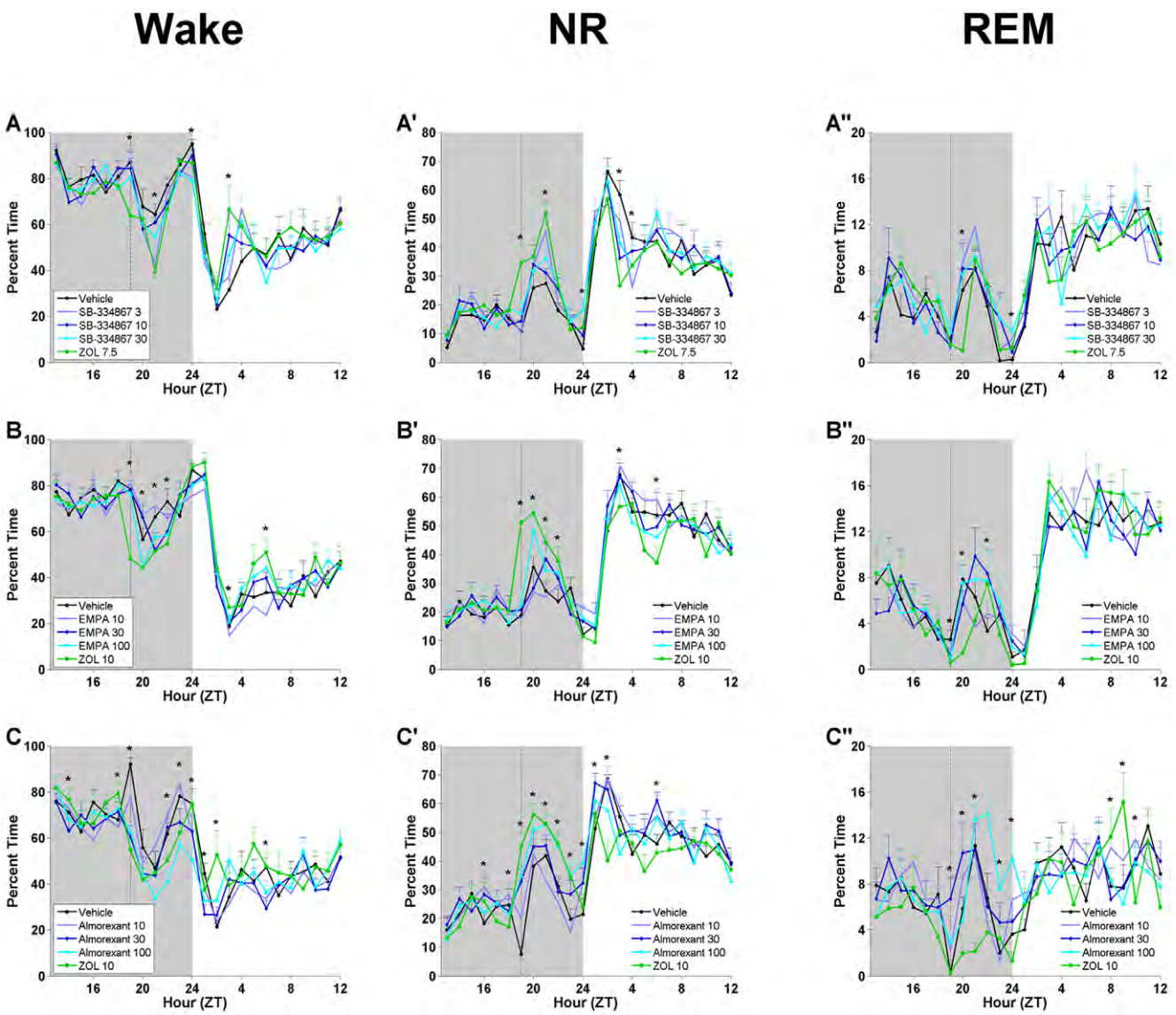

Figure 5. Hourly distribution of W, NR and REM sleep. W, NR and REM sleep for 6 h prior to and 18 h after administration of SB-334867 **(A)**, EMPA **(B)**, and almorexant **(C)** as compared to zolpidem (ZOL) and vehicle. Shaded area represents the dark phase; vertical dotted line in each panel indicates the time of injection. **(A)** Hourly amounts of wakefulness following SB 334867. **(A')** Hourly amounts of NR sleep following SB 334867. **(A'')** Hourly amounts of REM sleep following SB 334867. **(B)** Hourly amounts of wakefulness following EMPA. **(B')** Hourly amounts of NR sleep following EMPA. **(B'')** Hourly amounts of REM sleep following EMPA. **(C)** Hourly amounts of wakefulness following almorexant. **(C')** Hourly amounts of NR sleep following almorexant. **(C'')** Hourly amounts of REM sleep following almorexant. Data represent the mean±SEM (n = 8 rats per group). *, $p<0.05$. For detailed statistical results, see Text S1.
doi:10.1371/journal.pone.0039131.g005

Dual HCRTR Antagonists as Potential Hypnotic Medications

Dual HCRTR1/R2 antagonists are now well-established to induce sleep. In rats, almorexant administered po at the beginning of the dark phase promoted both NR and REM sleep and, at a higher dose, reduced NR and REM latency [27]. The effects on sleep duration but not sleep latency were confirmed when almorexant was administered sc [32]. Here, we report that almorexant given ip at the mid-dark phase also increases sleep duration. However, in contrast to Dugovic *et al.*, we found that almorexant at 30 and 100 mg/kg reduced NR latency and the 30 mg/kg dose also decreased REM latency. These differences likely reflect the greater sensitivity of the sleep/wake bioassay when injections occur in the mid-dark period after a sleep debt has accumulated. Recently, other dual HCRTR1/R2 antagonists have also been reported to reduce active wake and increase both NR or delta sleep and REM sleep when administered near the mid-dark phase [25,26,27,28,29,30,31,57]. Thus, multiple HCRTR1/R2 antagonists seem to be effective in inducing sleep.

Our results indicate some promising aspects of HCRT antagonists as hypnotic agents. First, in contrast to current hypnotics such as zolpidem which increase NR and suppress REM sleep, none of the three HCRTR antagonists affected the REM:NR ratio, indicating that both REM and NR increased proportionally. Second, in comparison to zolpidem, HCRTR antagonists only triggered a limited, physiological reduction of body temperature. Lastly, no excess wakefulness was observed during the subsequent light period. A proportional increase of

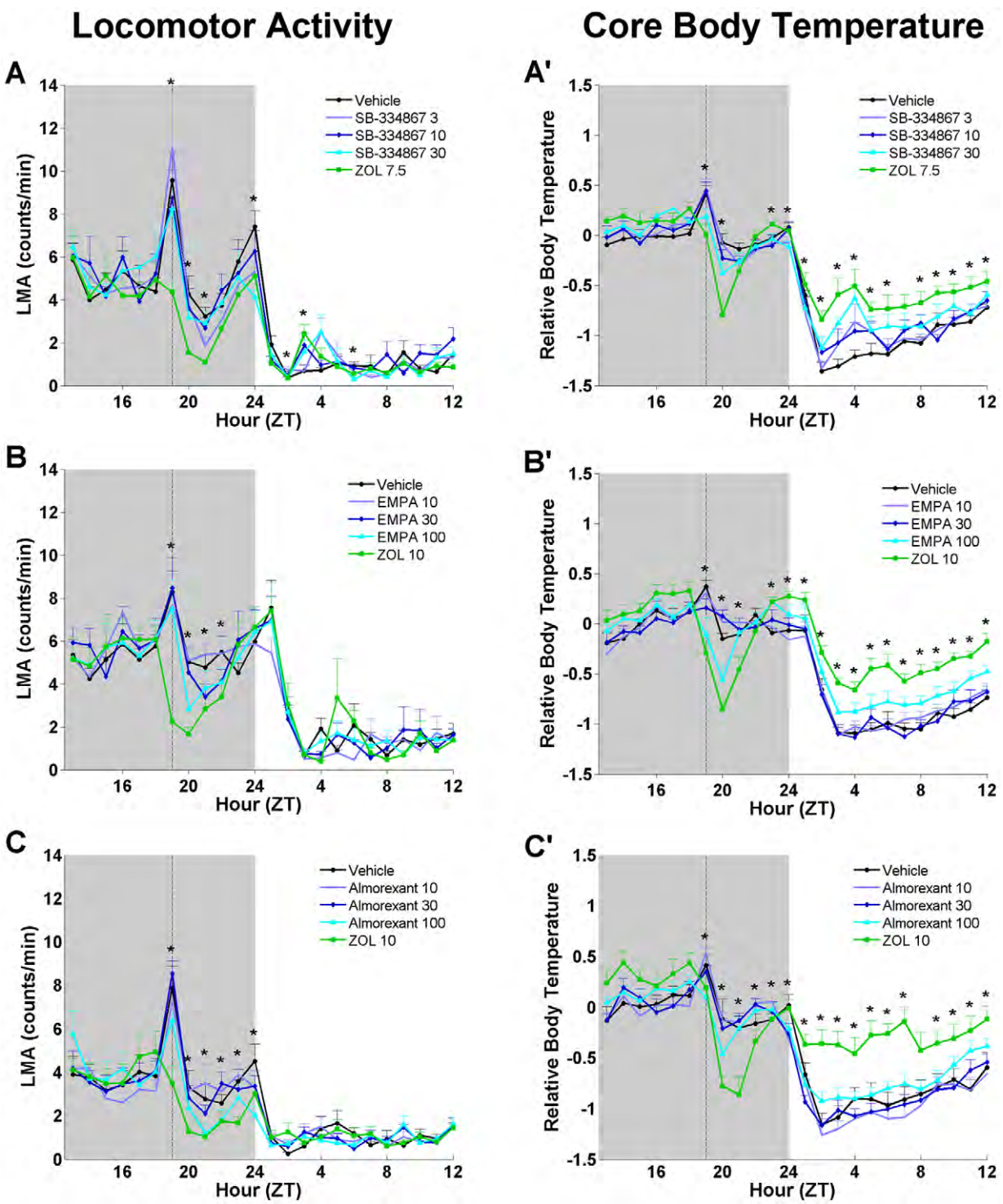

Figure 6. Average hourly LMA and relative T_{core}. LMA and relative T_{core} for 6 h prior to and 18 h after administration of SB-334867 (A), EMPA (B), and almorexant (C) as compared to zolpidem (ZOL) and vehicle. Shaded area represents the dark phase; vertical dotted line in each panel indicates the time of injection. (A) Average hourly LMA following SB-334867. (A′) The average hourly T_{core} following SB-334867. (B) The average hourly LMA following EMPA. (B′) The average hourly T_{core} following EMPA. (C) The average hourly LMA following almorexant. (C′) The average hourly T_{core} following almorexant. Data represent the mean±SEM (n = 8 rats per group). *, $p<0.05$. For detailed statistical results see Text S1.
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REM and NR sleep without rebound wakefulness and a mild change in core temperature are desirable properties of substances that induce "physiological" sleep.

On the other hand, the mechanism by which these HCRTR antagonists increased sleep duration suggests disruption of normal sleep/wake architecture. SB-334867 increased NR through a

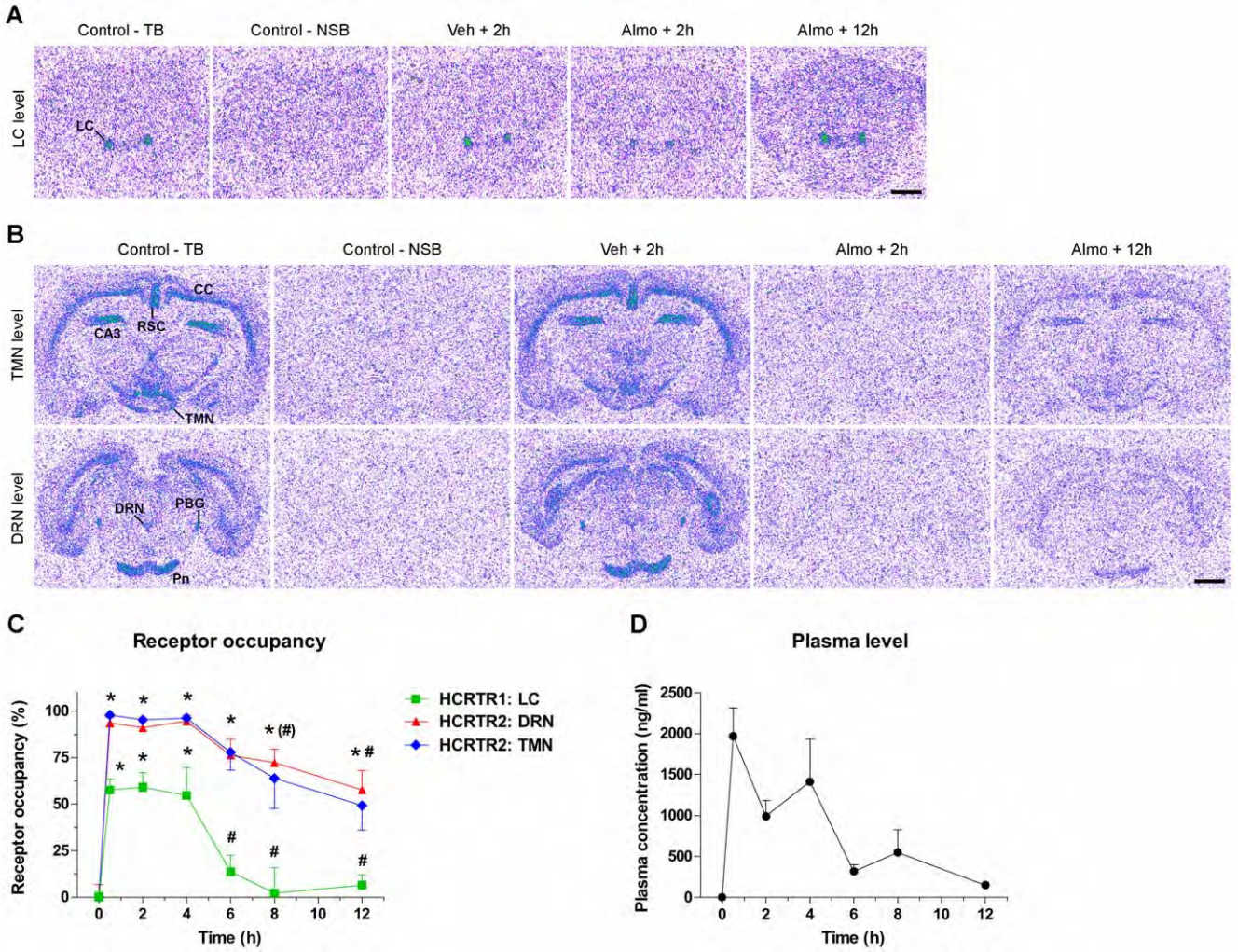

Figure 7. Time-course of HCRT1R and HCRT2R occupancies by almorexant. (A,B) Representative autoradiograms showing [3H]SB-674042 (5 nM) binding to HCRTR1 **(A)** and [3H]EMPA (1 nM) binding to HCRTR2 **(B)** in rat coronal brain sections. For both receptors, total binding (*TB*) was maximal in control animals (not injected) sampled at time 0 (*t0*). For HCRTR1 **(A)**, a clear signal was evident in the locus coeruleus (*LC*), which could be displaced by co-incubation with an excess of cold SB-674042 (10 µM) (non-specific binding, *NSB*). In contrast to vehicle administration (*Veh, 2 h*), almorexant (30 mg/kg injected intraperitoneally at ZT18) attenuated such specific signal after 2 h (*Almo, 2 h*), but not after 12 h (*Almo, 12 h*). For HCRTR2 **(B)**, signal was observed in various brain regions, including the tuberomammillary nuclei (*TMN*), cerebral cortex (*CC*), field CA3 of the hippocampus (*CA3*), retrosplenial cortex (*RSC*), dorsal raphe nuclei (*DRN*), pontine nuclei (*Pn*) and parabigeminal nuclei (*PBG*). [3H]EMPA could be displaced by co-incubation with an excess of Cp5 (10 µM) (*NSB*). HCRTR2 binding became minimal 2 h after almorexant (*Almo, 2 h*), but not after Vehicle (*Veh+2 h*), administration. After 12 h (*Almo, 12 h*), HCRTR2 binding was intermediate. Scale bars, 2 mm. **(C)** Time course of HCRTR1 and HCRTR2 occupancies by almorexant. Receptor occupancy was calculated by measuring the specific binding at various time points in the *LC* for HCRTR1, and in the *TMN* and *DRN* for HCRTR2. *, $p<0.001$ versus time 0; (#), $p<0.05$ (TMN only), #, $p<0.05$ (TMN) or $p<0.01$ (DRN), vs. time 30 min (one-way ANOVA followed by Dunnett's analysis). **(D)** Almorexant plasma concentrations. Data represent the mean±SEM (n = 5 rats per group).
doi:10.1371/journal.pone.0039131.g007

combination of small increases in both the number and duration of NR bouts that, although not significant for any particular hour, cumulatively summated into an overall significant NR increase at 4 and 6 h. For EMPA, a greater number of NR bouts underlie the overall increase in NR at the highest dose. For almorexant, NR augmentation resulted from an increased number of NR bouts without a change in bout duration, confirming previous results [32]. The increase in NR, however, was also associated with greater numbers of both W and REM bouts, particularly at the highest dose examined. Thus, although almorexant produces an overall increase in NR sleep that is greater than the other HCRTR antagonists, this is achieved through a fragmented sleep architecture. In this regard, almorexant-treated rats appear somewhat similar to *orexin* null mutant [4] or *orexin/ataxin-3* [12] mice which have disrupted sleep architecture (although these strains also exhibit cataplexy). However, the fragmentation of sleep architecture induced by dual HCRTR antagonists is consistent with the concept that the HCRT system stabilizes arousal states and minimizes the number of transitions between states [58]. Since drugs were administered to healthy animals during their active period, a more fragmented sleep architecture would be predicted. Rather than driving sleep *per se*, HCRTR antagonism seems to create a permissive neural environment for sleep to occur. Since the drive for sleep was low at the time of administration, more frequent sleep bouts without increases in bout durations could be expected.

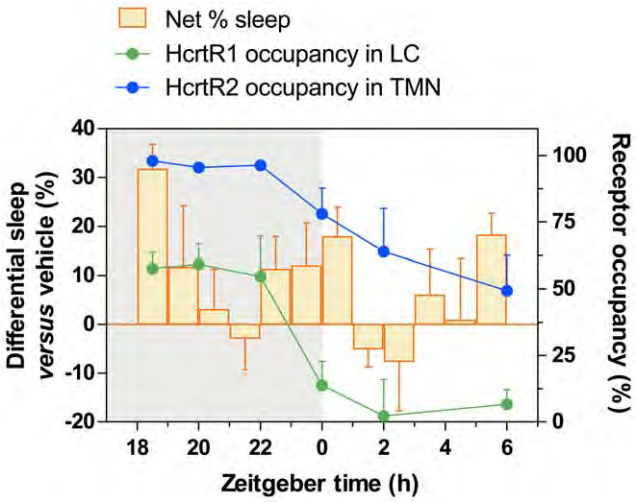

Figure 8. Net effect of almorexant on the percentage of sleep compared to HCRTR1 and HCRTR2 occupancies. The percentage of total sleep (%NR + %REM) in the vehicle-injected animals was subtracted from that of almorexant-treated rats (30 mg/kg) and was plotted over time. HCRTR1 occupancy in the locus coeruleus (*LC*) and HCRTR2 occupancy in the tuberomammillary nuclei (*TMN*) are shown in parallel. Injection occurred at ZT18. *Gray area,* dark phase; *White area,* light phase.
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Absence of Cataplexy but Facilitation of REM Sleep

One concern regarding the development of HCRTR antagonists is the possibility of inducing cataplexy as occurs in *HcrtR2* mutant dogs [3] or *HcrtR2* null mutant mice [59]. In the present study, we saw no evidence of cataplexy produced by any of the three compounds, even at the highest dose tested. However, almorexant significantly increased REM bout duration during the first hour after treatment and the highest dose – which presumably resulted in the most complete HCRTR blockade – produced 2 to 3 fold as many REM bouts during the latter half of the dark period when compared to vehicle. These observations indicate that HCRTR antagonism facilitates REM sleep occurrence, as noted by others [59].

Relationship between HCRTR Occupancy and Sleep

Whereas 30 mg/kg ip almorexant resulted in approximately 50% HCRTR1 occupancy, HCRTR2 occupancy was nearly complete in brain regions important for sleep/wake control. Moreover, while HCRTR1 occupancy declined after 4 h, HCRTR2 occupancy remained high even 12 h after treatment. While our results for HCRTR2 are consistent with a previous report, those for HCRTR1 differ [32]. A primary difference between these studies is the brain location used for determination of HCRTR1 occupancy: whereas Dugovic *et al.* used the *tenia tecta*, we measured HCRTR1 occupancy in the *LC*, an area implicated in sleep/wake control.

Figure 8 correlates RO with the net amount of sleep induced by almorexant at 30 mg/kg compared to vehicle. Since HCRTR2 occupancy is virtually 100% following this dose of almorexant while HCRTR1 occupancy is ~50%, it is likely that the stronger sleep-promoting effects observed at 100 mg/kg are due to greater HCRTR1 blockade. Figure 8 demonstrates that the sleep-promoting effects of almorexant do not simply mirror the RO data. The greatest amount of sleep occurred in the first hour after almorexant administration when occupancy of HCRTRs was maximal. Surprisingly, despite elevated occupancy of HCRTRs in subsequent hours, the hypnotic effect dissipated, suggesting that other arousal-promoting systems can overcome HCRTR blockade and produce wakefulness. In contrast, near the end of the dark phase when sleep pressure is elevated, partial HCRTR blockade was sufficient to produce sleep. These data highlight the contrasting sleep-promoting mechanisms between HCRTR antagonists and other hypnotic medications such as zolpidem. Whereas the latter compounds trigger long-lasting sleep and affect sleep intensity (sleep-inducing effect), HCRTR antagonists seem to merely antagonize wakefulness, generating conditions that allow sleep to occur (sleep permissive action).

Conclusion

Our results support the hypothesis that dual HCRTR1/R2 blockade is more effective in promoting sleep than selective blockade of either HCRTR alone. A similar conclusion was reached in a recent study of HCRT receptor knockout mice [45]. Although both HCRTR1 (SB-334867) and HCRTR2 (EMPA) antagonists produced somnogenic effects, neither promoted sleep to the levels of the dual HCRTR antagonist almorexant. Furthermore, since the lowest doses of almorexant that were sleep-promoting (30 mg/kg) bind virtually 100% of the HCRTR2s while only 50% of the HCRTR1s are occupied at that dose, the stronger sleep-promoting effects of higher doses are likely due to additional blockade of HCRTR1. These data support the notion that HCRTR antagonists are a promising avenue for sleep/wake therapeutics, with the qualifications stated above. However, given the involvement of the HCRT system in many physiological functions [9,60] including respiratory control [61,62,63,64], careful screening for side effects of HCRTR antagonists will be needed.

Supporting Information

Figure S1 Chemical structures of the compounds used in this study. Receptor selectivity is indicated into parentheses. All compounds except zolpidem are selective HCRTR antagonists. Zolpidem is a gama-aminobutyric acid (GABA) A-receptor agonist.
(TIF)

Figure S2 Hourly delta power normalized to the 24 h average vehicle control. A: 3 concentrations of SB-334867 vs. ZOL and vehicle. ANOVA is significant for treatment by time only ($F = 3.80$, $p<0.0001$). For treatment by time: **ZT19:** SB-334867 at 3 mg/kg > vehicle; ZOL >334867 at 3 and 10 mg/kg and vehicle. **ZT24:** 334867 at 3 and 10 mg/kg > ZOL; Vehicle >334867 at 10 and 30 mg/kg and ZOL **B:** 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA is significant for treatment (see legend, $F = 13.47$, $p<0.0001$) and for treatment by time ($F = 11.86$, $p<0.0001$). For treatment by time: **ZT19:** ZOL > all other conditions. **ZT20:** ZOL > all other conditions. **ZT21:** EMPA at 30 mg/kg > vehicle; ZOL > EMPA at 100 mg/kg and vehicle. **ZT22:** EMPA at 30 mg/kg > vehicle. **ZT23:** EMPA at 10 mg/kg > ZOL. **C:** 3 concentrations of almorexant vs. ZOL and vehicle. ANOVA is significant for treatment by time only ($F = 2.63$, $p = 0.0005$). For treatment by time: **ZT20:** Vehicle > almorexant at 100 mg/kg. **ZT23:** Almorexant at 10 mg/kg > vehicle. **ZT24:** Vehicle > almorexant at 100 mg/kg.
(TIF)

Figure S3 Hourly distribution of Wake Bout Duration and the Number of Wake Bouts. Wake Bout Duration (left) and Number of Wake Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**)

as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The Wake Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. No significant differences were found. **A':** The Wake Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT1-ZT6 is significant for treatment by time ($F = 1.82$, $p = 0.02341$). For treatment by time: **ZT2:** SB 334867 at 10 mg/kg and vehicle < ZOL vehicle < SB 334867 at 30 mg/kg **ZT4:** SB 334867 at 30 mg/kg and ZOL < vehicle **B:** The Wake Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. No ANOVA's were significant. **B':** The Wake Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 3.65$, $p = 0.01350$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 4.24$, $p = 0.00647$) For treatment by time: **ZT19:** vehicle < ZOL **ZT20:** vehicle < EMPA at 30 mg/kg **ZT22:** vehicle < ZOL **ZT24:** vehicle < EMPA at 10, 30 and 100 mg/kg **ZT7:** EMPA at 10 mg/kg < ZOL **ZT11:** vehicle < ZOL **C:** The Wake Bout Duration for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 4.01$, $p = 0.01077$) and for treatment by time ($F = 2.32$, $p = 0.00234$). For treatment by time: **ZT20:** Almorexant at 100 mg/kg < ZOL **ZT21:** Almorexant at 30 and 100 mg/kg < ZOL **ZT22:** Almorexant at 100 mg/kg < ZOL and vehicle **C':** The Wake Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 8.82$, $p = 0.00001$) and for treatment by time ($F = 2.07$, $p = 0.00769$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 3.39$, $p = 0.02208$). For treatment by time: **ZT19:** vehicle < Almorexant at 30 and 100 mg/kg **ZT20:** ZOL < Almorexant at 10, 30 and 100 mg/kg **ZT21:** ZOL < Almorexant at 30 and 100 mg/kg **ZT22:**ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg/kg **ZT24:** vehicle < Almorexant at 100 mg/kg **ZT9:** Almorexant at 10 and 30 mg.kg < vehicle.
(TIF)

Figure S4 Hourly distribution of NR Bout Duration and Number of NR Bouts. NR Bout Duration (left) and Number of NR Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**) as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The NR Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 12.46$, $p<0.00001$) and for treatment by time ($F = 4.57$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.70$, $p = 0.00498$) and for treatment by time ($F = 3.16$, $p = 0.00004$). For treatment by time: **ZT19:** SB 334867 at 3 mg/kg and vehicle < ZOL **ZT20:** all other conditions < ZOL **ZT21:** vehicle < SB 334867 at 30 mg/kg and ZOL **ZT24:** vehicle < SB 334867 at 3 mg/kg **ZT1:** ZOL < SB 334867 at 3 and 10 mg/kg and vehicle SB 334867 at 3 mg/kg < vehicle **ZT3:** SB 334867 at 30 mg/kg and ZOL < vehicle **A':** The NR Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT1-ZT6 is significant for treatment by time ($F = 1.81$, $p = 0.02532$). For treatment by time: **ZT1:** vehicle < SB 334867 at 3 and 30 mg/kg and ZOL **ZT4:** SB 334867 at 3 mg/kg < vehicle **B:** The NR Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 13.46$, $p<0.00001$) and for treatment by time ($F = 5.34$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 7.99$, $p = 0.00010$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 3.03$, $p = 0.02981$). For treatment by time: **ZT19:** all other conditions < ZOL **ZT20:** all other conditions < ZOL **ZT23:** ZOL < EMPA at 10 mg/kg **ZT24:** ZOL < EMPA at 30 mg/kg **ZT2:** ZOL < EMPA at 30 mg/kg **ZT3:** ZOL < EMPA at 10 and 100 mg/kg and vehicle **ZT5:** ZOL < EMPA at 10 and 100 mg/kg and vehicle EMPA at 30 and 100 mg/kg < vehicle **ZT6:** ZOL < EMPA at 10 mg/kg and vehicle EMPA at 100 mg/kg < vehicle **B':** The NR Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. No ANOVA's were significant. **C:** The NR Bout Duration for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 16.44$, $p<0.00001$) and for treatment by time ($F = 5.34$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.83$, $p = 0.00433$) and for treatment by time ($F = 2.24$, $p = 0.00341$). For treatment by time: **ZT19:** all other conditions < ZOL vehicle < Almorexant at 100 mg/kg **ZT20:** all other conditions < ZOL **ZT21:** all other conditions < ZOL **ZT22:** Almorexant at 10 and 30 mg/kg < ZOL **ZT2:** ZOL < Almorexant at 10 and 30 mg/kg and vehicle Almorexant at 100 mg/kg < vehicle **ZT3:** ZOL < Almorexant at 10 mg/kg **ZT4:** ZOL < Almorexant at 10 mg/kg **ZT5:** ZOL < Almorexant at 10 mg/kg **ZT6:** ZOL < Almorexant at 30 mg/kg **C':** The NR Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 12.58$, $p<0.00001$) and for treatment by time ($F = 2.41$, $p = 0.00149$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.18$, $p = 0.00890$). For treatment by time: **ZT19:** vehicle < Almorexant at 30 and 100 mg/kg **ZT20:** ZOL < Almorexant at 10, 30 and 100 mg/kg vehicle < Almorexant at 100 mg/kg **ZT21:** ZOL < Almorexant at 10, 30 and 100 mg/kg **ZT22:** ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg.kg **ZT24:** vehicle < Almorexant at 100 mg.kg **ZT1:** vehicle < ZOL.
(TIF)

Figure S5 Hourly distribution of REM Sleep Bout Duration and the Number of REM Sleep Bouts. REM Sleep Bout Duration (left) and the Number of REM Sleep Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**) as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The REM Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 4.40$, $p = 0.00692$) and treatment by time ($F = 2.16$, $p = 0.00500$). For treatment by time: **ZT19:** ZOL < SB 334867 at 3 mg/kg **ZT20:** ZOL < all other conditions **ZT23:** vehicle < all other conditions **ZT24:** SB 334867 at 10 mg/kg < ZOL vehicle < SB 334867 at 3 mg/kg **A':** The REM Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time only ($F = 4.49$, $p = 0.00625$). For treatment by time: **ZT20:** ZOL < all other conditions **ZT24:** vehicle < SB 334867 at 30 mg/kg **B:** The REM Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time ($F = 1.71$, $p = 0.03515$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.88$, $p = 0.00015$) and for treatment by time ($F = 2.81$, $p = 0.00015$). For treatment by time: **ZT21:** ZOL < EMPA at 100 mg/kg **ZT24:** EMPA at 100 mg/kg < vehicle **ZT1:** EMPA at 100 mg/kg < ZOL all other conditions < vehicle **ZT4:** EMPA at 10 and 30 mg/kg < vehicle **ZT5:** ZOL < EMPA at 10 mg/kg **B':** The REM Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 3.99$, $p = 0.00888$) and for treatment by time ($F = 1.96$, $p = 0.01112$). For treatment by time: **ZT20:** ZOL < all other conditions **ZT22:** vehicle < ZOL **ZT23:** ZOL < vehicle **C:** The REM Bout Duration for 3 concentrations of Almorexant vs.

ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time ($F = 6.91$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.45$, $p = 0.00657$). For treatment by time: **ZT19:** ZOL and vehicle < Almorexant at 10, 30 and 100 mg/kg **ZT20:** all other conditions < ZOL **ZT24:**ZOL < Almorexant at 10 and 100 mg/kg and vehicle Almorexant at 30 mg/kg < vehicle **C':** The REM Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 9.29$, $p = 0.00007$) and for treatment by time ($F = 2.96$, $p = 0.00010$). For treatment by time: **ZT19:** ZOL and vehicle < Almorexant at 30 mg/kg **ZT20:** ZOL < Almorexant at 10 and 30 mg/kg and vehicle **ZT21:** ZOL < all other conditions **ZT22:** ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg/kg **ZT24:** ZOL and vehicle < Almorexant at 100 mg/kg.
(TIF)

Figure S6 Brain concentration of almorexant. Time course of almorexant concentration in the brain of rats injected intraperitoneally with 30 mg/kg at the mid-dark phase (same animals as in Figures 7). Data are the mean±SEM (n = 5 rats per group).
(PDF)

Figure S7 HCRTR2 occupancy in the cerebral cortex, retrosplenial cortex, pontine nuclei, and hippocampus. Data are the mean±SEM (n = 5 rats per group). *, $p<0.001$ vs. time 0; ##, $p<0.01$, #, $p<0.05$ vs. time 30 min (one-way ANOVA followed by Dunnett's analysis). Almorexant plasma concentrations (data from Figure 7) are shown for comparison.
(TIF)

Materials and Methods S1 Expanded materials and methods for both *in vitro* and *in vivo* experiments as referenced in the text.
(DOCX)

Text S1 Expanded legends for Figures 5 and 6 that include detailed statistical results.
(DOCX)

Table S1 Pharmacokinetic assessment of almorexant, SB-334867 and SB-408124 after i.v. and p.o. administration to Wistar rat.
(DOCX)

Table S2 CEREP selectivity screen in the broad radioligand binding assays were undertaken to determine the pharmacological activity of SB-334867.
(DOCX)

Table S3 Measures of state consolidation for 6 h following the administration of SB-334867.
(DOCX)

Table S4 Measures of state consolidation for 6 h following the administration of EMPA.
(DOCX)

Table S5 Measures of state consolidation for 6 h following the administration of almorexant.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SRM FGR JLM JGW TSK EB PM. Performed the experiments: SRM FGR DV EB. Analyzed the data: SRM FGR DV JLM TSK EB PM. Wrote the paper: SRM FGR TSK.

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The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats

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The hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but little is known about neurocognitive performance in the presence of ALM. HcrtR antagonists are hypothesized to induce sleep by disfacilitation of wake-promoting systems whereas $GABA_A$ receptor modulators such as zolpidem (ZOL) induce sleep through general inhibition of neural activity. To test the hypothesis that less functional impairment results from HcrtR antagonist-induced sleep, we evaluated the performance of rats in the Morris Water Maze in the presence of ALM vs. ZOL. Performance in spatial reference memory (SRM) and spatial working memory (SWM) tasks were assessed during the dark period after equipotent sleep-promoting doses (100 mg/kg, po) following undisturbed and sleep deprivation (SD) conditions. ALM-treated rats were indistinguishable from vehicle (VEH)-treated rats for all SRM performance measures (distance traveled, latency to enter, time within, and number of entries into, the target quadrant) after both the undisturbed and 6 h SD conditions. In contrast, rats administered ZOL showed impairments in all parameters measured compared to VEH or ALM in the undisturbed conditions. Following SD, ZOL-treated rats also showed impairments in all measures. ALM-treated rats were similar to VEH-treated rats for all SWM measures (velocity, time to locate the platform and success rate at finding the platform within 60 s) after both the undisturbed and SD conditions. In contrast, ZOL-treated rats showed impairments in velocity and in the time to locate the platform. Importantly, ZOL rats only completed the task 23–50% of the time while ALM and VEH rats completed the task 79–100% of the time. Thus, following equipotent sleep-promoting doses, ZOL impaired rats in both memory tasks while ALM rats performed at levels comparable to VEH rats. These results are consistent with the hypothesis that less impairment results from HcrtR antagonism than from $GABA_A$-induced inhibition.

Keywords: hypocretins/orexins, cognitive impairment, memory impairment, hypnotics, water maze, spatial reference memory, spatial working memory, EEG

INTRODUCTION

Insomnia is a highly prevalent condition affecting 10–30% of the general population; (NIH, 2005; Roth, 2007; Mai and Buysse, 2008). Sleep loss and sleep disruption can lead to a degradation of neurocognitive performance as assessed by objective and subjective measures (Wesensten et al., 1999; Belenky et al., 2003; Lamond et al., 2007). Prescription sleep medications are often used to treat insomnia and obtain desired amounts of sleep. Presently, nonbenzodiazepine, positive allosteric modulators of the $GABA_A$ receptor such as zolpidem (ZOL) are the most widely prescribed hypnotic medications. Although known to induce sleep, these compounds have been shown to significantly impair psychomotor and memory functions in rodents (Huang et al., 2010; Uslaner et al., 2013; Zanin et al., 2013), non-human primates (Makaron et al., 2013; Soto et al., 2013; Uslaner et al., 2013) and humans (Balkin et al., 1992; Wesensten et al., 1996, 2005; Mattila et al., 1998; Mintzer and Griffiths, 1999; Verster et al., 2002; Storm et al., 2007; Otmani et al., 2008; Gunja, 2013). Such impairment can be particularly troubling when there is an urgent need for highly functional performance in the presence of drug such as with first responders, military personnel, and caregivers. Further, complex behaviors during the sleep period (e.g., eating, cooking, driving, conversations, sex) have been associated with these medications (Dolder and Nelson, 2008). Therefore, more effective hypnotics are needed that facilitate sleep that is easily reversible in the event of an unexpected awakening that demands unimpaired cognitive and psychomotor performance.

Recently, antagonism of the hypocretin (Hcrt; also called orexin) receptors has been identified as a target mechanism for the next generation of sleep medications (Brisbare-Roch et al., 2007; Dugovic et al., 2009; Whitman et al., 2009; Hoever et al., 2010, 2012a,b; Coleman et al., 2012; Herring et al., 2012; Winrow et al., 2012; Betschart et al., 2013). The Hcrt system is well known to play an important role in the maintenance of wakefulness (de Lecea, 2012; Inutsuka and Yamanaka, 2013; Mieda and Sakurai, 2013; Saper, 2013). Hcrt fibers project throughout the central nervous system (CNS), with particularly dense projections and receptor expression found in arousal centers including the locus

coeruleus, the tuberomammilary nucleus, dorsal raphe nuclei, laterodorsal tegmentum, pedunculopontine tegmentum, and the basal forebrain (Peyron et al., 1998; Marcus et al., 2001). The excitatory effects of the Hcrt peptides on these arousal centers is hypothesized to stabilize and maintain wakefulness. Therefore, blockade of the Hcrt system should disfacilitate these arousal centers, creating conditions that are permissive for sleep to occur.

The current study tests the hypothesis that the dual Hcrt receptor antagonist almorexant (ALM) produces less functional impairment than ZOL. The rationale that underlies this hypothesis is that ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. We tested this hypothesis using tests of spatial reference memory (SRM) and spatial working memory (SWM) in the Morris Water Maze. Although the concentrations of ALM and ZOL administered prior to these tests were equipotent in hypnotic efficacy, the performance of rats treated with ALM were superior to that of rats treated with ZOL.

MATERIALS AND METHODS

ANIMALS

One hundred fifty three male Sprague Dawley rats (300 g at time of purchase; Charles River, Wilmington, MA) were distributed among the 12 groups as described in **Table 1**. All animals were individually housed in temperature-controlled recording chambers (22 ± 2°C, 50 ± 25% relative humidity) under a 12:12 light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

SURGICAL PROCEDURES

Rats were instrumented with sterile telemetry transmitters (F40-EET, Data Sciences Inc., St Paul, MN) as previously described (Morairty et al., 2008, 2012; Revel et al., 2012, 2013). Briefly, under isoflurane anesthesia, transmitters were placed intraperitoneally and biopotential leads were routed subcutaneously to the head and neck. Holes were drilled into the skull at 1.5 mm anterior to bregma and 1.5 mm lateral to midline, and 6 mm posterior to bregma and 4 mm lateral to midline on the right hemisphere. Two biopotential leads used as EEG electrodes were inserted into the holes and affixed to the skull with dental acrylic. Two biopotential leads used as EMG electrodes were positioned bilaterally through the nuchal muscles.

IDENTIFICATION OF SLEEP/WAKE STATES

After at least 3 weeks post-surgical recovery, EEG, and EMG were recorded via telemetry using DQ ART 4.1 software (Data Sciences Inc., St Paul, MN). Following completion of data collection, the EEG, and EMG recordings were scored in 10 s epochs as waking (W), rapid eye movement sleep (REM), or non-rapid eye movement sleep (NREM) by expert scorers blinded to the treatments using NeuroScore software (Data Sciences Inc., St Paul, MN). Sleep latency was defined as the first 60 s of continuous sleep following drug administration. Recordings were started at Zeitgeber time (ZT) 12 (lights off) and continued until animals performed the water maze tests.

Table 1 | The number of rats tested for each of the 12 experimental groups.

Test	No SD			6 h SD		
	VEH	ALM	ZOL	VEH	ALM	ZOL
Reference memory	14	13	17	16	16	8
Working memory	11	12	12	12	11	11

SLEEP DEPRIVATION PROCEDURES

Animals were sleep deprived (SD) from ZT12-18 by progressive manual stimulation concurrent with EEG and EMG recording. The rats were continuously observed and, when they appeared to attempt to sleep, progressive interventions were employed to keep them awake: removal of cage tops, tapping on cages, placement of brushes inside the cage, or stroking of vibrissae or fur with an artist's brush.

DRUGS

Almorexant (ALM; ACT-078573), was synthesized at SRI International (Menlo Park, CA. USA) according to the patent literature. Zolpidem (ZOL) was a gift from Actelion Pharmaceuticals Ltd. For the SWM task, rats were dosed with ALM (100 mg/kg, p.o.), ZOL (100 mg/kg, p.o.) or vehicle (VEH; 1.25% hydroxypropyl methyl cellulose, 0.1% dioctyl sodium sulfosuccinate, and 0.25% methyl cellulose in water) at ZT18 and left undisturbed until time to perform memory tasks (see below). For the SRM task, most rats were also administered ALM, ZOL, and VEH p.o. at the concentrations above. However, one cohort of rats was administered drugs i.p. For these rats, ALM was administered at 100 mg/kg ($N = 6$), ZOL at 30 mg/kg ($N = 8$) and VEH ($N = 7$). ZOL is approximately 3X more potent i.p. than p.o.(Vanover et al., 1999) while ALM is equipotent through both routes of administration. Analysis of the sleep/wake data confirmed the equipotent effects of both drugs through both routes of administration at the concentrations tested.

WATER MAZE

All water maze (WM) tasks occurred in a pool 68″ in diameter and 25″ in depth, containing water at 24 ± 2°C made opaque by the addition of non-toxic, water soluble black paint and milk powder. Since all tests took place during the dark period, distinctive spatial cues were made of small "rice" lights colored blue, yellow, and green. Patterns of lights in distinct shapes (circle, square, diamond, "T" shape) were clearly visible from within the pool. Preliminary studies determined the minimum number of lights that were needed for learning to occur. A 10 cm diameter platform was submerged approximately 1 cm below the surface of the water in one of 6 locations (**Figure 1**). The platform location determined the orientation of the 4 quadrants used for analysis. Both WM tasks were similar to previous reports (Wenk, 2004; Ward et al., 2009).

TEST OF SPATIAL REFERENCE MEMORY

The acquisition phase occurred in one session consisting of 12–15 consecutive trials with a 60 s inter-trial interval. For each trial,

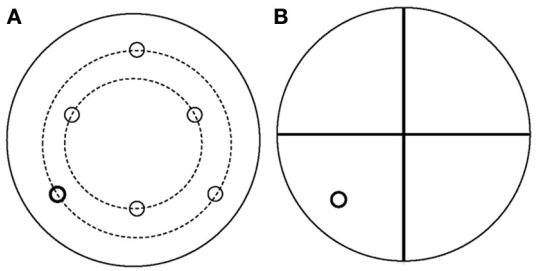

FIGURE 1 | Schematic of the water maze apparatus used for both spatial reference and spatial working memory tasks. (A) Schematic of the platform locations. **(B)** Example of quadrant orientations used for analysis used for the platform indicated in bold. Quadrant locations were always oriented so that the platform was central within a quadrant.

rats were placed in the WM facing the wall in one of three quadrants that did not contain the hidden platform. The location of the hidden platform remained constant across all trials. Rats were given 60 s to locate the platform. If the rats did not locate the platform within this period, they were guided to the platform location. When the rats reached the platform, they were allowed to remain on the platform for approximately 15 s before being placed in a dry holding cage for the next 60 s. This training sequence continued until the rats learned the task, typically 12–15 trials.

On the following day, rats were dosed with ALM, ZOL or VEH at ZT18 and a retention probe trial was performed 90 min later in which the rats were returned to the WM but the platform had been removed. A total of 40 rats were subjected to SD for 6 h prior to drug administration, and 42 were left undisturbed during this period (**Table 1**). Rats were started in the quadrant opposite the target quadrant and allowed to swim for 30 s. All trials were recorded by video camera and analyzed with Ethovision XT software (Noldus, Leesburg, VA). Test measures for the retention probe were time spent in target quadrant, latency to target quadrant, frequency of entrance into target quadrant, and total distance traveled. Swim speed was calculated to control for nonspecific effects.

TEST OF SPATIAL WORKING MEMORY

The SWM task consisted of 6 pairs of trials, one for each platform location (**Figure 1A**). In the first trial, a cued platform marked with a flag was placed in one of 6 positions in the WM. Rats were released facing the wall from one of the 3 quadrants not containing the platform and were allowed 120 s to locate the cued platform before the researcher guided the rats to the platform. This procedure provided all rats the opportunity to learn the platform location even if they did not find it on their own. After 15 s on the platform, the rats were removed from the WM and placed in a holding cage. The flag was then removed but the platform remained in the same location as in the first trial. Following a delay of 1, 5, or 10 min in the holding cage, the rats were placed back in the WM into one of the 2 quadrants that did not contain the platform and was not the starting quadrant during the first trial. Once the rats found the platform, they were removed after approximately 5 s and placed back in a holding cage for 10 min before a new pair of trials with a novel platform location was given. The order of delays was counterbalanced so that each rat was tested twice at 1, 5, or 10 min delays between the cued and hidden platforms. All trials were recorded by video camera and analyzed with Ethovision XT software (Noldus, Leesburg, VA). Test measures were time to locate the platform and the swim velocity during all tests.

STATISTICAL ANALYSIS

Statistical analyses were performed using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA). Sleep/wake data (W, NREM, and REM time) were analyzed in 30 min bins and compared between drug groups using Two-Way mixed-model ANOVA on factors "drug group" (between subjects) and "time" (within subjects). SRM performance parameters (latency, duration and frequency in target quadrant, total distance traveled) were analyzed using a One-Way ANOVA. SWM performance measures (velocity, time to platform, percent found) by delay time were analyzed using Two-Way mixed-model ANOVA on factors "drug group" (between subjects) and "time" (within subjects). Significance levels were set at $\alpha = 0.05$. When ANOVA indicated significance, Bonferroni *t*-tests were used for *post hoc* analyses.

RESULTS

Drug concentrations were chosen to be equipotent at sleep promotion based on our previous experience (Morairty et al., 2012). Although ZOL produced a more rapid onset to sleep under both SD and undisturbed conditions (No SD: ZOL = 6.6 min, VEH = 32.2 min, ALM = 25.4 min; SD: ZOL = 5.9 min, VEH = 20.0 min, ALM = 15.5 min), ALM- and ZOL-treated rats slept equivalent amounts during the last hour before the WM test (**Figure 2**; No SD: ZOL = 69.4%, ALM = 62.3%, VEH = 37.6%; SD: ZOL = 69.6%, ALM = 71.5%, VEH = 52.0%).

TEST OF SPATIAL REFERENCE MEMORY

For all performance measures analyzed, rats treated with ZOL showed significant impairments while ALM- and VEH-treated rats were indistinguishable (**Figure 3**). Following ZOL, the latency to the target zone increased (No SD: ZOL = 14.1 s, VEH = 5.7 s, ALM = 5.8 s; SD: ZOL = 18.4 s, VEH = 4.2 s, ALM = 3.6 s) and the duration in the target zone (No SD: ZOL = 5.5 s, VEH = 8.4 s, ALM = 7.9 s; SD: ZOL = 4.8 s, VEH = 7.7 s, ALM = 7.8 s), frequency entering the target zone (No SD: ZOL = 1.2, VEH = 2.7, ALM = 2.5; SD: ZOL = 0.9, VEH = 2.8, ALM = 2.9) and the distance traveled (No SD: ZOL = 472 cm, VEH = 666 cm, ALM = 725 cm; SD: ZOL = 343 cm, VEH = 709 cm, ALM = 775 cm) all decreased compared to VEH and ALM-treated rats. ALM-treated rats did not differ from VEH-treated rats on any of these four measures. Performance in the SRM task was not significantly affected by 6 h SD for any measure within any group.

Swim patterns in the WM were different for ZOL-treated rats compared to VEH- and ALM-treated rats (**Figure 4**). Both VEH and ALM rats repeatedly swam across the WM and typically swam through the area where the hidden platform was present on the previous day (**Figure 4A**). In contrast, ZOL-treated rats primarily swam around the perimeter of the WM, a pattern typical of a rat during its first exposure to the WM.

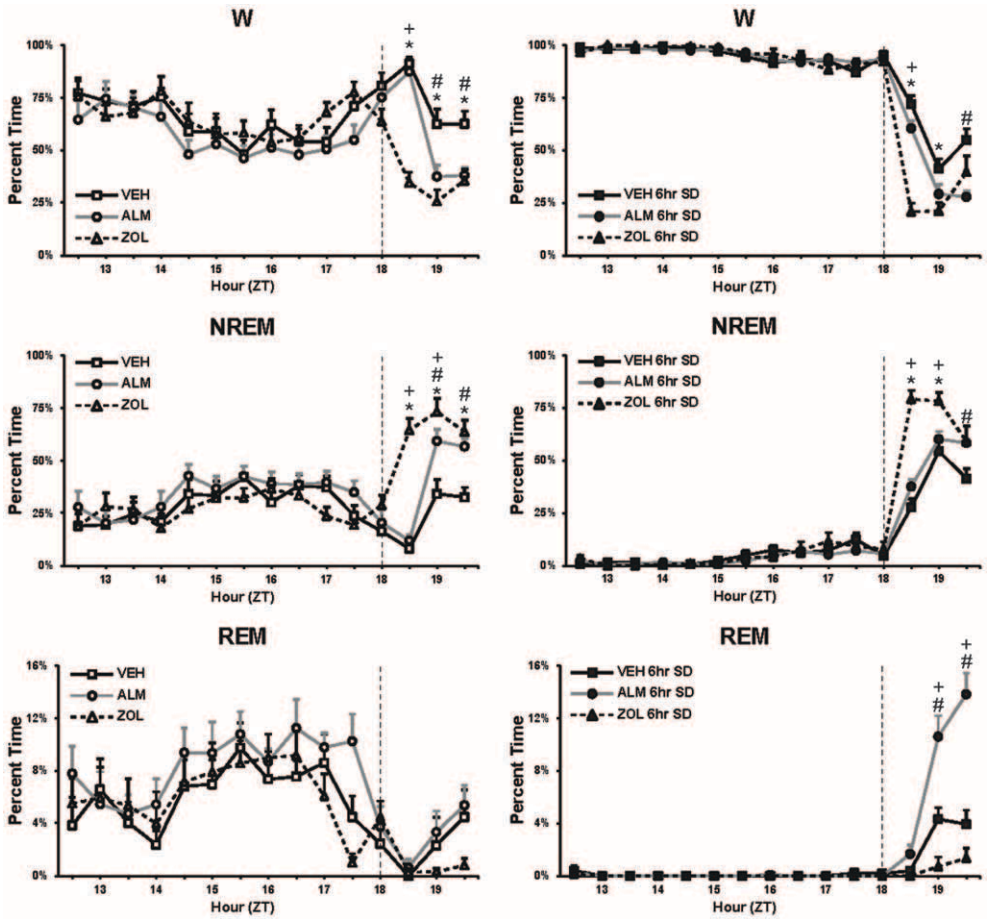

FIGURE 2 | Percent time spent in W, NREM, and REM during baseline (left panels) and during 6 h SD (right panels). The vertical line in each panel at ZT18 depicts the time of drug administration. At the end of the recording time displayed in these panels, rats were tested in the water maze. Note that, for the 60 min prior to testing (ZT19.5), the ALM and ZOL groups slept similar amounts. *, ZOL different from VEH; +, ZOL different ALM; #, ALM different from VEH; $p < 0.05$.

TEST OF SPATIAL WORKING MEMORY

ZOL-treated rats performed poorly in the SWM task compared to either VEH- or ALM-treated rats (**Figures 5**, **6**). ZOL-treated rats took longer to find the platform (No SD: ZOL = 43.4–47.3 s, VEH = 20.6–30.0 s, ALM = 22.5–30.7 s; SD: ZOL = 48.0–55.5 s, VEH = 26.9–31.0 s, ALM = 25.6–28.2 s) and swam more slowly (No SD: ZOL = 14.0–14.2 cm/s, VEH = 18.0–19.6 cm/s, ALM = 18.9–20.4 cm/s; SD: ZOL = 9.9–10.9 cm/s, VEH = 15.7–16.8 cm/s, ALM = 17.5–18.1 cm/s) than the VEH or ALM rats (**Figure 5**). These measures were not affected by increasing the delay from 1 to 5 min or 10 min for any of the 6 groups of rats.

The goal for the SWM task was to locate the platform. VEH- and ALM-treated rats found the platform the majority of the time in both SD and undisturbed conditions (83.3–100% for VEH and 79.2–87.5% for ALM; **Figure 6**). Conversely, ZOL-treated rats failed to find the platform most of the time (22.7–50.0% success rate). Interestingly, ZOL-treated rats also often failed to find the cued platform during the training phase of each pair of trials (**Figure 7**). The ZOL-treated rats in the baseline group found the cued platform 54.4% of the time while the SD ZOL-treated group were successful 53.8% of the time as compared to 98.6% for ALM-treated rats in the baseline group and 100% following SD and 100% of the time for all VEH-treated rats. A trend toward improved performance was observed with progressive trials in the ZOL-treated rats.

DISCUSSION

Though differing in the latency to induce sleep at the doses tested, ALM, and ZOL were equally effective at promoting sleep during the 90 min period prior to performance testing and both compounds significantly increased sleep compared to VEH. ALM-treated rats were indistinguishable from VEH-treated rats in their performance of both the SRM and SWM tasks. In contrast, ZOL caused significant impairments in both tasks. Specifically, in the SRM task, ZOL increased the latency to, the duration in, and the frequency of entering the target zone. In the SWM task, ZOL increased the time to find the platform, decreased the swim velocity and decreased the success rate in finding the platform. These results support the hypothesis that dual Hcrt receptor antagonism

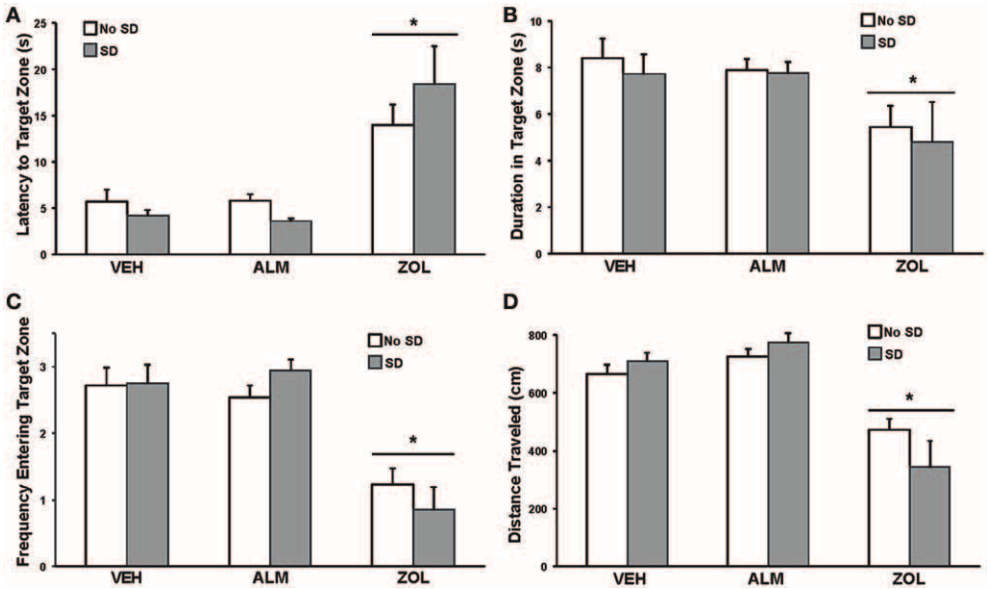

FIGURE 3 | Measures of performance in the spatial reference memory task. For all measures, ZOL-treated rats performed poorly compared to VEH- and ALM-treated rats. For all measures, the ALM-treated rats were indistinguishable from the VEH-treated rats. **(A)** Latency to the target zone. **(B)** Duration in the target zone. **(C)** Frequency entering the target zone. **(D)** Total distance traveled. For all measures, ANOVA revealed an effect of drug condition without an effect of SD. *, $p < 0.05$.

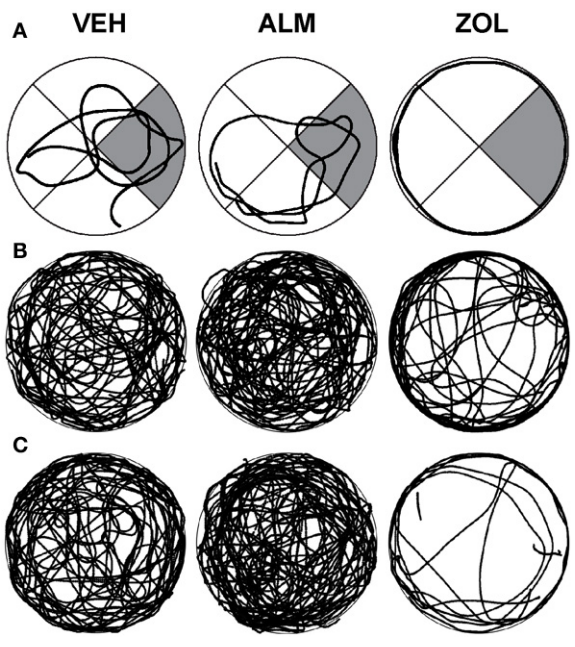

FIGURE 4 | Swim patterns during the spatial reference memory probe trials following VEH (left columns), ALM (center columns) and ZOL (right columns). (A) Examples of individual rats. The target quadrant is highlighted in gray. **(B)** Traces for all rats in the undisturbed condition. **(C)** Traces for all rats in the 6 h SD condition. Note that the searching pattern for VEH and ALM are similar while the pattern following ZOL remains primarily around the perimeter of the maze.

effectively promotes sleep without the functional impairments observed following $GABA_A$ receptor modulation.

An alternative explanation of the results obtained is that ZOL-treated rats were not motivated to perform the tasks rather than having memory/cognitive deficits. ZOL-treated rats had decreased distance traveled during the SRM task and decreased velocity during the SWM task, which could indicate a lack of motivation to escape the WM. Further, the lower success rate in finding the cued platform during the training trials for the SWM task could be interpreted as an absence of motivation to escape. However, ZOL rats did not simply float in the WM; they swam continuously, primarily circling the perimeter of the WM. As mentioned above, this swim pattern is typical of an untrained rat during its first exposure to the WM. Although not measured in this study, it is possible that the decreased distance traveled during the SRM task and decreased velocity during the SWM task are due to motor deficits produced by ZOL. This hypothesis is supported by previous studies that found prominent motor effects following ZOL administration (Depoortere et al., 1986; Steiner et al., 2011; Milic et al., 2012).

The SD protocol in these studies was included to assess whether moderate increases in sleep drive would exacerbate any cognitive deficits found following ALM or ZOL administration and also produce deficits in VEH-treated rats. While the primary active period of nocturnal rodents such as the rat is during the dark phase, rats still sleep approximately 30% of the time during this period and increasing wake duration during the dark period should create a mild sleep deficit (see **Figure 2**). Therefore, a portion of our experimental protocol involved SD during the 6 h of the dark period just prior to drug administration at ZT18.

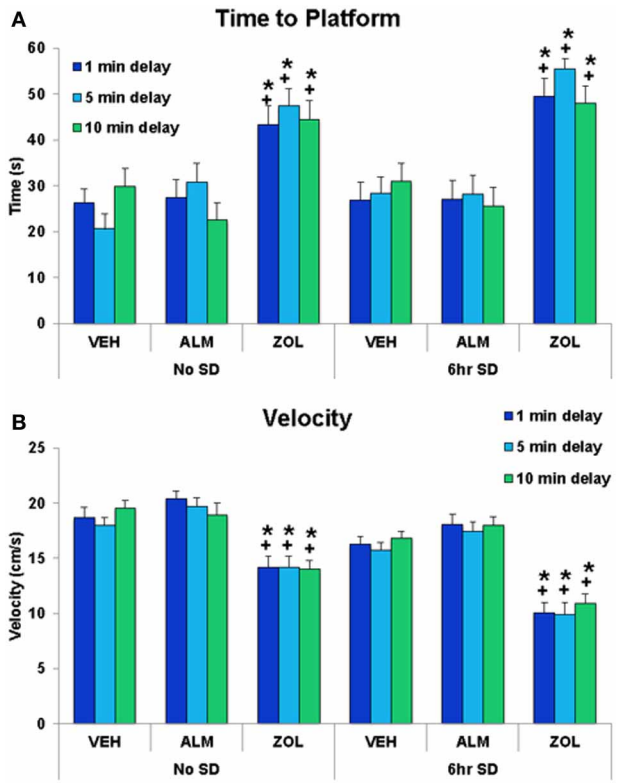

FIGURE 5 | The time to platform and the velocity swam during the spatial working memory task. (A) ZOL-treated rats found the platform significantly slower than VEH- or ALM-treated rats for all three delays following either undisturbed or SD conditions. The ALM-treated rats were not significantly different from VEH-treated rats for any condition. **(B)** ZOL-treated rats swam more slowly than either VEH- or ALM-treated rats. *, different from VEH; +, different from ALM; $p < 0.05$.

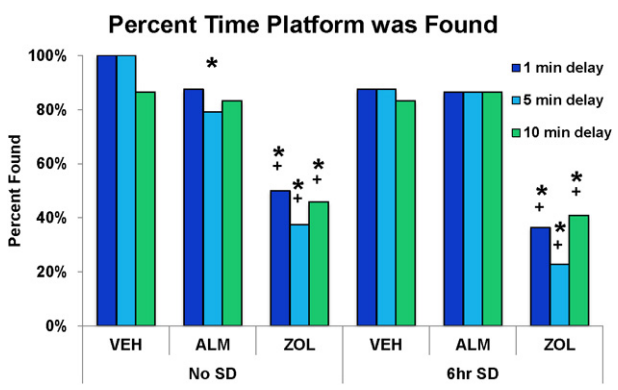

FIGURE 6 | Success rate in locating the platform during the test trials in the spatial working memory task. ZOL-treated rats found the platform significantly fewer times compared to VEH- or ALM-treated rats for all three delays and following both the undisturbed and SD conditions. In each trial, an individual rat either found or didn't find the platform; thus, there is no variation to represent as error bars in the graphs. *, different from VEH; +, different from ALM; $p < 0.05$.

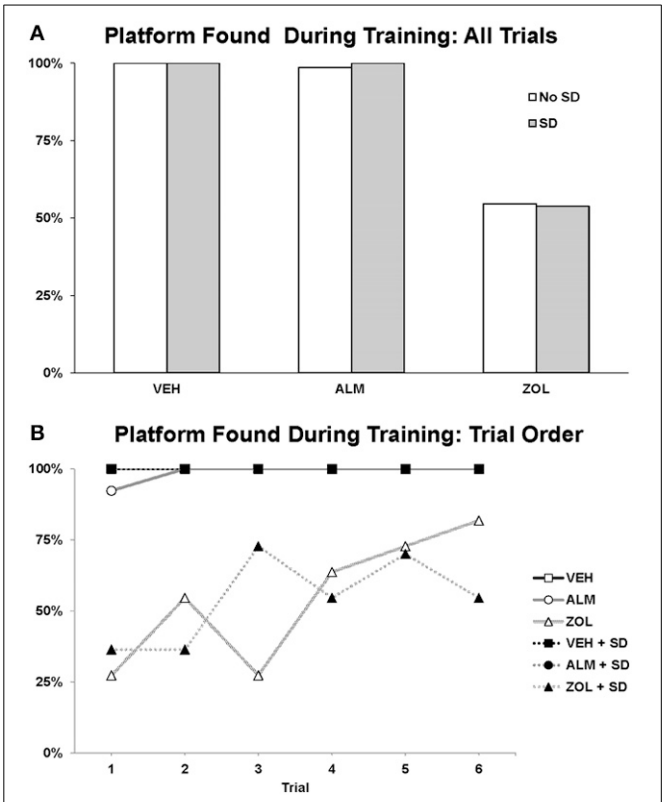

FIGURE 7 | Success rate in locating the platform during the training trials in the spatial working memory task. The platform was cued during these training trials by a flag. **(A)** The percentage of times the platform was found across all 6 training trials. **(B)** The percentage of times the platform was found trial by trial. Note that the ZOL rats tended to progressively improve across trials. In each trial, an individual rat either found or didn't find the platform; thus, there is no variation to represent as error bars in the graphs.

Although we did not find significant effects of SD vs. non-SD within any of the 3 dosing conditions, these results are likely due to the fact that we allowed the rats to sleep after drug administration until water maze testing began. This undisturbed period lasted only 60–90 min but provided an opportunity for the experimental subjects to recover from this mild sleep deprivation. If the SD were continued until testing, increased memory deficits might have been observed. Further studies are needed to determine whether this is indeed to case.

ZOL is a widely prescribed hypnotic medication that can be well-tolerated when taken as directed (Greenblatt and Roth, 2012). However, numerous adverse effects associated with ZOL usage have been reported including driving impairment (Verster et al., 2006; Gunja, 2013), effects on balance and postural tone (Zammit et al., 2008), interference with memory consolidation (Balkin et al., 1992; Wesensten et al., 1996, 2005; Mintzer and Griffiths, 1999; Morgan et al., 2010) and increased incidence of complex behaviors during sleep (Hoever et al., 2010). Some studies investigated the effects of daytime administration of ZOL and tested psychomotor function upon arousal from naps (Wesensten et al., 2005; Storm et al., 2007), a protocol which our experiments closely mimic. In these studies, ZOL or melatonin was

administered at either 10:00 or 13:00. Following a 1.5–2 h nap opportunity, subjects were awakened and required to perform a series of psychomotor and cognitive tests. Significant performance decrements were observed following ZOL in cognitive, vigilance and memory tasks while little to no decrements were found following melatonin. The results of ZOL administration on rat cognitive performance in the current study correlate well with these deficits found in humans.

In contrast, the high level of performance following ALM in both of our memory tasks suggests a high degree of safety at concentrations with hypnotic efficacy. Indeed, a recent study found no performance decrements in a variant of the WM SRM task at three-fold the concentration of ALM that we used (Dietrich and Jenck, 2010). Furthermore, another recent study found no effect of ALM at 300 mg/kg on motor function (Steiner et al., 2011). In humans, however, psychometric test battery assessment of the effect of ALM administered in the daytime found reductions in vigilance, alertness, and visuomotor and motor coordination at dose concentrations of 400–1000 mg (Hoever et al., 2010, 2012a). Notably, 400 mg ALM is within the therapeutic dose range required to improve sleep in patients with primary insomnia (Hoever et al., 2012b). Therefore, performance deficits following ALM occur within the range of hypnotic efficacy in humans. In one report, pharmacokinetic/pharmacodynamic modeling suggests that doses of 500 mg ALM and 10 mg ZOL are equivalent with respect to subjectively assessed alertness (Hoever et al., 2010). Since we find hypnotic efficacy to be achieved at roughly similar dose concentrations, there may be species differences in pharmacokinetic/pharmacodynamics of ALM and/or ZOL. While not uncommon, this makes direct translational interpretations of the present data more difficult. Regardless, in both rodents and humans, ALM appears to have a significantly better safety profile than ZOL with regards to cognitive/memory domains.

CONCLUSION

ALM and ZOL are effective hypnotics in multiple mammalian species (Brisbare-Roch et al., 2007; Hoever et al., 2010, 2012a,b; Morairty et al., 2012). They act through entirely different mechanisms of action, and their effects on cognition, psychomotor vigilance and memory are in stark contrast to one another. We found that at equipotent hypnotic concentrations, ZOL impaired SRM and SWM but ALM did not. These results support the hypothesis that antagonism of the Hcrt system can provide hypnotic efficacy without the impairments found by inducing sleep through $GABA_A$ modulation.

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ORIGINAL ARTICLE

Hypocretin/orexin antagonism enhances sleep-related adenosine and GABA neurotransmission in rat basal forebrain

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Abstract Hypocretin/orexin (HCRT) neurons provide excitatory input to wake-promoting brain regions including the basal forebrain (BF). The dual HCRT receptor antagonist almorexant (ALM) decreases waking and increases sleep. We hypothesized that HCRT antagonists induce sleep, in part, through disfacilitation of BF neurons; consequently, ALM should have reduced efficacy in BF-lesioned (BFx) animals. To test this hypothesis, rats were given bilateral IgG-192-saporin injections, which predominantly targets cholinergic BF neurons. BFx and intact rats were then given oral ALM, the benzodiazepine agonist zolpidem (ZOL) or vehicle (VEH) at lights-out. ALM was less effective than ZOL at inducing sleep in BFx rats compared to controls. BF adenosine (ADO), γ-aminobutyric acid (GABA), and glutamate levels were then determined via microdialysis from intact, freely behaving rats following oral ALM, ZOL or VEH. ALM increased BF ADO and GABA levels during waking and mixed vigilance states, and preserved sleep-associated increases in GABA under low and high sleep pressure conditions. ALM infusion into the BF also enhanced cortical ADO release, demonstrating that HCRT input is critical for ADO signaling in the BF. In contrast, oral ZOL and BF-infused ZOL had no effect on ADO levels in either BF or cortex. ALM increased BF ADO (an endogenous sleep-promoting substance) and GABA (which is increased during normal sleep), and required an intact BF for maximal efficacy, whereas ZOL blocked sleep-associated BF GABA release, and required no functional contribution from the BF to induce sleep. ALM thus induces sleep by facilitating the neural mechanisms underlying the normal transition to sleep.

Keywords Microdialysis · Saporin lesions · Arousal state · Hypocretin · Orexin

Introduction

The hypocretin (HCRT or orexin) system influences multiple physiological processes including sleep–wakefulness, energy metabolism, reward and addiction (Sakurai and Mieda 2011). Deficiency in HCRT signaling underlies narcolepsy in humans and animal models (Chemelli et al. 1999; Thannickal et al. 2000; Lin et al. 1999). HCRT neurons, located in the tuberal hypothalamus (de Lecea et al. 1998; Sakurai et al. 1998), project to multiple subcortical wake-promoting regions, including the basal forebrain (BF) (Peyron et al. 1998).

BF cholinergic neurons are cortically projecting (Rye et al. 1984) and are most active during waking and rapid eye movement (REM) sleep (Berntson et al. 2002; Manns et al. 2000b). Acetylcholine (ACh) release in both the BF (Vazquez and Baghdoyan 2001) and cortex (Jasper and Tessier 1971; Marrosu et al. 1995) is increased during waking and REM sleep. In contrast, BF GABAergic and

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glutamatergic (GLU) release and neuronal discharge are neither preferentially wake nor sleep related (Hassani et al. 2009; Manns et al. 2000a; Vanini et al. 2012). BF neurons express HCRT receptors (Marcus et al. 2001), HCRT modulates cholinergic and noncholinergic BF neuronal excitability in vitro (Eggermann et al. 2001; Arrigoni et al. 2010) and increases waking when infused into the BF (Espana et al. 2001; Fadel et al. 2005; Methippara et al. 2000). However, how this network regulates sleep–wake state in vivo is only partially understood.

BF adenosine (ADO) levels increase during extended wakefulness and decreases during subsequent recovery sleep (RS) (Porkka-Heiskanen et al. 1997). ADO regulates sleep via A1 (Alam et al. 1999; Strecker et al. 2000) and A2a ADO receptors in the BF (Satoh et al. 1996, 1998) and other regions (Arrigoni et al. 2006; Rainnie et al. 1994). BF cholinergic neuron lesions decrease wake-related BF ADO release (Blanco-Centurion et al. 2006a; Kalinchuk et al. 2008); reports differ on whether such lesions attenuate (Kalinchuk et al. 2008; Kaur et al. 2008) or have no effect on sleep (Blanco-Centurion et al. 2006a, 2007). BF cholinergic neurons are not necessary for HCRT-induced wakefulness (Blanco-Centurion et al. 2006b) and HCRT neuron ablation blocks wake-related BF ADO release (Murillo-Rodriguez et al. 2008). Thus, noncholinergic transmission, including GABA, GLU and ADO, may be a key component of HCRT–BF interactions.

The dual HCRT receptor antagonist almorexant (ALM) blocks the excitatory effects of the HCRT peptides at both HCRT receptors (HCRTR1 and HCRTR2), eliciting somnolence, decreasing active wake, and increasing non-REM (NREM) and REM sleep time (Brisbare-Roch et al. 2007). In contrast, zolpidem (ZOL; trade name Ambien®) induces somnolence by activating $GABA_A$ receptors, thereby causing widespread neuronal inhibition (Dang et al. 2011). We hypothesized that ALM induces sleep by disfacilitating subcortical wake-promoting regions such as the BF, whereas ZOL acts via generalized inhibition throughout the brain. To test this hypothesis, we lesioned the cholinergic neurons of the BF and evaluated the efficacy of sleep induction by ALM and ZOL, determined the effects of ALM and ZOL on BF release of ADO, GABA and GLU in intact animals, and infused ALM and ZOL into the BF and measured cortical release of ADO, GABA and GLU. We found that ALM, but not ZOL, required an intact BF for maximal hypnotic efficacy and that ALM, but not ZOL, induced neurochemical events typically associated with the transition to normal sleep. The sleep-inducing effect persisted with central infusion of ALM into the BF and resulted in enhanced cortical release of ADO. Together, these results demonstrate that HCRT input modulates ADO signaling in the BF and the cerebral cortex.

Materials and methods

Animals

Male Sprague–Dawley rats ($n = 69$; 200–250 g; Harlan Laboratories) were housed under constant temperature (22 ± 2 °C, 50 ± 25 % relative humidity) on a 12-h dark/light cycle with food and water ad libitum. All experimental procedures occurring during the dark phase (e.g., dosing, microdialysis, sleep deprivation) were carried out under dim red light. All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at SRI International. Every effort was made to minimize animal discomfort throughout the experimental protocols.

Chemicals

Almorexant was synthesized by the Medicinal Chemistry Laboratory at SRI International using previously reported methods (Koberstein et al. 2005). ZOL was purchased from IS Chemical Company (Shanghai, China). All drugs that were delivered orally were suspended and sonicated for 1 h in 1.25 % hydroxypropyl methyl cellulose (HPMC) with 0.1 % dioctyl sodium sulfosuccinate (DOSS; 2.24 mM) in sterile water (hereafter referred to as 'VEH'). All drug solutions were made on the day of the experiment and serially diluted to their final concentrations. Tetrodotoxin (TTX; 1 μM) was purchased from Abcam Chemical (Cambridge, UK). Microdialysis artificial cerebrospinal fluid (aCSF) contained in mM: NaCl, 148; KCl, 3; $MgCl_2 \cdot 6H_2O$, 0.8; $CaCl_2$, 1.4; Na_2HPO_4, 1.5; $0NaH_2PO_4$, 0.225; pH 6.5 ± 0.5.

Telemetry surgery

All rats ($n = 69$) were surgically implanted with a sterile abdominal transmitter (F40-EET, DSI, St Paul, MN, USA) for continuous telemetric recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_b), and locomotor activity (LMA) as described previously (Morairty et al. 2008, 2012). Briefly, the wires from the transmitter were subcutaneously channeled rostrally to the head. Two biopotential leads (used as EEG electrodes) were inserted into drilled holes over the skull and affixed with dental acrylic. Two additional biopotential leads (EMG electrodes) were sutured into the neck musculature and closed with non-absorbable suture.

192-IgG-saporin lesions

Under isoflurane anesthesia, rats ($n = 16$) were placed into a stereotaxic apparatus (Kopf Instruments, Tujunga,

CA, USA), the skull was exposed and two burr holes were drilled. Animals were injected bilaterally with 1 μL of 192-IgG-saporin (SAP; 0.5 μg/μL; Advanced Targeting Systems, San Diego, CA, USA) (Wiley et al. 1991) or sterile saline via glass micropipettes (inner tip diameter 20–25 μm) using a Picospritzer (Parker Hannifin, Cleveland, OH) at −0.3 mm AP and ±1.5 mm ML relative to bregma, and 8.5 mm below dura. Injectate volume was measured via precalibrated marks on the barrel of the pipette. Injections lasted ~10 min/side; the pipette was left in place for 5 min after the injection. Rats were then instrumented for EEG/EMG telemetry as described above. Animals were allowed to recover for at least 3 weeks before recording. One rat was euthanized due to postoperative weight loss prior to the start of data collection, resulting in $n = 15$ rats for experimentation.

Microdialysis cannulation

Rats ($n = 53$) were surgically implanted with an abdominal telemetry transmitter as described above and with a microdialysis cannula in the BF. The guide cannula (CMA/12; CMA Microdialysis, Chelmsford, MA, USA) was stereotaxically implanted 2 mm above the BF at −0.3 mm AP, +2.0 mm ML, and −7.0 mm DV, calculated relative to bregma (Paxinos and Watson 2007). The guide cannula and EEG/EMG electrodes were then affixed to the head with dental cement. Animals were allowed to recover for at least 3 weeks before recording behavior or dialysis.

Experimental protocol 1

Assessment of hypnotic efficacy in 192-IgG-saporin-lesioned rats

Rats ($n = 15$) were kept in their home cages for the duration of the study in ventilated, light-tight and sound-attenuated chambers in 12:12 LD. Prior to initiation of sleep recordings, animals were acclimated to handling and oral gavage with VEH for approximately 1 week, then left undisturbed for 2 days after acclimation was complete. At the start of the experiment, a 24-h undisturbed baseline was recorded, followed by a 6-h sleep deprivation (SD) and an 18-h recovery period; both baseline and SD recordings started at lights-off (Zeitgeber Time (ZT) 12, where ZT 0 = lights-on). During SD, animals were observed by an experimenter; if a rat showed behavioral signs of sleep (closed its eyes or assumed a sleep-typical posture), it was awoken by light cage-tapping, brief rotation of the home cage, or lightly stroking the animal's back with a soft brush. To eliminate any residual effects of the SD procedure, rats were left undisturbed for 48 h before dosing. Rats were administered ALM (30, 100 and 300 mg/kg), ZOL (10, 30 and 100 mg/kg), or VEH p.o. at lights-out (ZT 12). EEG was recorded for 24 h following dosing. ALM, ZOL and VEH were given in balanced order with at least 3 days between treatments in a cross-over study design; previous work from our lab has shown that this dosing regimen allows sufficient time for washout between doses (Morairty et al. 2012).

To confirm the extent of BF lesions, rats were deeply anesthetized and transcardially perfused with heparinized 0.1 M phosphate-buffered saline followed by 4 % paraformaldehyde. Brains were removed and postfixed overnight in 4 % paraformaldehyde, then transferred to 30 % sucrose until sectioning. Brains were sectioned at 40 μm on a freezing microtome. Free-floating sections containing the BF were incubated with 1 % H_2O_2 for 15 min to quench endogenous peroxidase activity, followed by (1) 1 h in blocking buffer containing 3 % normal donkey serum, (2) overnight in goat anti-ChAT (1:6,000; Millipore), (3) 2 h in biotinylated donkey anti-goat IgG (1:500; Jackson Immunoresearch), and (4) 1 h in avidin–biotin complex (ABC; Vector Laboratories). ChAT was visualized by reacting sections in 0.05 % diaminobenzidine tetrahydrochloride and 0.01 % H_2O_2. Sections were then mounted, dehydrated and coverslipped. ChAT-positive neurons were counted bilaterally in the magnocellular preoptic nucleus (MCPO), SI and basal nucleus of Meynert (NBM) in three sections by an observer blind to the treatment of the animals.

Experimental protocol 2: assessment of hypnotic effects on BF neurotransmitter levels and sleep–wake states

Rats ($n = 19$) were handled 4–6 h daily for 1 week to acclimate to the procedures and given 1 mL doses of VEH on 2 days at least 1 week before the first experimental day. Rats were freely moving in microdialysis chambers (CMA 120 System, Harvard Apparatus, Co.) that were positioned over telemetry receiver boards (RPC-1, DSI, St. Paul, MN, USA).

Baseline EEG and EMG recordings were collected for 48 h via implanted telemetry devices concurrent with video recordings and microdialysis sampling. For experiments, a microdialysis probe (2 mm length, 0.5 mm diameter, 20 kDa cutoff; CMA 12, CMA Microdialysis) was inserted into the cannula ~18 h prior to sample collection to allow for neurotransmitter stabilization and perfused with aCSF at a rate of 1 μL/min. Upon experiment initiation (4.5 h into the dark period; ZT 16.5), three 30-min samples (1 μL/min flow rate, 30 μL total) were collected from the animals to assess basal levels of ADO, GABA, and GLU in conjunction with baseline EEG and EMG data. Following baseline collection, animals were administered ALM (100 mg/kg), ZOL (100 mg/kg), or VEH in 1 mL volume

p.o., 6 h into the dark period (ZT 18) and then 12 additional 30-min samples were collected to assess the effects of the drug on sleep–wake and BF neurotransmitter release. Each rat randomly received one of three drug treatments (minimum 1 week apart with no more than two different drugs or dialysis attempts per animal). The drug doses used were previously shown to be the minimum doses required to induce equivalent levels of somnolence in rats (Morairty et al. 2012, 2014).

Experimental protocol 3: assessment of hypnotic effects on BF neurotransmitter levels during sleep deprivation and recovery sleep

Animals ($n = 26$) were prepared as described in protocol 2 above. 30-min baseline samples (1 μL/min flow rate, 30 μL total) were collected 4 h into the dark period (ZT 16), from animals under extended wakefulness conditions. The animals were kept awake for 6 h (gentle handling, tapping cage) beginning at ZT 16 and then permitted 2 h of RS from ZT 22 to ZT 24. As in protocol 2, animals received one of three drugs (100 mg/kg ALM, 100 mg/kg ZOL, or VEH, in 1 mL p.o.) 6 h into the dark period (ZT 18). Nine 30-min samples were then collected to assess the effects of the drug on extended wakefulness and BF neurotransmitter release and four additional samples were collected during the 2-h RS period.

Experimental protocol 4: effects of BF administration of hypnotics on sleep–wake states and cortical neurotransmission

Rats ($n = 15$) were surgically prepared as described above with a dual microinjection cannula (Plastics One, Roanoke, VA, USA) stereotaxically implanted into the BF and a single CMA guide cannula into the prefrontal cortex at +3.2 mm AP, −0.6 mm ML, and −2.0 mm DV relative to bregma (Paxinos and Watson 2007) and acclimated for at least 1 week before the first experimental day. Baseline EEG and EMG recordings were collected for 48 h via implanted telemetry devices concurrent with video recordings and microdialysis sampling. For experiments, five 30-min baseline samples (1 μL/min flow rate, 30 μL total) were collected beginning at 3.5 h into the dark period (ZT 15.5) to assess basal levels of ADO, GABA, and GLU in conjunction with baseline EEG and EMG data. Following baseline collection, rats received bilateral BF microinjections of either ALM (10 ng/200 nL), ZOL (60 ng/200 nL), or VEH in physiological aCSF at ZT 18 and then twelve additional 30-min samples were collected to assess the effects on sleep–wake and cortical neurotransmitter release. Each rat randomly received one of three drug treatments (minimum 1 week apart with no more than two different drugs or dialysis attempts per animal). All samples in protocols 2–4 were collected in refrigerated fraction collectors at 4 °C and stored at −80 °C at the end of the experiment until analysis by HPLC.

Experimental protocol 5: reverse dialysis with TTX and high KCl in anesthetized animals

To determine whether the neurotransmitter changes measured by microdialysis reflected synaptic-mediated events that could be affected by drug administration and not simply by changes in vigilance state (John et al. 2008; Kodama and Honda 1999; Kodama et al. 1992; Lena et al. 2005; Nitz and Siegel 1997a; Vazquez and Baghdoyan 2001; Vazquez et al. 2002), a series of experiments was performed to evaluate the effects of TTX and high KCl perfusion on BF ACh, GABA, and GLU release in rats under general anesthesia. Since ADO concentrations are modulated by production and transport of ATP released by both neurons and astrocytes (Ben Achour and Pascual 2012), ADO was not measured under this protocol.

Rats ($n = 8$) were implanted with telemeters and BF cannulae as described above and allowed 1 week of recovery prior to experiments. Animals were used only once per experiment. Sample collection for all experiments began between ZT 18–ZT 20 and lasted 4–5.5 h. Rats were anesthetized with isoflurane (3 %) and, once all autonomic signs were stable, the anesthesia was lowered and maintained at 1.5 %. A microdialysis probe was inserted 2 h prior to the onset of the experiment and continuously perfused with aCSF containing neostigmine (10 μM), a cholinesterase inhibitor used to prevent ACh degradation. Five sequential 12-min samples (2.5 μL/min flow rate, 30 μL total) were collected under anesthesia to assess basal levels of BF ACh, GLU, and GABA. A CMA/110 liquid switch was then activated to deliver TTX (1 μM, in aCSF) through the probe during the sixth sample (a transition sample). Five additional samples were collected during TTX perfusion followed by another transition sample (via liquid switch) to a high concentration of KCl (100 mM, in aCSF). Five subsequent samples were collected during aCSF plus high KCl. All samples were collected on ice and immediately stored at −80 °C until analysis by HPLC.

Quantification of ADO, GLU, GABA, and ACh

Microdialysis samples from experimental protocols 2–4 were split for ADO (10 μL), GLU and GABA (20 μL) analysis. Samples (10 μL total volume) containing ADO were separated using mobile phase (10 mM monosodium phosphate, 7 % acetonitrile, pH 4.50) pressurized through a U3000 isocratic pump with a flow of 0.8 mL/min across a

reversed-phase C18 column (150 mm ID × 4.6 mm, 2.6 μm, Phenomenex) and detected by UV at 254 nm. GLU and GABA content (20 μL total volume) were separated by HPLC with electrochemical detection (EC) using mobile phase (100 mM Na_2HPO_4, 22 % MeOH, and 3.5 % acetonitrile, pH 6.75; 0.7 mL/min flow rate) on a U3000 biocompatible isocratic pump. GLU and GABA were detected by precolumn derivatization using 2.2 mM *O*-phthalaldehyde and 0.8 mM 2-mercaptoethanol (β-ME) mixed by automation with the sample at 10 °C for 2 min prior to injection into the HPLC. Separation was achieved through a reversed-phase C18 column (3.0 mm ID × 75 mm, 3 μm, Shiseido Capcell Pak) and electrically detected on a CouloChem III (E1; +250 mV, E2; +550 mV, Guard +650 mV) at 45 °C. Calibration curves for ADO and GLU/GABA were constructed using Chromeleon 6.8.0 software (Dionex Corp., Sunnyvale, CA, USA).

In protocol 5, ACh was analyzed in a subset of microdialysis samples (10 μL) in addition to GLU/GABA (20 μL; described above). Dialysates were automatically injected via a refrigerated (4 °C) autosampler into a CoulArray HPLC/EC system (ESA-Dionex). Samples were carried in mobile phase (100 mM Na_2HPO_4, 2 mM 1-octanesulfonic acid, pH 8.0; 0.6 mL/min flow rate) through an enzyme reactor attached to the column (ACH-250 × 3.0-mm, ESA-Dionex) to eliminate the choline peak. The samples were then converted to hydrogen peroxide (H_2O_2) by a solid-phase reactor (containing immobilized choline oxidase and acetylcholinesterase enzymes) and detected amperometrically and quantified on a platinum (Pt) working electrode set to +300 mV with a solid-state palladium reference electrode. ACh calibration curves were constructed using CoulArray Data Station 3.0 software (ESA, Inc.).

EEG and EMG analyses and sleep/wake determinations

In protocols 1–4, EEG and EMG were recorded via telemetry on a PC running Dataquest ART 3.1 (Data Sciences). All recordings were first screened for artifact and then manually scored offline in 10-s epochs as Wake, NREM, or REM sleep using NeuroScore 2.1 (Data™, St. Paul, MN, USA). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. Individual state data were quantified as time spent in each state per 30 min, 1 h, or 6 h. Latency to NREM and REM onset for each animal was calculated from the time of drug injection. Bouts were defined as a minimum of three consecutive epochs of wake or NREM, and two consecutive epochs of REM sleep. NREM delta power was normalized to the average total spectral power for the 24-h baseline.

Quantification of vigilance state-dependent neurochemical release patterns

Since sleep/wake is polyphasic and highly fragmented throughout both the light and dark periods in rodents, multiple bouts of wake and sleep typically occurred during a 30-min dialysis sample collection period. Consequently, to attribute changes in neurotransmitter level as a function of vigilance state, all 30-min dialysis samples were classified in one of three state categories: (1) wake-dominated states (WAKE), consisting of samples during which wakefulness constituted >75 % of the 30-min period; (2) mixed states (MIXED), sampling during which either 2 or 3 states occurred (Wake and NREM, or Wake, NREM and REM) and each state constituted less than 75 % of the 30-min period; and (3) NREM/REM (NR/R) states, consisting of samples during which NREM and REM sleep combined were >75 % of the 30-min period.

For microdialysis samples collected in protocol 2, the mean concentration for each neurotransmitter was calculated during baseline wakefulness prior to drug administration at ZT 18 and was considered to represent the basal Waking level (100 %). Relative changes in neurotransmitters after oral drug delivery were then calculated for WAKE, MIXED and NR/R compared to this basal Waking level. For samples collected during SD and RS in protocol 3, the mean concentration for Wake during SD was calculated prior to drug administration at ZT 18 and was considered to represent basal Waking levels (100 %) for each neurotransmitter; relative changes in neurotransmitters after oral drug delivery were calculated for SD and RS compared to this basal Waking level. For samples collected in protocol 4, the mean concentration for each neurotransmitter was calculated for baseline release prior to the onset of drug microinjections at ZT 18 and was considered to represent basal levels (100 %); relative changes in neurotransmitters after drug microinjections were calculated for each hour compared to this basal level. The microdialysis data presented here were obtained from experiments that showed no statistically significant change in probe recovery (∼10 %, data not shown), ensuring that measured changes in ACh, ADO, GLU, and GABA resulted from changes in either vigilance states and/or drug delivery and were not due to changes in the dialysis membrane.

Histological confirmation of BF dialysis probes

After the final microdialysis experiment in protocols 2–5, rats were deeply anesthetized, decapitated and brains removed and fixed in 10 % formalin. The brains were postfixed in 30 % sucrose–formalin and serial coronal sections (30 μm) were later cut on a freezing microtome

and stained with cresyl violet. All tissue sections containing a dialysis probe-induced lesion were digitized and compared with sections in the rat brain atlas (Paxinos and Watson 2007).

Statistical analyses

Descriptive statistics and two-way analysis of variance (ANOVA) were performed on all EEG measures as a function of time, drug and/or lesion condition as appropriate (GBSTAT v8.0 and Statistica). Where ANOVA indicated a probability (P) value <0.05, Fisher's LSD post hoc multiple comparisons were used to determine significance between groups. Because we predicted specific effects of lesion treatment on ALM and ZOL efficacy, planned comparisons (F test) were also used to compare the effects of lesion at each drug dose, and the effects of each drug dose compared to VEH independently of the omnibus ANOVA results. Neurochemical data were subjected to a two-way ANOVA to determine the effect of drug(s) on vigilance states (WAKE, MIXED, NR/R, SD, or RS) or time (hours post-microinjection) for ADO, GABA, and GLU, followed by multiple comparison tests with statistical significance set at $P < 0.05$.

Results

BF lesions attenuate sleep induction by ALM but not by ZOL

To evaluate the effects of hypocretin receptor antagonism on sleep/wake control, we lesioned the cholinergic neurons of the BF and examined the changes in the animals' vigilance states in response to oral administration of ALM or ZOL compared to VEH. Figure 1a shows the area targeted by the saporin lesions; the gray box denotes the area depicted in Fig. 1b. ChAT-ir cells were plentiful in the SI, NBM and MCPO of saline-injected rats (Shams; Fig. 1b, top panel), whereas only a few scattered ChAT-ir cells were visible in the BF of 192-IgG-saporin-injected rats (BFx; Fig. 1b, bottom panel). There were 917 ± 88 ChAT-ir cells in the SI, NBM and MCPO of Shams ($n = 8$), compared to 100 ± 37 ChAT-ir cells in the BF of BFx rats ($n = 7$). Average ChAT-ir cell loss in BFx rats was 89.9 ± 4.1 % of the total number of ChAT-ir cells in Sham rats with no systematic differences in the extent of cell loss from anterior to posterior or between right and left hemispheres. Noncholinergic cell loss was also apparent in some BFx rats in the form of degraded or necrotic tissue in the BF. Such nonspecific damage was not observed in any Sham rats, suggesting that it was most likely caused by the saporin itself.

BFx decreased baseline NREM sleep time in the dark phase compared to Sham rats (Fig. 1c; $F_{1,13} = 9.14$; $P = 0.01$). Decreased NREM sleep time was attributable to shorter NREM bout durations ($F_{1,13} = 13.54$; $P = 0.003$). BFx also decreased REM bout duration ($F_{1,13} = 12.7$; $P = 0.003$), which was associated with a borderline effect on REM sleep time in the dark phase (Fig. 1d; $F_{1,13} = 4.59$; $P = 0.052$). BFx did not affect the number of either NREM or REM bouts. BFx also had no effect on sleep/wake state in the light phase (Fig. 1c, d). Following 6-h SD (ZT 12 to ZT 18), BFx rats exhibited less NREM during RS compared to Shams (Fig. 1e; $F_{1,13} = 19.22$; $P = 0.0007$), as well as an attenuated increase in NREM delta power over baseline (Fig. 1f; $F_{5,65} = 2.58$; $P = 0.03$). These results are indicative of an attenuated homeostatic sleep response. Similar to baseline REM sleep, REM time during RS exhibited a borderline decrease that did not reach statistical significance ($F_{1,13} = 3.98$; $P = 0.068$).

Both ALM and ZOL increased NREM sleep time compared to VEH from ZT 12 to ZT 18 at all doses tested (Fig. 2a, b; main effect of drug; $F_{6,78} = 15.882$; $P = 0.002$). As observed in the baseline, BFx rats also spent less time in NREM sleep compared to Sham rats, independent of drug treatment (main effect of lesion; $F_{1,13} = 20.078$; $P < 0.0001$). A planned comparison of lesion condition (BFx vs Sham) at each drug treatment dose revealed that NREM time was decreased in BFx rats compared to Shams for the VEH, ALM-30, ALM-300 and ZOL-10 conditions (Fig. 2a). While not statistically significant, average NREM time following ALM-100 trended towards a decrease in BFx rats compared to Shams, consistent with the low and high ALM doses. Thus, ALM increased NREM sleep time over VEH in BFx and Sham rats, but ALM was unable to compensate for the basal attenuation of NREM sleep in BFx rats, even at the highest dose (Fig. 2a). By contrast, ZOL exhibited full efficacy in both lesion groups at mid to high doses (30 and 100 mg/kg, respectively), increasing NREM sleep time in BFx rats to levels similar to that of Shams (Fig. 2b).

ALM increased REM sleep time compared to VEH at all three doses (main effect of drug; $F_{6,78} = 40.475$; $P < 0.0001$). Planned comparisons revealed that ALM increased REM sleep time compared to VEH in both BFx and Sham rats (Fig. 2c), whereas ZOL at 100 mg/kg decreased REM time in Sham rats, but not in BFx rats (Fig. 2d). Thus, ZOL suppressed REM sleep in Sham rats, but BFx abolished this effect.

Oral administration of ALM promotes sleep and BF ADO and GABA release

To further test the hypothesis that ALM induces sleep, in part, by disfacilitating the neurons in the wake-promoting

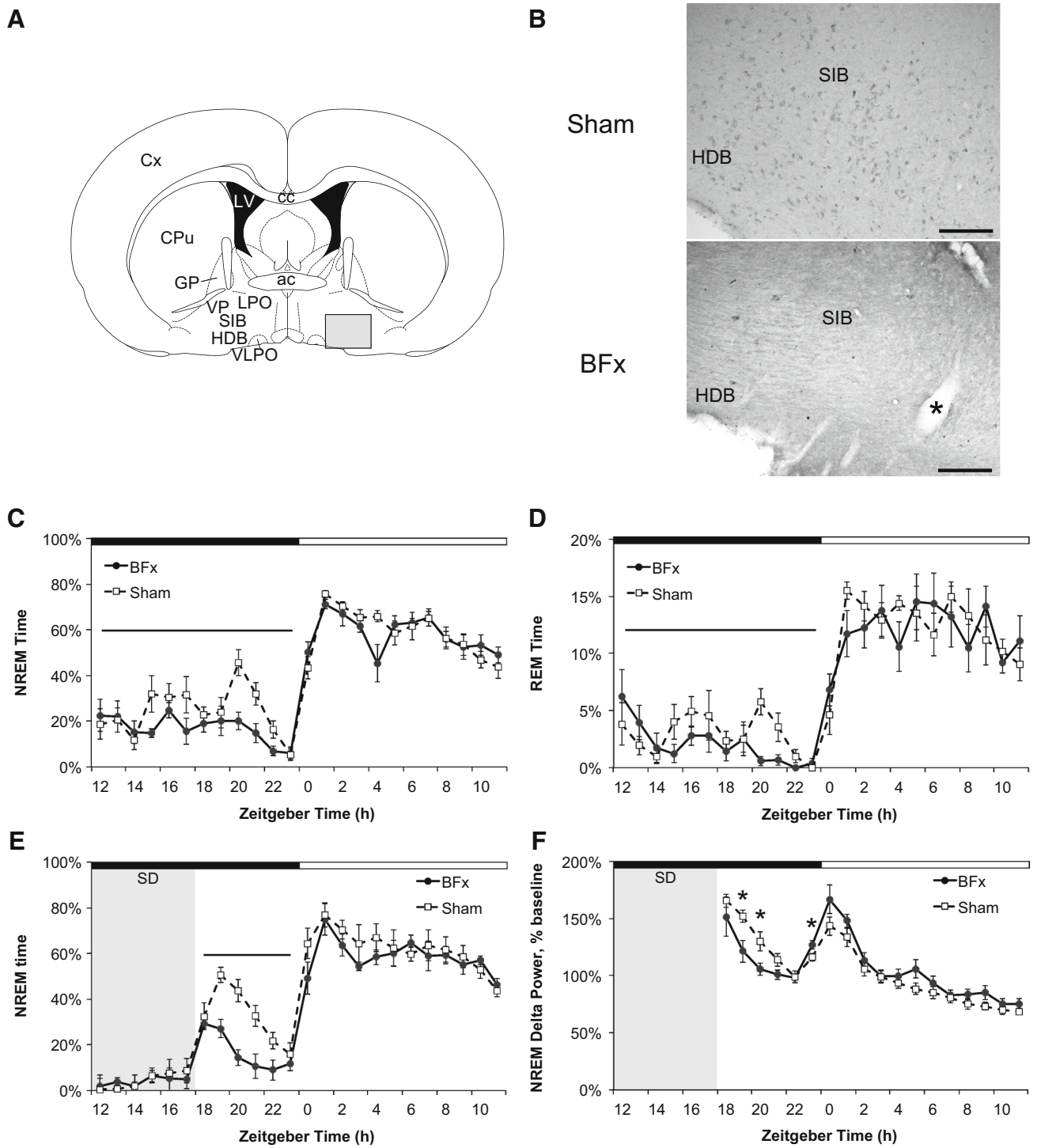


Fig. 1 BF lesions decrease sleep time in the dark phase. **a** Schematic of rat brain (Paxinos and Watson 2007) showing the BF target area (*gray box*). **b** ChAT immunoreactivity (-ir) in the BF of rats given bilateral microinjections of sterile saline (*upper panel*) and 192-IgG-saporin (*lower panel*). ChAT-ir cells decreased by 90 % in saporin-treated rats. Baseline NREM (**c**) and REM (**d**) sleep time in BFx and Sham (saline) rats. *Horizontal lines* indicate a significant lesion effect on NREM and REM sleep time in the dark phase ($P < 0.05$). BFx rats spent less time in NREM and REM sleep in the dark phase compared to shams; however, neither NREM nor REM sleep was affected during the light phase. **e**, **f** 6-h SD induced a smaller increase in NREM sleep (**e**) and NREM delta power (f) in BFx rats compared to Shams. *Asterisks* indicate significant lesion × ZT interaction ($P < 0.05$). *Horizontal black* and *white bars* above graphs indicate LD cycle, with lights-on at ZT 0. *ac* anterior commissure, *cc* corpus collosum, *CPu* caudate putamen, *Cx* cortex, *GP* globus pallidus, *HDB* diagonal band of Broca, horizontal limb, *LV* lateral ventricle, *LPO* lateral preoptic area, *SIB* substantia innominata, basal nucleus of Meynert, *VLPO* ventrolateral preoptic area, *VP* ventral palladium, *asterisk* blood vessel. *Scale bar* in **a** is 200 μm

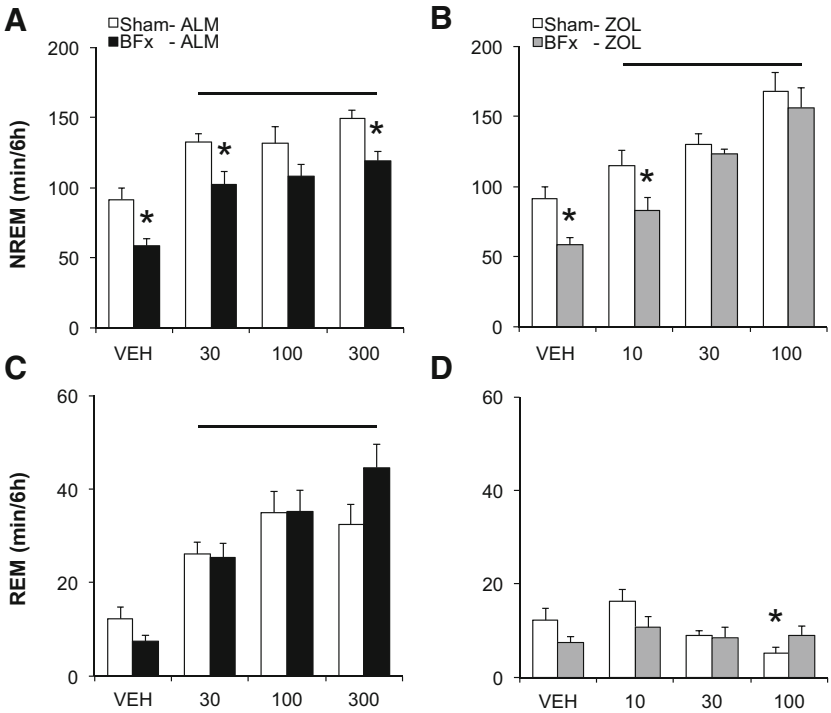


Fig. 2 BF lesions attenuate efficacy of ALM, but not ZOL. **a**, **b** NREM sleep time in BFx and Sham rats following VEH, ALM or ZOL at doses (mg/kg) indicated on x-axes. ALM and ZOL data were graphed separately for clarity; VEH data are repeated for comparison. **a** ALM increased NREM sleep time compared to VEH (main effect of drug, $P < 0.001$), but was unable to compensate for the basal attenuation of NREM sleep in BFx rats compared to Shams at any dose (*$P < 0.05$, planned comparison). **b** ZOL exhibited full efficacy at 30 and 100 mg/kg, increasing NREM sleep time in BFx rats to similar levels as Shams. **c** ALM increased REM sleep time compared to VEH (main effect of drug, $P < 0.001$) with no effect of lesion. **d** ZOL 100 mg/kg suppressed REM sleep compared to VEH in Sham rats, but not in BFx rats (*$P < 0.05$, planned comparison). All values represent total time in each state for the first 6 h immediately following dosing at lights-out

region of the BF, whereas ZOL promotes a generalized inhibition throughout the brain, we determined the effects of ALM and ZOL on BF release of ADO, GABA and GLU in intact, behaving animals. Histological analyses from all experiments performed in this study showed that the probe sites were primarily localized to the substantia innominata region of the BF (Fig. 3a). All dialysis site coordinates ($n = 53$ rats) ranged from AP −0.8 to AP 0.36 (−0.53 ± 0.04; mean ± SEM), L 1.5 to L 2.5 (2.16 ± 0.04; mean ± SEM), and V 8.0 to V 9.0 (8.60 ± 0.05; mean ± SEM) based on the rat atlas (Paxinos and Watson 2007).

Figure 3b–d presents representative hypnograms highlighting the differential effects of oral VEH, ALM (100 mg/kg), and ZOL (100 mg/kg) on vigilance states. Consistent with published data (Morairty et al. 2012) and the lesion study (Fig. 2), ALM (Fig. 3c) and ZOL (Fig. 3d) promoted both NREM and REM sleep, demonstrating that microdialysis procedures did not alter the effects of either drug on sleep–wake. Figure 3e illustrates how microdialysis samples were assigned to one of three vigilance state categories.

The changes in waking, NREM, and REM sleep following oral delivery of either VEH ($n = 8$), ALM (100 mg/kg; $n = 9$), or ZOL (100 mg/kg; $n = 9$) are summarized in Fig. 4a, b. ZOL increased the latency to the onset of REM relative to ALM ($F_{5,51} = 6.6$; $P < 0.0001$; Fig. 4a). Both drugs also significantly affected Wake, NREM, and REM sleep time ($F_{8,77} = 27.8$; $P < 0.0001$; Fig. 4b). ALM and ZOL decreased Wake time and increased NREM sleep relative to VEH control (Fig. 4b; $P < 0.01$). ALM significantly increased the amount of time spent in REM sleep compared to VEH ($P < 0.01$).

ADO levels in the BF were significantly increased by ALM in samples collected during WAKE and MIXED states compared to VEH ($F_{2,81} = 33.3$; $P < 0.0001$; Fig. 4c). ALM also significantly elevated BF ADO relative to ZOL during all three states (Fig. 4c; $P < 0.05$). By contrast, ZOL did not alter BF ADO levels compared to VEH in any state (Fig. 4c).

BF GABA levels changed as a function of vigilance state and drug administration ($F_{2,83} = 8.4$; $P = 0.0005$; Fig. 4d). ALM significantly increased BF GABA levels (Fig. 4d) during WAKE and MIXED states compared to

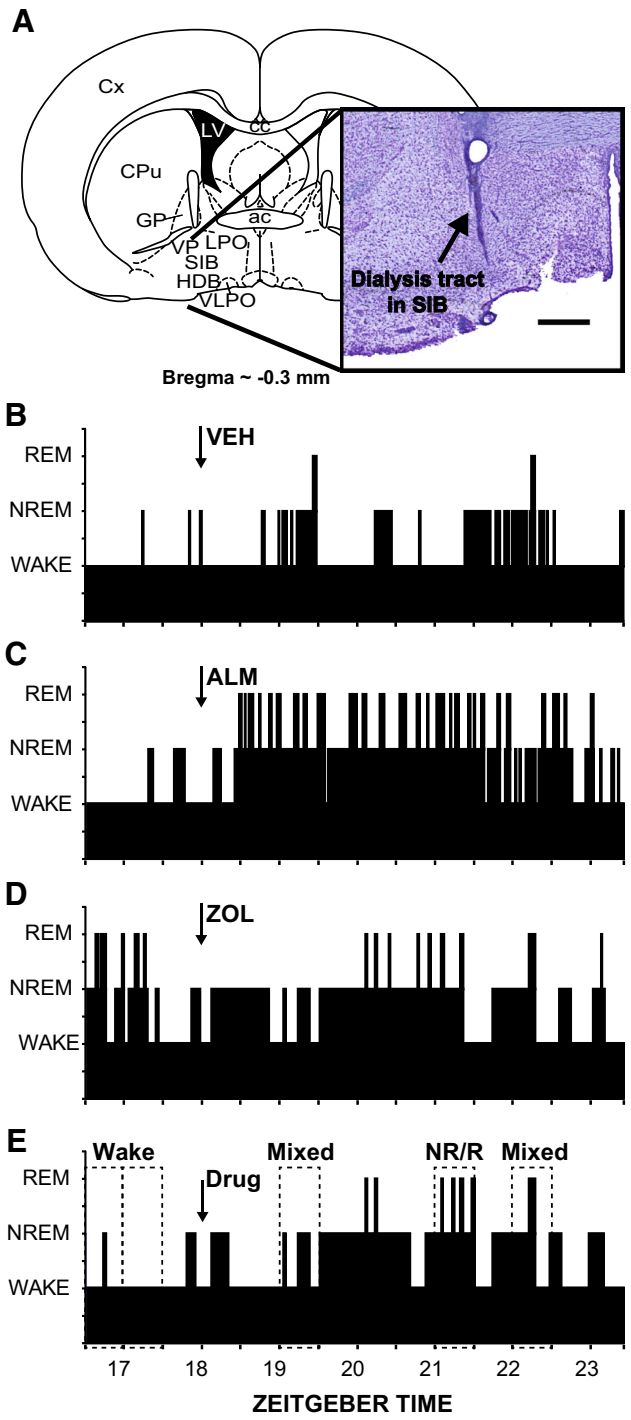


Fig. 3 Dialysis probe placement in the BF and sleep–wake architecture following drug delivery. **a** Representative probe tract shown in brain with arrow indicating dialysis probe was located within the substantia innominata. Hypnograms in **b** through **d** present the effects of orally delivered VEH (**b**), ALM (**c**), or ZOL (**d**) for 6 h subsequent to drug treatment (*arrow* denotes drug delivery at ZT 18). **e** Schematic showing microdialysis samples assigned to one of three behavioral state categories. *ac* anterior commissure, *cc* corpus callosum, *CPu* caudate putamen, *Cx* cortex, *GP* globus pallidus, *HDB* diagonal band of Broca, horizontal limb, *LPO* lateral preoptic area, *LV* lateral ventricle, *SIB* substantia innominata, basal nucleus of Meynert, *VLPO* ventrolateral preoptic nucleus, *VP* ventral pallidum

VEH ($P < 0.05$). A significant decrease in GABA levels was also observed during NR/R states following ZOL compared to VEH and ALM ($P < 0.05$).

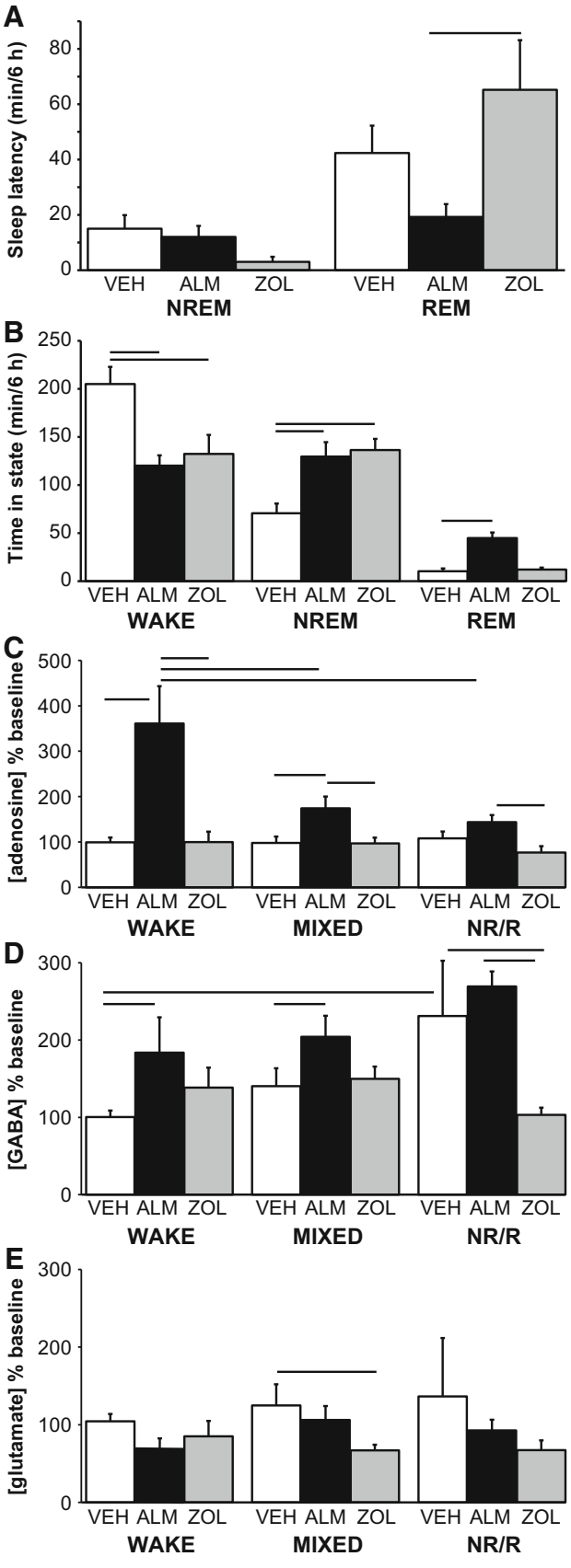
GLU levels in the BF were significantly reduced by ZOL during MIXED states compared to VEH ($F_{2,85} = 3.7$; $P = 0.02$; Fig. 4e). There were no other effects of any drug on GLU.

Oral administration of ALM promotes BF ADO and GABA release during extended wakefulness

Next, we determined whether ALM-induced increases in ADO and GABA persisted during a period of forced wakefulness and subsequent recovery. During the 6-h SD challenge, ZOL animals appeared to struggle to remain alert following drug administration compared to rats that had received ALM; however, no significant differences were observed in total percent time spent awake (VEH; 95.18 ± 1.15 % wake, ALM; 92.48 ± 1.39 % wake, and ZOL; 92.20 ± 1.78 % wake), indicating the efficacy of our SD procedure. Figure 5 summarizes the effects of VEH ($n = 10$ rats), ZOL ($n = 10$), and ALM ($n = 11$) administration on vigilance states in rats allowed a 2-h RS opportunity after 6 h of SD. ANOVA revealed a significant drug effect on the latency to the onset of NREM and REM sleep following cessation of SD ($F_{5,48} = 8.8$; $P < 0.0001$; Fig. 5a). ZOL significantly decreased NREM latency relative to VEH and significantly increased REM sleep latency compared to VEH and ALM ($P < 0.05$).

Figure 5b presents the cumulative time that the rats spent in waking, NREM, and REM sleep states after cessation of 6 h of SD combined with drug administration of either VEH, ALM (100 mg/kg), or ZOL (100 mg/kg). ANOVA revealed a significant drug effect on the cumulative time spent in vigilance states. During the 2-h RS opportunity, ALM and ZOL significantly decreased wakefulness and increased NREM sleep compared to VEH ($F_{8,80} = 11.7$; $P < 0.0001$). No effects on cumulative time spent in REM sleep were observed.

Figure 6a presents the percentage of time in wakefulness during SD and RS relative to the baseline wakefulness and demonstrates the efficacy of our SD procedure; the RS data are replotted from Fig. 5b relative to basal wakefulness values. The time spent in wakefulness during RS was significantly reduced ($F_{6,42} = 210.7$; $P < 0.0001$) when the animals were given ALM or ZOL relative to waking under VEH conditions ($P < 0.05$). BF neurotransmitters were affected by drug treatment delivered during SD, and these effects persisted into RS (Fig. 6b–e). ALM (Fig. 6b; black triangles) dramatically increased BF ADO during SD relative to the increase following VEH (open squares) or ZOL (inverted triangles); ADO returned to baseline levels when the animals were permitted to sleep. Group data are



◀**Fig. 4** Effects of oral drug administration on vigilance state and BF neurotransmission. **a** Latency to the onset of NREM and REM sleep following ALM (100 mg/kg; p.o.) as compared to ZOL (100 mg/kg; p.o.). *Horizontal line* indicates that ZOL produces a significant increase ($P < 0.05$) in latency to REM sleep relative to ALM. **b** Cumulative time in each vigilance state (mean ± SEM) for VEH-, ALM-, and ZOL-treated rats. *Horizontal lines* denote significant drug effects ($P < 0.05$) on each vigilance state relative to VEH. **c** ALM significantly increased BF ADO during waking, mixed states, and NR/R compared to VEH or ZOL ($P < 0.05$). ADO levels following ALM progressively decreased from Wake to Mixed states to NR/R sleep. **d** GABA levels increased during NR/R sleep relative to wakefulness ($P < 0.05$). ALM significantly increased BF GABA relative to VEH during waking and mixed states, and relative to ZOL during NR/R ($P < 0.05$). **e** Post hoc comparisons showed that ZOL decreased GLU relative to VEH during mixed states ($P < 0.05$)

summarized in Fig. 6c. ALM promoted a significant increase in BF ADO levels during SD relative to Waking prior to drug administration and relative to rats that received VEH ($F_{6,42} = 4.0$; $P = 0.0035$). ADO levels following ALM administration during SD returned to basal levels during RS (Fig. 6b, c). By contrast, ADO levels were not altered by ZOL during SD or RS (Fig. 6b, c).

GABA levels were significantly increased from SD to RS in the VEH condition ($F_{6,42} = 3.9$; $P = 0.004$; Fig. 6d). ALM elevated GABA during SD compared to VEH and this elevation persisted during RS. ZOL also enhanced GABA levels during RS (Fig. 6d) relative to the levels during SD. Thus, ZOL did not inhibit GABA increases during RS as it did during spontaneous sleep (Fig. 4d). In contrast to ADO and GABA, GLU levels (Fig. 6e) decreased significantly during RS compared to SD following VEH and ZOL ($F_{6,42} = 3.5$; $P = 0.0073$).

Central administration of ALM promotes sleep and cortical ADO release

To further evaluate the BF as a potential site of hypocretin receptor antagonism on sleep/wake control, hypnotics were microinjected into the BF and their effects on sleep/wake and cortical neurotransmitter release were determined. Central administration of ALM and ZOL into the BF significantly increased the amount of time the rats spent in NREM sleep relative to VEH control (Fig. 7a; $F_{2,113} = 10.6$; $P < 0.001$). Sleep induction by ALM lasted throughout the 6-h recording and dialysis collection period, whereas NREM sleep returned to control levels in the 6th hour of recording for animals that received ZOL.

BF administration of ALM increased cortical ADO levels which persisted for the duration of the 6-h recording and dialysis collection period ($F_{2,125} = 4.1$; $P < 0.05$;

Fig. 7b). By contrast, ZOL did not alter cortical ADO levels compared to VEH until the last hour of recording. Neither BF microinjections of ALM or ZOL affected cortical GABA levels (Fig. 7c). Similarly, cortical GLU levels were unaffected by ALM, although ZOL increased cortical GLU levels during the last hour of recording when compared to ALM ($F_{5,101} = 9.1$; $P < 0.0001$; Fig. 7d).

TTX and KCl evoke BF neurotransmitter release under anesthesia

To determine whether the various neurotransmitter changes observed in protocols 2–4 were of neuronal origin, protocol 5 ($n = 8$ rats) was performed while holding vigilance state constant. Under isoflurane anesthesia, 1 μM TTX was delivered by reverse dialysis followed by stimulation with 100 mM KCl to the BF. In Fig. 8a, c, e an individual animal's response over time (dialysates sampled every 12 min) to reverse perfusion with TTX (1 μM) followed by KCl (100 mM) into the BF for GLU (Fig. 8a), GABA (Fig. 8c), and ACh (Fig. 8e) relative to baseline release (white bars) is shown. Figure 7b, d, and f shows mean concentrations of GLU (Fig. 8b), GABA (Fig. 8d), and ACh (Fig. 8f) in response to 1 μM TTX followed by 100 mM KCl. ANOVA analyses of neurotransmitter levels during reverse dialysis with TTX and KCl revealed a significant drug effect on neurotransmitter release. Although neither TTX nor KCl evoked a significant response in BF GLU (Fig. 8b; $F_{2,20} = 1.06$; $P = 0.36$), BF GABA (Fig. 8d; $F_{2,20} = 4.9$; $P = 0.02$) and BF ACh (Fig. 8f; $F_{2,20} = 9.2$; $P = 0.002$) release were significantly increased by stimulation with KCl relative to basal levels and compared to TTX. In addition, 1 μM TTX significantly decreased BF ACh relative to baseline ($P < 0.05$), indicating that the concentrations used in this study for TTX and KCl were sufficient to induce action potential-mediated events.

Discussion

Almorexant, a dual HCRT receptor antagonist, induced neurochemical events typically associated with the transition to normal sleep under conditions of both low and high sleep pressure, and required an intact BF for maximum efficacy. By contrast, ZOL blocked the normal sleep-associated increase in GABA and was equally effective in promoting sleep in BFx and sham-lesioned rats. These data are consistent with the hypothesis that HCRT antagonism induces sleep by facilitating the neural mechanisms that underlie the transition to normal sleep, including disfacilitation of wake-promoting BF neurons.

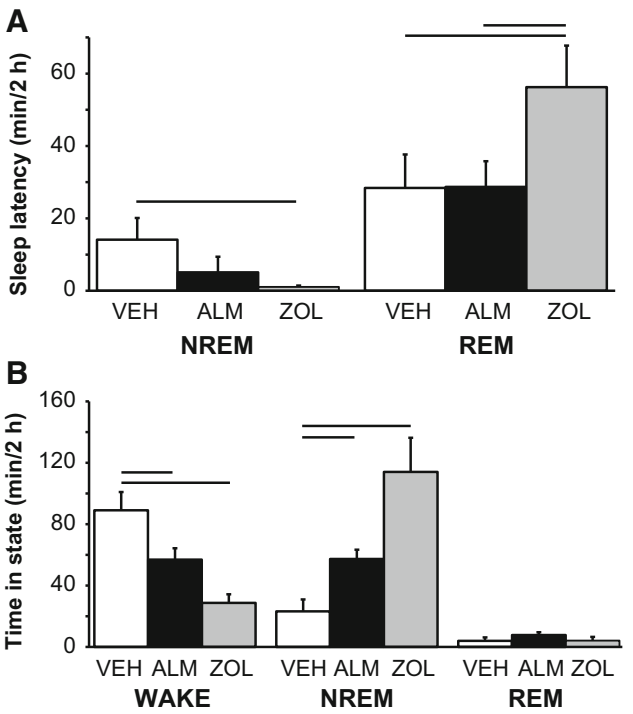


Fig. 5 Effects of oral drug administration on sleep–wakefulness during the 2-h RS opportunity after cessation of SD. **a** Latency to NREM and REM sleep after 6 h of SD following ALM (100 mg/kg) or ZOL (100 mg/kg). *Horizontal lines* indicate that ZOL significantly decreased the latency to the onset of NREM relative to VEH ($P < 0.05$) and increased REM latency ($P < 0.05$) relative to both ALM and VEH. **b** Cumulative time spent in each vigilance state (mean ± SEM) during the 2-h RS opportunity after cessation of SD for VEH-, ALM-, and ZOL-treated rats. *Horizontal lines* denote a significant drug effect ($P < 0.05$) on waking and NREM sleep relative to VEH control

Basal forebrain lesions attenuate hypnotic effects of hypocretin antagonism

Previous studies using 192-IgG-conjugated saporin to selectively lesion BF cholinergic neurons (Wiley et al. 1991) reported minimal, transient or no effects on spontaneous or baseline sleep (Blanco-Centurion et al. 2006a; Kaur et al. 2008; Murillo-Rodriguez et al. 2008). In our study, BF lesions resulted in normal sleep during the light phase, but decreased baseline sleep in the dark phase. To our knowledge, such a time-of-day-specific reduction in spontaneous sleep has not previously been reported in a BFx model. A transient increase in NR sleep time in 192-IgG-lesioned rats following lesion surgery followed by subsequent return to baseline values (Kaur et al. 2008) was attributed to compensation from other elements of the sleep–wake regulatory network, leading to re-stabilization of sleep–wake regulation. Similarly, we cannot rule out that other elements of this regulatory network may have altered their activity following the lesion; since our lesions

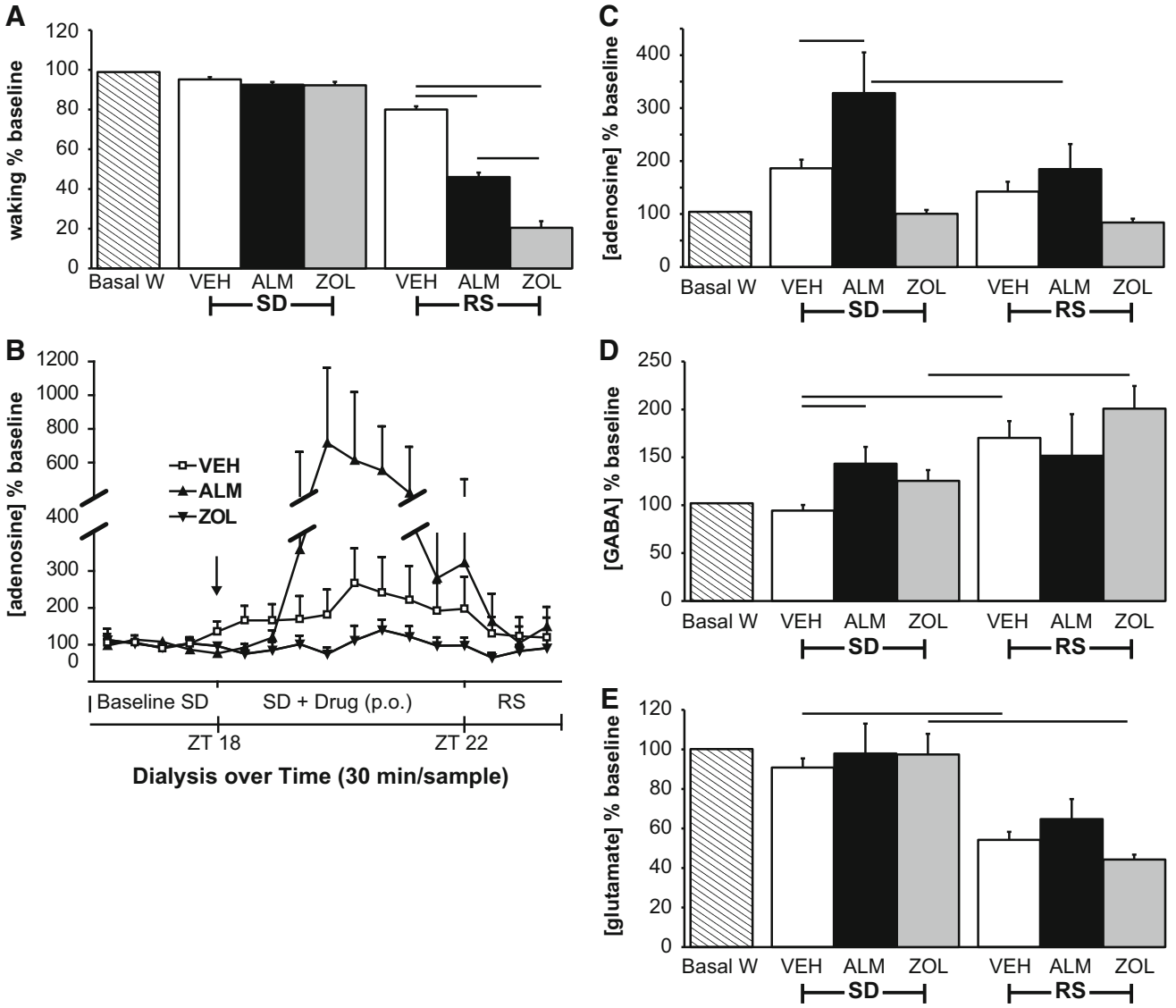


Fig. 6 BF ADO increases during SD and is affected by oral drug administration. **a** When rats are given ALM or ZOL, waking time during RS is significantly reduced relative to VEH ($P < 0.05$). **b** BF ADO levels rose during SD after VEH (*open squares*) administration relative to baseline levels of SD. However, within 2 h after ALM (*black triangles*) administration at ZT 18, BF ADO levels increased dramatically and remained elevated until the rats were permitted to sleep (RS) at ZT 22. ADO levels did not change following ZOL administration (*inverted triangles*). **c** ALM during SD increased BF ADO levels relative to VEH ($P < 0.05$); ADO levels returned to baseline during RS ($P < 0.05$). **d** BF GABA levels rose significantly during RS under VEH and ZOL conditions relative to SD ($P < 0.05$). ALM also increased GABA levels during SD compared to VEH ($P < 0.05$). **e** Post hoc tests show that GLU levels decreased during RS compared to SD under VEH and ZOL conditions ($P < 0.05$)

likely damaged both cholinergic and noncholinergic neurons, such compensatory activity could have resulted in 'overcompensation'.

Following a 6-h sleep deprivation in the dark phase, lesioned rats exhibited attenuated NREM recovery sleep time and NREM delta power compared to shams. This result is consistent with several published studies examining sleep regulation during the light phase in rats with cholinergic BF lesions. Sleep homeostasis was reported to be both normal (Blanco-Centurion et al. 2006a) and impaired (Kalinchuk et al. 2008; Kaur et al. 2008) following saporin-based cholinergic BF lesions. Ibotenic acid-based BF lesions, which target noncholinergic neurons, increased basal NREM delta power and attenuated its homeostatic regulation (Kaur et al. 2008). More recently, selective lesions of either cholinergic or noncholinergic BF neurons were shown to have little to no effect on basal sleep–wake regulation, whereas destroying both populations induced an unresponsive coma-like state (Fuller et al. 2011). Together, these data suggest that cholinergic and

noncholinergic neurons in the BF contribute to the normal expression of sleep and waking in a complex manner (Arrigoni et al. 2010; Szymusiak et al. 2000; Zaborszky and Duque 2003). Similarly, the sleep–wake deficits observed in our animals may reflect the combined effects of substantial cholinergic cell loss in the BF with additional noncholinergic collateral damage.

ALM increased NREM sleep time compared to VEH at all doses, although not enough to fully compensate for BFx-induced decreases in NREM sleep. HCRTR2 is expressed in the BF (Marcus et al. 2001), and HCRTergic fibers project to the BF (Espana et al. 2005) and form synapses with cholinergic neurons (Fadel et al. 2005). HCRT activates BF cholinergic neurons (Eggermann et al. 2001), and infusing HCRT into the various BF regions promotes waking (Espana et al. 2001; Fadel et al. 2005; Methippara et al. 2000). On the other hand, HCRT-conjugated saporin destroys noncholinergic neurons in the BF (Fuller et al. 2011), and intracerebroventricular HCRT-1 administration increases wakefulness in 192-IgG-SAP-lesioned rats comparable to unlesioned rats (Blanco-Centurion et al. 2006b). Together, these findings suggest that in the BF, a dual HCRT receptor antagonist such as ALM may act on both cholinergic and noncholinergic targets (Arrigoni et al. 2010; Eggermann et al. 2001), which may explain why our lesions were only partially effective at blocking ALM-induced sleep. Furthermore, the HCRT system also projects to other arousal-promoting brain regions, including the noradrenergic locus coeruleus, histaminergic tuberomammillary nuclei and the Raphe nuclei (Peyron et al. 1998); ALM would have still acted at these sites in our rats. The BF thus appears to be an important site of action for ALM to influence sleep, but not an indispensable one. By contrast, ZOL increased NREM sleep time to similar total duration in BFx and Sham rats at the two highest doses tested, suggesting that the BF is entirely unnecessary for induction of NREM sleep by ZOL, or that the loss of the BF could even facilitate ZOL's actions. Interestingly, ALM-induced REM sleep was unaffected by BFx, suggesting that this action is mediated elsewhere in the brain.

Hypocretin receptor antagonism facilitates BF adenosine and GABA release

To better understand the effects of ALM and ZOL on BF neurotransmission, we selected one concentration each of ALM (100 mg/kg) and ZOL (100 mg/kg). We have previously demonstrated that the selected concentration (100 mg/kg by oral administration) was the minimum dose required for ALM and ZOL to induce equivalent levels of somnolence in rats (Morairty et al. 2012, 2014). Our results

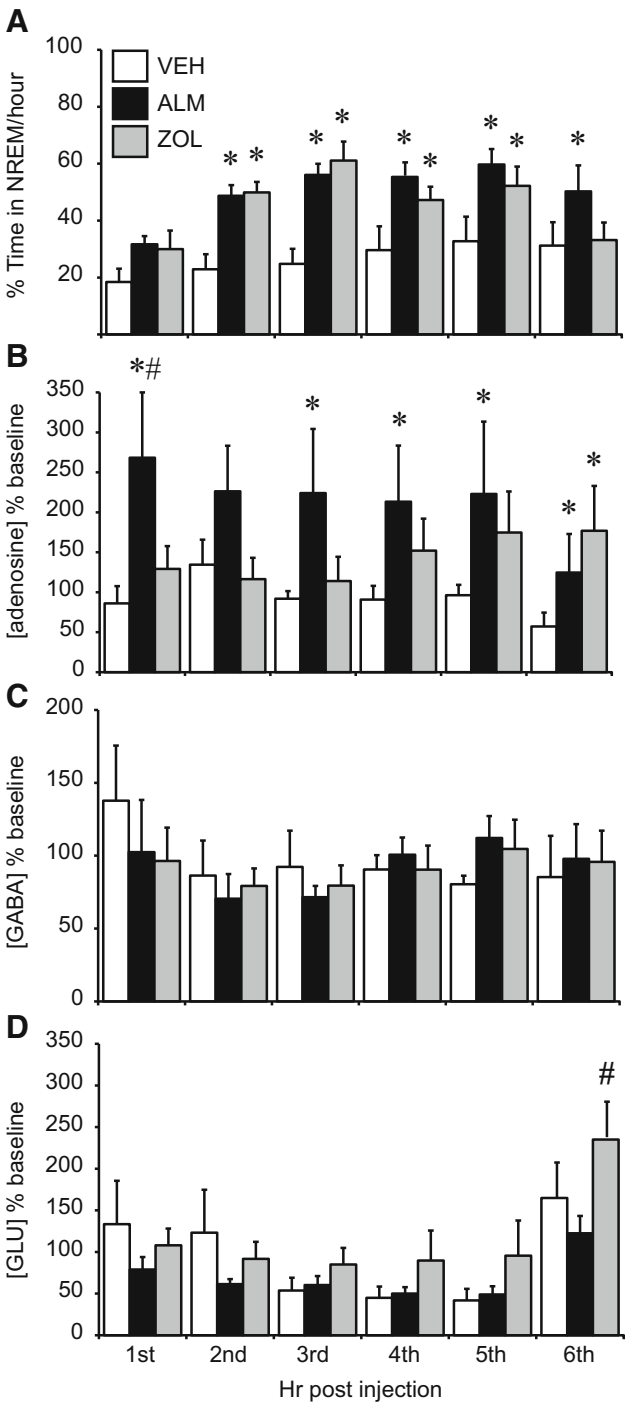


Fig. 7 BF microinjections induce sleep and facilitate cortical ADO release by ALM. **a** Percent time in NREM sleep (mean ± SEM) following BF microinjection of VEH, ALM or ZOL. *Asterisk* denotes significant drug effects ($P < 0.05$) relative to VEH. **b** BF administration of ALM significantly increased cortical ADO levels compared to VEH ($P < 0.05$). *Asterisk* denotes significant difference from VEH. *Hash symbol* denotes significant difference from ZOL. **c** Cortical GABA levels were not significantly altered by either ALM or ZOL compared to VEH. **d** Post hoc tests showed that ZOL caused a significant increase ($P < 0.05$) in cortical GLU relative to ALM at the end of the 6th hour post-drug administration

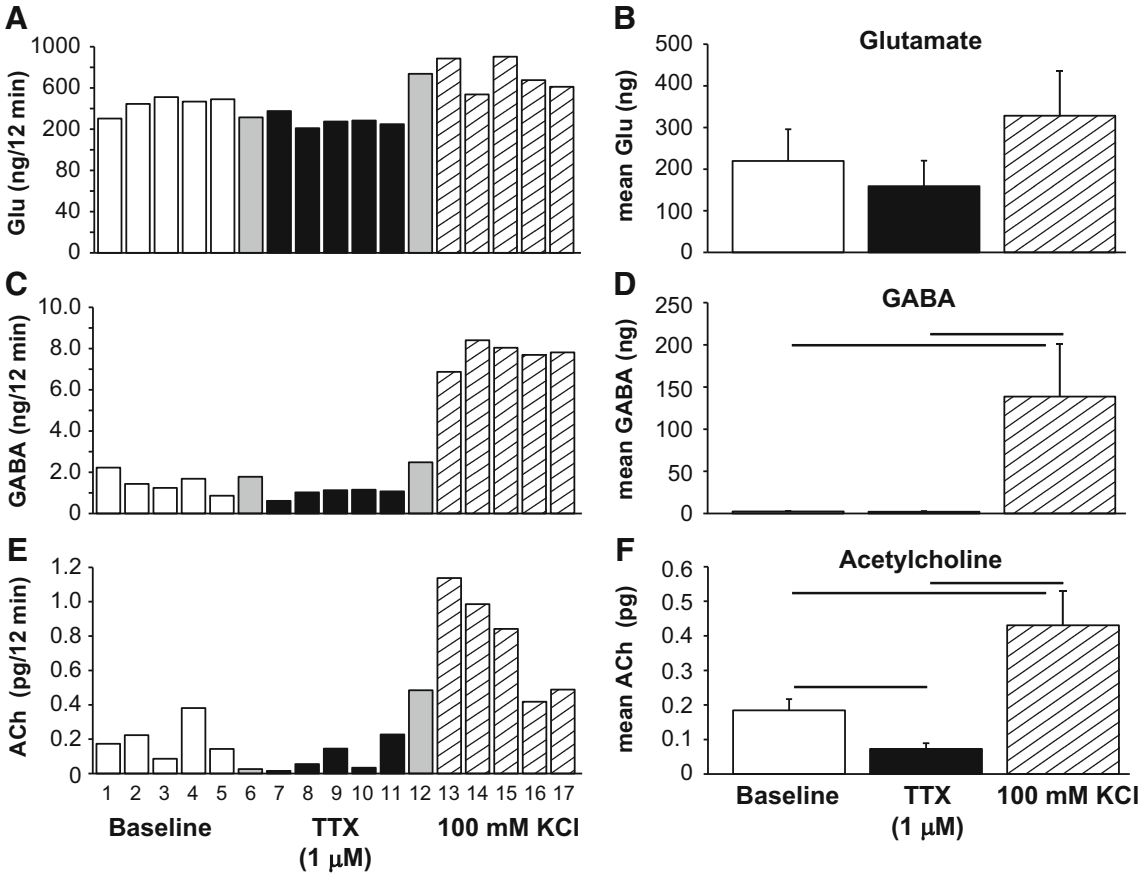


Fig. 8 BF neurotransmission in response to reverse dialysis of TTX under steady-state conditions. **a**, **c**, **e** Effects of TTX (1 μM; reverse dialysis) in the BF of an anesthetized rat. The *x*-axes show the temporal distribution of BF GLU (**a**), GABA (**c**), and ACh (**e**) levels collected every 12 min during aCSF perfusion (baseline; *white bars*) followed by perfusion with TTX (1 μM in aCSF; *black bars*), and subsequent perfusion with a high concentration of KCl (100 mM in aCSF; *hatched bars*). *Gray bars* (at 6 and 12) indicate samples that were transitions between changes in perfusion fluid (data not included). *Panels* on the *right* (**b**, **d**, **f**) present the overall mean concentrations (+SEM) of GLU, GABA, and ACh in response to TTX (1 μM) followed by high KCl. GLU neurotransmission was not significantly altered in the presence of either TTX or high KCl (**b**). Both BF GABA (**c**, **d**) and ACh (**e**, **f**) release were significantly increased by 100 mM KCl relative to basal levels and compared to TTX. TTX also caused a significant decrease in BF ACh relative to baseline ($P < 0.05$)

confirm that ALM and ZOL at this particular dose were equally effective at reducing wakefulness and inducing NREM sleep in intact rats. ALM also increased REM sleep compared to VEH and ZOL, as previously reported (Brisbare-Roch et al. 2007; Morairty et al. 2012).

Systemic ALM increased BF ADO during WAKE and MIXED sleep states under conditions of low (Fig. 4) and high (Fig. 6) sleep pressure, while ZOL had no effect on ADO in the BF. To our knowledge, this represents the first report of increased ADO release following blockade of HCRT signaling. When microinjected into the BF, ALM also increased NREM sleep time and evoked ADO release in the cortex (Fig. 7a, b), whereas microinjected ZOL increased NREM sleep without altering cortical ADO release (Fig. 7c). Thus, sleep-inducing doses of ALM elicit ADO release and this action is mediated by the BF. Notably, ADO levels in the BF also declined during ALM-induced sleep as occurs during normal sleep. ADO, a downstream metabolite of ATP, regulates sleep in the BF (Basheer et al. 2004). ADO inhibits synaptic transmission and hyperpolarizes membrane potential (Rainnie et al. 1994). In the BF, ADO acts on A1 (Alam et al. 1999; Strecker et al. 2000) and A2a ADO receptors (Satoh et al. 1996, 1998), inhibiting excitatory inputs onto cholinergic and noncholinergic BF neurons and presynaptically inhibiting local GABA interneurons (Arrigoni et al. 2006; Hawryluk et al. 2012; Yang et al. 2013). Adenosinergic modulation of sleep–wake state in the BF is thus complex and involves multiple neuronal and possibly non-neuronal (glial) cell types (Halassa and Haydon 2010; Scharf et al. 2008; Halassa et al. 2009).

ADO is released in the BF as a consequence of prolonged waking (Porkka-Heiskanen 1999); this release appears to depend on the presence of cholinergic BF

neurons (Blanco-Centurion et al. 2006a; Kalinchuk et al. 2008) and has been hypothesized to be a critical component of the sleep homeostat (Kalinchuk et al. 2008, 2011; Kaur et al. 2008). In our studies, ADO evoked by systemic ALM was of similar magnitude under conditions of low and high sleep pressure and was significantly greater than that evoked by SD alone (Figs. 4, 6), suggesting that ALM-induced BF ADO release does not depend on homeostatic sleep drive. Rather, the acute removal of HCRT tone during waking by ALM may disinhibit ADO release, thereby pushing the BF network towards sleep. Thus, HCRTergic tone during waking in the dark phase may suppress BF ADO release. Although the mechanism is presently unknown, one possibility is that HCRT terminals may innervate local inhibitory neurons in the BF that, in turn, downregulate ADO accumulation.

On the other hand, ablation of HCRT neurons in the LH has been reported to abolish SD-induced ADO increases (Murillo-Rodriguez et al. 2008). This apparent discrepancy may be explained by the facts that HCRT neurons co-release GLU (Henny et al. 2010; Rosin et al. 2003; Schone et al. 2014) and that GLU evokes ADO release in BF (Sims et al. 2013; Wigren et al. 2007) whereas HCRT does not (Sims et al. 2013). Destruction of the HCRT neurons would, therefore, reduce overall glutamatergic input to the BF, thereby possibly impairing ADO release. In contrast, acute HCRT receptor blockade by ALM would be expected to leave glutamatergic signaling—including that from the HCRT neurons themselves—unaffected. Since it is likely that HCRT terminals in the BF also contain GLU as they do elsewhere in the brain (Henny et al. 2010; Schone et al. 2014), the HCRT neurons may, via co-released GLU, drive accumulation of ADO in the BF in the presence of ALM.

Microinjection of ALM into the BF significantly increased NREM sleep time similar to oral administration (present study; Dugovic et al. 2009; Morairty et al. 2012). Microinjection of ZOL also increased NREM sleep time comparably to ALM; however, these effects are likely mediated by different mechanisms. When infused into the BF, ALM evoked a significant enhancement of cortical ADO release whereas ZOL had no effect (Fig. 7b), suggesting that HCRTergic input represents a novel pathway for mediating ADO signaling by the BF. To our knowledge, only one other study has examined the effects of HCRT antagonism in the BF; BF microinjections of the HCRT receptor1 (Ox1R) antagonist SB-334867A delayed the emergence from propofol anesthesia (Zhang et al. 2012). Our findings extend these observations by showing that HCRT antagonism in the BF is important for sleep–wake maintenance as well as the transition from anesthesia to behavioral arousal.

Sleep-associated increases in GABA release were preserved following ALM (Fig. 4d); however, orally administered ALM also increased BF GABA release during Waking and Mixed states compared to VEH under conditions of low and high sleep pressure. The BF contains sleep- and wake-active noncholinergic neurons (Hassani et al. 2009), suggesting that GABAergic effects on sleep–wake state are complex. However, endogenous GABA release in the BF is high during NREM sleep compared to waking and REM sleep (Nitz and Siegel 1996; Vanini et al. 2012). Infusion of GABA agonists into the BF promotes sleep (Manfridi et al. 2001), infusion of $GABA_A$ receptor antagonists increases ACh release (Vazquez and Baghdoyan 2003), and SD increases GABA receptor expression on ACh neurons (Modirrousta et al. 2007), suggesting that GABA released in the BF has a net sleep-promoting influence. Consequently, the ALM-induced GABA release that we observed during Waking and Mixed states likely comprises an important part of ALM's ability to induce sleep, and does so in a way that mimics the neurochemical events normally associated with the transition to sleep. One possible route for increased BF GABA release could be disinhibition of local GABAergic neurons; alternatively, increased GABA seen following systemic ALM could originate outside the BF. Dual HCRTR1/R2 antagonism with ALM thus enhanced GABAergic transmission in the BF facilitating the transition to sleep. In contrast, systemic ZOL blocked sleep-associated BF GABA release under conditions of low, but not high, sleep pressure, consistent with the idea that ZOL induces sleep in part by circumventing the endogenous subcortical sleep–wake regulatory network.

ZOL significantly decreased BF GLU release in Mixed states, consistent with the idea that it induces sleep via generalized inhibition. However, there were no other overt changes in BF GLU transmission as a function of spontaneous sleep–wakefulness, drug administration, or increased sleep pressure. Others have shown (Wigren et al. 2007) that glutamatergic tone in the BF is increased during waking and contributes to sleep pressure. However, our studies were conducted during the middle of the animals' active period when sleep pressure is low; higher endogenous waking drive in the dark (active) phase may have obscured such effects compared to the light phase. Under these conditions, HCRTR1/R2 antagonism and traditional GABAergic hypnotics do not appear to substantially modulate GLU release levels in the BF. Nonetheless, as discussed above, GLU released by HCRT neurons may still play an important role in ALM-mediated ADO release.

Hypnotic-induced BF neurotransmitter release is likely of neuronal origin

Sodium channel blockade induced by TTX or potassium depolarization is a commonly used tool in microdialysis to

address whether or not neurotransmitters sampled in the extracellular space are likely derived from synaptic events (Timmerman and Westerink 1997; van der Zeyden et al. 2008). Given that neurotransmitter release can vary significantly with vigilance state (John et al. 2008; Kodama and Honda 1999; Kodama et al. 1992; Lena et al. 2005; Nitz and Siegel 1997b) including in the BF (Vazquez and Baghdoyan 2001; Vazquez et al. 2002), we held vigilance state constant under isoflurane anesthesia to address whether changes in BF GLU, GABA and ACh levels were of neuronal origin. Our results demonstrate that treatment with TTX or KCl failed to evoke a significant effect on GLU, whereas KCl significantly stimulated GABA. TTX also produced a significant decrease in ACh that was immediately reversed in the presence of KCl. While GLU release may be affected by vigilance state and/or drug effects, the extracellular concentrations obtained by dialysis are likely derived from both glial and neuronal sources (van der Zeyden et al. 2008) and caution should be used when interpreting in vivo release of GLU. In contrast, BF GABA and ACh efflux responded in a robust manner to TTX and KCl, consistent with previous studies confirming that these neurotransmitters are of neuronal origin (van der Zeyden et al. 2008). The neurotransmitter changes we observed in the BF thus reflect synaptic-mediated events that can be modulated both by drug administration and arousal state.

Dual HCRT receptor antagonists have emerged as promising new therapeutics for insomnia (Bettica et al. 2012; Uslaner et al. 2013; Winrow et al. 2011; Gotter et al. 2013), and ALM has proved to be a useful tool to mechanistically examine the role of the HCRT system in sleep/wake control. We have shown that ALM elicited neurochemical release profiles in the BF similar to those associated with normal sleep and required an intact BF for maximum efficacy, whereas ZOL induced sleep without eliciting a 'sleep-typical' neurochemical profile and did not require functional contributions from the BF to induce sleep. These data are consistent with prior studies showing that GABAergic activity in the BF plays a major role in promoting sleep. In addition, our finding that ALM induces ADO release in both the BF and the cerebral cortex suggests a novel mechanism whereby HCRT neuronal activity modulates adenosinergic tone, possibly in concert with co-released transmitters such as GLU. Overall, these results are consistent with the hypothesis that ALM selectively inhibits the endogenous subcortical sleep–wake regulatory network to induce sleep.

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Homeostatic Sleep Pressure is the Primary Factor for Activation of Cortical nNOS/NK1 Neurons

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Cortical interneurons, immunoreactive for neuronal nitric oxide synthase (nNOS) and the receptor NK1, express the functional activity marker Fos selectively during sleep. NREM sleep 'pressure' is hypothesized to accumulate during waking and to dissipate during sleep. We reported previously that the proportion of Fos^+ cortical nNOS/NK1 neurons is correlated with established electrophysiological markers of sleep pressure. As these markers covary with the amount of NREM sleep, it remained unclear whether cortical nNOS/NK1 neurons are activated to the same degree throughout NREM sleep or whether the extent of their activation is related to the sleep pressure that accrued during the prior waking period. To distinguish between these possibilities, we used hypnotic medications to control the amount of NREM sleep in rats while we varied prior wake duration and the resultant sleep pressure. Drug administration was preceded by 6 h of sleep deprivation (SD) ('high sleep pressure') or undisturbed conditions ('low sleep pressure'). We find that the proportion of Fos^+ cortical nNOS/NK1 neurons was minimal when sleep pressure was low, irrespective of the amount of time spent in NREM sleep. In contrast, a large proportion of cortical nNOS/NK1 neurons was Fos^+ when an equivalent amount of sleep was preceded by SD. We conclude that, although sleep is necessary for cortical nNOS/NK1 neuron activation, the proportion of cells activated is dependent upon prior wake duration.

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INTRODUCTION

Although the functions of sleep remain controversial, one of the strongest arguments for its fundamental importance is its homeostatic regulation. Sleep homeostasis refers to compensatory increases in sleep amount, sleep consolidation and/or sleep intensity that occur in response to a period of extended wakefulness (Borbely and Achermann, 2000). In mammals and birds, sleep intensity, usually assessed by measuring the spectral power of the EEG in the delta frequency range (0.5–4.5 Hz) during non-rapid eye movement sleep (NREM), is used as an index of the hypothetical sleep 'pressure' that has accumulated during wakefulness (Borbely and Achermann, 2000; Rattenborg *et al*, 2009).

Despite the widespread occurrence of sleep homeostasis among animal species, our understanding of the underlying mechanisms is incomplete. Current hypotheses implicate sleep factors such as adenosine or cytokines that accumulate during waking, and increase the propensity and depth of sleep (Coulon *et al*, 2012; Krueger *et al*, 2008; Landolt, 2008; Porkka-Heiskanen and Kalinchuk, 2011; Szymusiak and McGinty, 2008). Sleep factors are thought to act by inhibiting wake-promoting neurons (Porkka-Heiskanen and Kalinchuk, 2011; Rainnie *et al*, 1994), but may also act directly on the cerebral cortex (Clinton *et al*, 2011; Szymusiak, 2010). We have recently described a population of cortical GABAergic interneurons that is specifically activated during sleep (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). These neurons are identified by colocalized immunoreactivity for neuronal nitric oxide synthase (nNOS) and the substance P (SP) receptor NK1 (Dittrich *et al*, 2012). Activation of these neurons, assessed by immunoreactivity for the functional activity marker Fos, correlates with time spent in NREM sleep as well as with NREM delta power (Gerashchenko *et al*, 2008; Morairty *et al*, 2013). Prior wake duration 'dose-dependently' increased the proportion of Fos-labeled nNOS neurons when rats were subjected to 2, 4, or 6 h of sleep deprivation (SD) followed by a 2-h recovery sleep (RS) opportunity (Morairty *et al*, 2013). Based on these observations, we have suggested that cortical nNOS/NK1 neurons are inhibited by wakefulness and activated by sleep pressure (Kilduff *et al*, 2011).

In our previous studies, we increased sleep pressure by increasing the amount of prior wakefulness, which resulted in a compensatory increase in the amount of NREM sleep during RS. Therefore, we could not distinguish whether the time spent in NREM sleep or the magnitude of sleep pressure produced by prolonging wakefulness was the primary factor driving Fos expression in cortical nNOS/NK1 neurons. Fos expression typically reflects neuronal activity occurring during the prior 1–2 h to killing (Hoffman

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and Lyo, 2002; Zangenehpour and Chaudhuri, 2002). If cortical nNOS/NK1 neurons are uniformly activated throughout NREM sleep and inactive during wakefulness, Fos expression in these neurons should depend on the time spent in NREM during the 1–2 h before transcardial perfusion and thus would only indirectly correlate with measures of sleep pressure. Here, we sought to distinguish between these alternatives by dissociating the occurrence of NREM from the magnitude of sleep pressure using hypnotic medications to pharmacologically control NREM sleep duration under conditions in which the prior sleep/wake history was varied. To ensure that our results were not drug-specific, we utilized hypnotics with different mechanisms of action: the dual hypocretin/orexin receptor antagonist almorexant (ALM) and the $GABA_A$ receptor modulator zolpidem (ZOL). We find that, when time spent in NREM sleep is held constant, the proportion of cortical nNOS/NK1 cells activated is dependent upon prior sleep/wake history and that Fos expression in cortical nNOS/NK1 neurons reflects time kept awake (and, presumably, the accompanying sleep pressure) more robustly than any other parameter of NREM sleep.

MATERIALS AND METHODS

Animals

A total of 39 male Sprague–Dawley rats were studied. Animals were housed in separate cages in temperature-controlled recording chambers (20–24°C, 30–70% relative humidity) under a 12/12 light/dark cycle with food and water available *ad libitum*. The weights at experiment were 587 g ± 63 (mean ± SD). All experimental procedures involving animals were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

Surgical Procedures

Surgical procedures involved implantation of sterile telemetry transmitters (F40-EET, Data Sciences International, St Paul, MN, USA) as previously described (Morairty *et al*, 2013, 2008, 2012). Briefly, transmitters were placed intraperitoneally under isoflurane anesthesia. Biopotential leads were routed subcutaneously to the head and neck. EEG electrodes were placed epidurally 1.5 mm anterior to bregma and 1.5 mm lateral to midline, and 6 mm posterior to bregma and 4 mm lateral to midline on the right hemisphere. EMG leads were positioned bilaterally through the nuchal muscles.

Identification of Sleep/Wake States and Sleep/Wake Data Analyses

Behavioral state determinations and data analyses were conducted as previously described (Morairty *et al*, 2013, 2012). After at least 3-week postsurgical recovery, EEG and EMG were recorded via telemetry using DQ ART 4.1 software (Data Sciences International). Following completion of data collection, the EEG and EMG recordings were scored in 10 s epochs as waking, rapid eye movement sleep (REM), or NREM by expert scorers who examined the recordings visually using NeuroScore software (Data Sciences International). For calculation of bout durations, a bout was defined as consisting of a minimum of two consecutive epochs of a given state and ended with any single state change epoch. EEG spectra were analyzed with a fast Fourier transform algorithm using a Hanning Window without overlap (NeuroScore software, Data Sciences International) on all epochs without artifact. For comparisons of EEG spectra, average spectra of a specific state were normalized to the average spectra of the respective state during a 6 h baseline recording (Zeitgeber time 0–6, or ZT0-ZT6). For calculation of NREM EEG delta power (NRD), the mean of the power between 0.5–4.5 Hz of the averaged NREM spectra was calculated and normalized to the respective value of the 6 h baseline recording. NRD energy (NRDE) was calculated by multiplying the time (h) spent in NREM sleep by the normalized NRD power.

Detection of individual slow waves was adapted from (Vyazovskiy *et al*, 2007). Raw EEG was bandpass filtered (0.5–4.5 Hz) using the bandpassfilter.m function from the FieldTrip toolbox (http://www.ru.nl/neuroimaging/fieldtrip) in MATLAB (Mathworks, Natick, MA, USA). The first positive peak after a zero crossing was identified as a single slow wave. The slope was approximated as a straight line between that peak and the last negative peak preceding the zero crossing. All slopes from artifact-free NREM epochs were averaged for each rat. Slopes were normalized to the average NREM slopes from the respective baseline recordings.

SD Procedures

Animals were continuously observed while EEG and EMG were recorded and, when inactive and appeared to be entering sleep, cage tapping occurred. When necessary, an artist's brush was used to stroke the fur or vibrissae. After ZOL, it was sometimes necessary to touch rats to keep them awake.

Experimental Protocol

The rats were assigned to six groups: (1) VEH with low sleep pressure ($n=6$); (2) VEH with high sleep pressure ($n=7$); (3) ZOL with low sleep pressure ($n=6$); (4) ZOL with high sleep pressure ($n=7$); (5) ALM with low sleep pressure ($n=6$); and (6) ALM with high sleep pressure ($n=7$). Dosing occurred at ZT12, 100 mg/kg p.o. in 2 ml/kg for both drugs. Perfusion occurred at ZT14 for VEH and ZOL groups and at ZT14.5 for ALM groups due to its longer latency to sleep onset (Black *et al*, 2013; Morairty *et al*, 2012, 2014). Rats in the high sleep pressure conditions were sleep deprived during the 6 h prior to dosing (Figure 1).

Immunohistochemical Procedures

Rats were killed with an overdose of euthanasia solution i.p. (SomnaSol, Butler-Schein, Dublin, OH, USA) and transcardially perfused with heparinized phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were postfixed overnight in the same fixative and then immersed in 30% sucrose in phosphate-buffered saline until they sank. Coronal brain sections were cut at 40-μm thickness. Double immunohistochemistry for Fos and nNOS was performed on serial sections of rat brain as described previously (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). Sections were first incubated overnight with

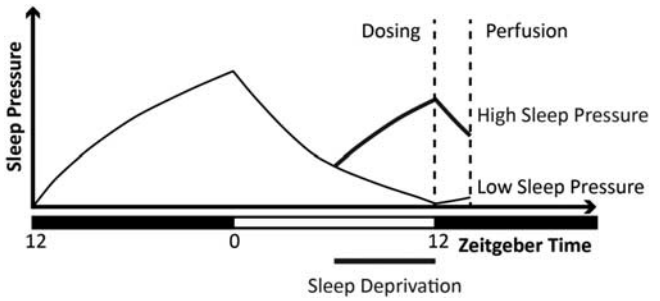

Figure 1 Experimental design. Sleep pressure increases during the active phase and decreases during the inactive phase. Rats were dosed at ZT12 (first vertical dashed line), when sleep pressure is lowest. We assume that sleep pressure increases slowly, if at all, during hypnotic-induced sleep; therefore, the corresponding curve remains low after dosing. To increase sleep pressure but keep the circadian conditions identical, rats in a second group were subjected to 6 h of SD starting at ZT6 and dosed at ZT12. Rats in both conditions were perfused 2 or 2.5 h after dosing (second vertical dashed line). Black and white bars indicate light conditions.

rabbit anti-Fos antibody (1:4000–5000, sc-52, Santa Cruz Biotechnology, Dallas, TX, USA), then with biotinylated donkey anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA, USA), followed by avidin-biotinylated horseradish peroxidase complex (1:200, PK-6100, Vector Laboratories, Burlingame, CA, USA), and nickel-enhanced 3,3′ diaminobenzidine (SK4100, Vector Laboratories) for a black reaction product. nNOS neurons were stained in the same sections by overnight incubation in rabbit-nNOS antibody (1:2000, 61–7000, Invitrogen, Camarillo, CA, USA), followed by biotinylated donkey anti-rabbit antibody and avidin-biotinylated horseradish peroxidase complex, and visualized with NovaRED (SK-4805, Vector Laboratories) for a red-brown reaction product.

Cell Counting

Single-labeled nNOS and double-labeled Fos^+/nNOS cells were counted in one hemisection each at 1.4 mm anterior, 0.5 mm posterior, and 3.0 mm posterior to bregma (Paxinos *et al*, 1999). The percentage of nNOS neurons expressing Fos was calculated as described previously (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). Micrographs for publication were taken at $\times 200$ magnification on a Leica DM 5000B microscope (Leica Microsystems, IL, USA) with a Microfire S99808 camera (Optronics, CA, USA) in Stereoinvestigator (MBF Bioscience, Williston, VT, USA). Adjustments of brightness, color or contrast were applied to the whole image and performed in Photoshop (Adobe Systems, San Jose, CA, USA).

Statistics

Statistical tests were performed using Excel (Microsoft, Redmond, WA, USA), MATLAB and R (R Foundation for Statistical Computing). For each studied variable, we used Mann–Whitney *U*-tests to test whether it significantly distinguished between the high sleep pressure and low sleep pressure groups within the same drug treatment condition. The NREM bout duration histograms (Figure 2) were tested with two-way permutation ANOVA (Manly, 2007) with 5000 iterations of the factors 'bout duration' and 'sleep pressure'. If an interaction was found, the sleep pressure conditions were compared for each bout duration using Holm–Sidak-corrected *t*-tests. For comparison of EEG power spectra, we first performed two-way permutation ANOVA with 5000 iterations with factors 'frequency bin' and 'sleep pressure'. If interactions were found, the source of the interaction was evaluated through bin-by-bin (0.122 Hz) uncorrected *t*-tests between the sleep pressure conditions. Only changes that affected a range of frequencies were considered potentially meaningful, whereas isolated bins with significant changes were ignored. To determine how strongly different physiological parameters distinguished the high *vs* low sleep pressure groups, we calculated Hedges' g (difference of the means divided by pooled standard deviation) and the 95% confidence intervals as an effect size measure (Hedges and Olkin, 1985). As Hedges' g is a parametric measure, data were first transformed to achieve a normal distribution. The percent time in NREM and the $\%Fos^+$/nNOS neurons were arcsine transformed. NREM bout durations, NRD, slow wave slopes (each normalized by respective baseline values), and NRDE were $\log_{10}$ transformed. Normal distributions after data transformation were verified visually using normal probability plots. To test whether Hedges' g for %Fos/nNOS was significantly different from Hedges' g for any other variable, g was first transferred to Fisher's z (Borenstein *et al*, 2009). The z for %Fos/nNOS was then compared with z for every other variable (Meng *et al*, 1992) and the *p*-values were Holm–Sidak-corrected for multiple comparisons.

RESULTS

Sleep Time can be Dissociated from Sleep/Wake History Using Hypnotics

After experimental manipulation of sleep pressure as illustrated in Figure 1, the VEH-dosed rats showed the expected influence of waking history on sleep propensity: whereas undisturbed rats showed an increased time spent awake beginning at lights off (ZT12), rats that were sleep deprived during the preceding 6 h showed strongly reduced wakefulness at the same time of day (Figure 2a). ALM decreased the time spent awake in both groups but, at the dose used, the sleep-deprived rats showed a stronger reduction of wakefulness than rats that were undisturbed for the 6 h preceding dosing, indicating an additive effect of ALM and sleep pressure (Figure 2b). In contrast, ZOL caused a strong reduction of wakefulness irrespective of the preceding wake history (Figure 2c). As we previously found that Fos expression in rat cortical nNOS neurons is dependent on NREM time during the 90 min preceding transcardial perfusion (Morairty *et al*, 2013), we focused on that time window for the following analyses. Figure 2d–f depicts the time each rat spent in wake, NREM, and REM during the 90 min immediately before transcardial perfusion. Whereas the time spent in wake, NREM and REM differed between the low and high sleep pressure groups treated with either VEH or ALM, these physiological parameters did not differ between the groups treated with ZOL, indicating a decoupling between prior sleep/wake history and vigilance states with ZOL treatment.

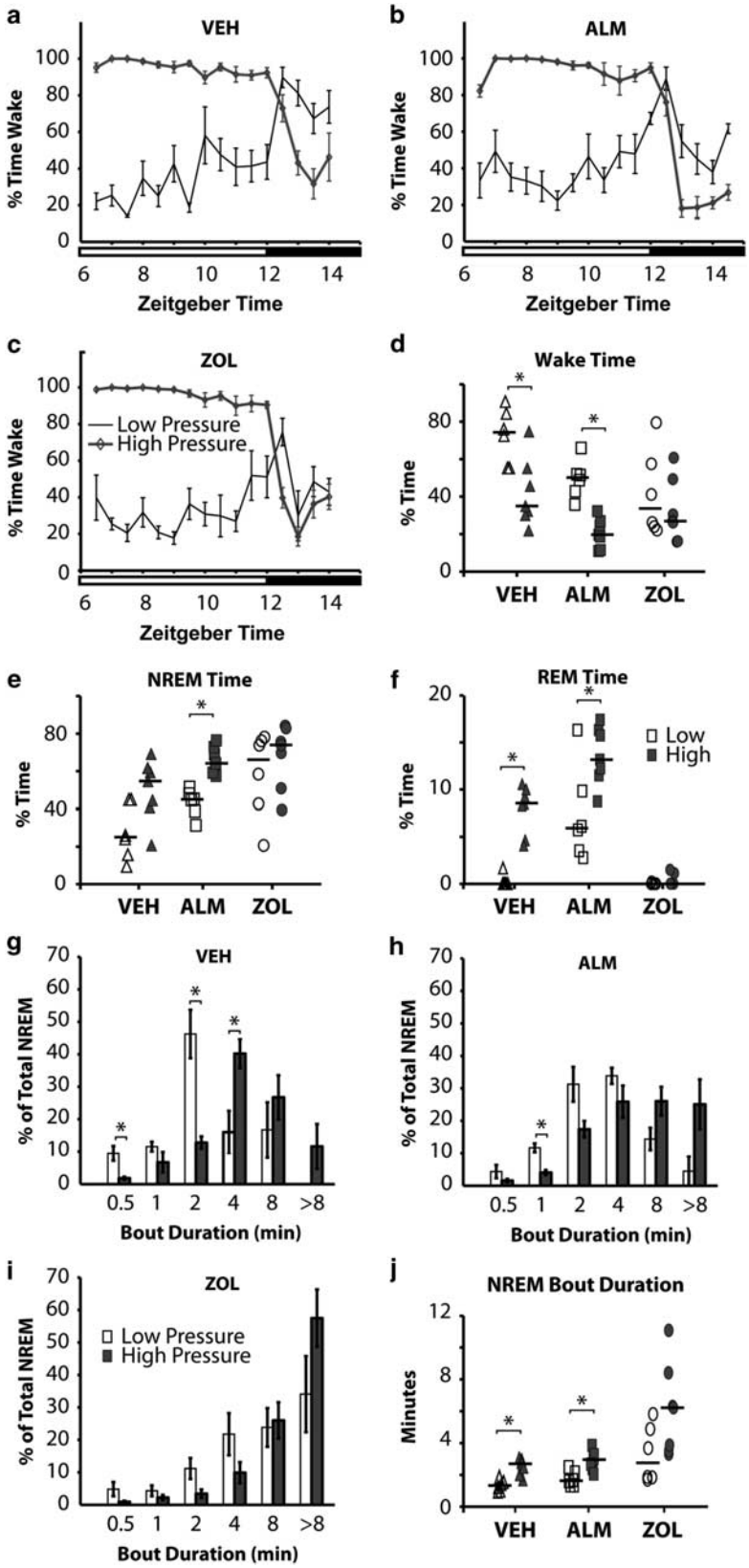

Figure 2 Sleep/wake parameters in the 6 experimental conditions. (a–c) Time spent awake between ZT6 and the time of transcardial perfusion. High sleep pressure groups were sleep deprived from ZT6-ZT12, low sleep pressure groups were left undisturbed so that the amount of sleep pressure differed. Lighting conditions are indicated below the panels. Dosing occurred at ZT12. (d–f) Time spent in wake, NREM, and REM during the 90 min before transcardial perfusion. After ZOL dosing, the time spent in any state did not differ between the sleep pressure conditions. Horizontal lines indicate group medians. *$p < 0.05$, *U*-test. (g–i) NREM bout duration frequency histograms during the 90 min before transcardial perfusion following VEH (g), ALM (h) and ZOL (I) dosing. *$p < 0.05$, Holm–Sidak test after significant interaction in permutation ANOVA. (j) Average NREM bout durations. *$p < 0.05$, *U*-test.

ZOL Disrupts Established Measures of Sleep Pressure

Given the results in Figure 2d–f, we evaluated whether the high and low sleep pressure groups could be distinguished after ALM and ZOL treatment using four established measures of sleep pressure: NREM bout duration, NRD, NRDE, and slow wave slopes. NREM bout duration frequency histograms were shifted towards longer bout durations in the sleep-deprived groups (Figure 2g–i). Although there was a significant interaction between the factors 'bout duration' and 'sleep pressure' following VEH ($F_{5,55} = 6.74$, $p = 0.007$) and ALM ($F_{5,55} = 4.60$, $p = 0.0008$), this interaction did not reach statistical significance for ZOL ($F_{5,55} = 2.28$, $p = 0.056$). Accordingly, the average NREM bout durations were longer for sleep-deprived than undisturbed rats following VEH ($p = 0.002$) and ALM ($p = 0.008$), but not for ZOL ($p = 0.073$; Figure 2j).

As expected, the NREM EEG power spectra showed an elevated power in the delta range in the high sleep pressure group for VEH-dosed rats (Figure 3b). The wake and NREM spectra for ALM-dosed rats resembled those of VEH-dosed rats, including the increased NREM delta power in the high sleep pressure group (Figure 3c and d). Following ZOL, wake and NREM spectra were strongly altered compared with the corresponding baseline recordings, as indicated by the deviations from the basal value 1 in Figure 3e and f. In contrast to VEH and ALM, neither a main effect of 'sleep pressure' nor an interaction of 'frequency bin' and 'sleep pressure' was found following ZOL for either wake or NREM spectra, indicating that spectral power after ZOL did not depend on prior sleep/wake history. (REM spectra are not shown because, in some groups, too little REM occurred to calculate representative spectra). Figure 3g and h depicts normalized NRD (0.5–4.5 Hz) and NRDE for each rat. Both measures significantly distinguished the high *vs* low sleep pressure groups following VEH ($p = 0.005$ for NRD, $p = 0.005$ for NRDE) as well as ALM ($p = 0.022$ for NRD, $p = 0.001$ for NRDE) treatment, whereas no difference was found following ZOL ($p = 1$ for NRD, $p = 0.63$ for NRDE).

Finally, we measured the average slopes of individual EEG slow waves during NREM. As expected, the slow wave slopes were steeper for sleep-deprived rats than for undisturbed rats following VEH (Figure 4b). This difference was preserved after ALM (Figure 4c) but not after ZOL (Figure 4d). Consequently, the average slow wave slope was significantly greater in the high sleep pressure than in the low sleep pressure group following VEH ($p = 0.008$) and ALM ($p = 0.001$) but not following ZOL ($p = 0.366$; Figure 4e).

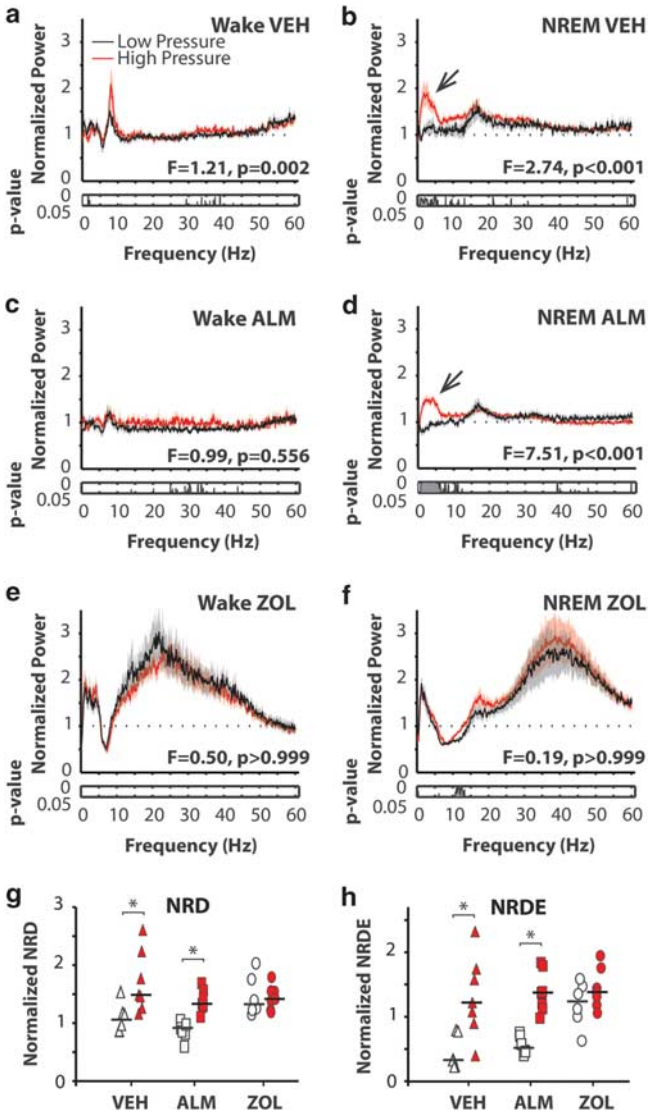

Figure 3 Spectral analyses. Wake (a, c, e) and NREM (b, d, f) EEG power spectra for the 90 min before transcardial perfusion were normalized by the respective baselines (ZT0–6). Interactions of factors 'frequency' and 'sleep pressure' (permutation ANOVA) are indicated for each panel. The degrees of freedom are 491 and 5401 for all interactions. The *p*-values for *post hoc* uncorrected bin-by-bin *t*-tests are indicated below the spectra. Following VEH and ALM dosing, increased sleep pressure coincided with increased NREM delta power (arrows) whereas, after ZOL dosing, NREM delta power was increased irrespective of sleep pressure. (g) Average NREM delta power (NRD) during the 90 min before transcardial perfusion. (h) NREM delta energy (NRDE) during 90 min before transcardial perfusion. Both NRD and NRDE distinguished between the high and low sleep pressure groups following VEH and ALM but not following ZOL dosing. Horizontal lines indicate group medians. $^*p < 0.05$, *U*-test.

Fos Expression in nNOS Neurons Depends on Prior Sleep/Wake History and Resultant Sleep Pressure

To determine whether the percentage of Fos^+ cortical nNOS neurons depends on prior sleep/wake history or only on NREM time during the 90 min before transcardial perfusion, we performed double immunohistochemistry for Fos and nNOS. As depicted in Figure 5a–e, sleep-deprived rats showed higher levels of %Fos/nNOS than undisturbed rats irrespective of drug treatment. Consequently, %Fos/nNOS significantly distinguished between the high *vs* low sleep pressure conditions following VEH ($p = 0.001$), ZOL ($p = 0.001$) and ALM ($p = 0.001$; Figure 5e). Notably, the separation between conditions was absolute after each drug treatment, i.e., there were no overlapping data points.

Fos/nNOS is the Best Indicator of Sleep/Wake History and Resultant Sleep Pressure

To determine which physiological measure was most closely related to prior sleep/wake history, we quantified the effect sizes (Hedges' g) for the difference between the high *vs* low

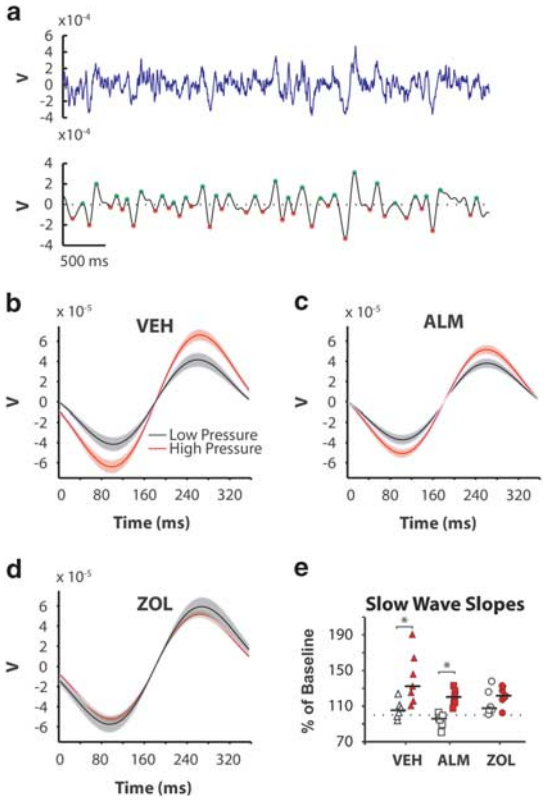

Figure 4 Slopes of NREM EEG slow waves during the 90 min before transcardial perfusion. (a) The raw EEG trace (top) was bandpass filtered in the slow wave range 0.5–4.5 Hz (bottom). Positive (green) and negative peaks (red) were identified. A straight line between a negative and a positive peak encompassing a zero crossing was defined as the slope of the respective slow wave. (b, c, d) Average slow waves ± SEM for the experimental groups. (e) Average NREM slow wave slopes. The slopes distinguished between the low and high sleep pressure groups following VEH and ALM but not following ZOL dosing. Horizontal lines indicate group medians. *$p < 0.05$, *U*-test.

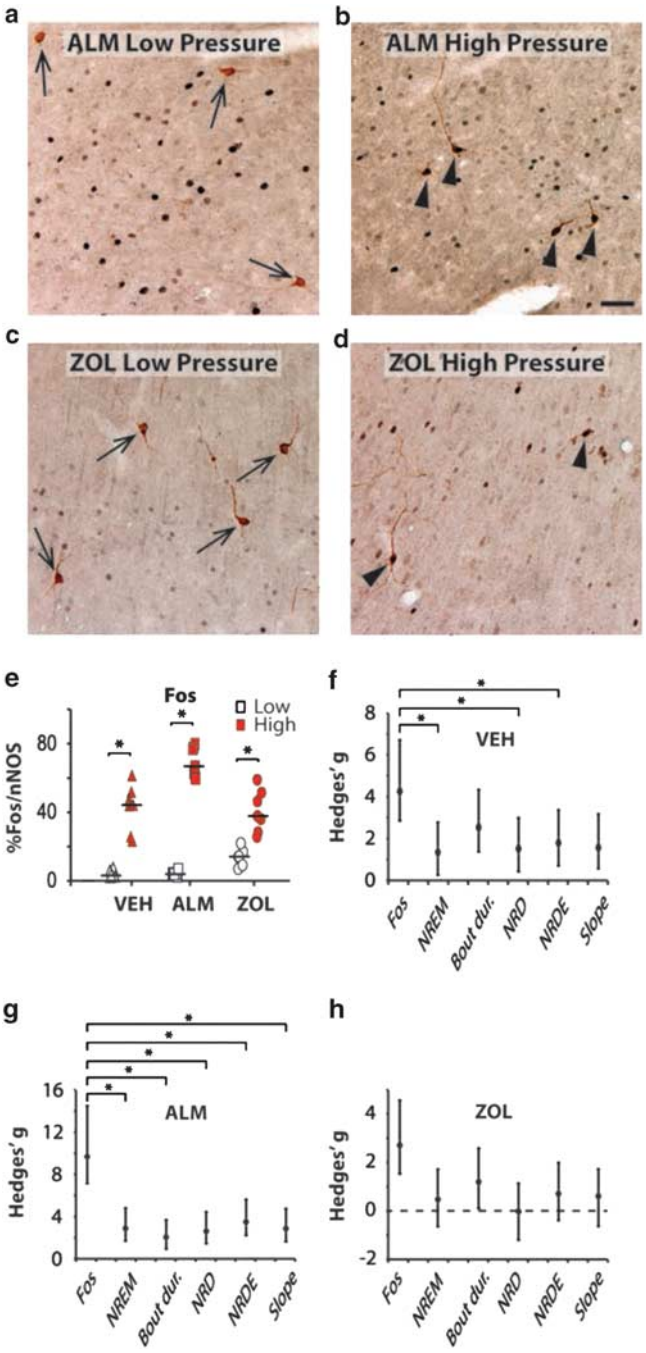

Figure 5 Fos expression in cortical nNOS neurons depends on sleep pressure. (a–d) Example micrographs of Fos/nNOS double immunohistochemistry. Following both hypnotics, nNOS neurons were single-labeled (arrows) in low sleep pressure conditions. (a, c) In the high sleep pressure conditions (b, d), many nNOS neurons were double-labeled for Fos (black triangles) irrespective of the drug treatment. Scale bar indicates 50 μm. (e) Proportion of Fos^+ cortical nNOS neurons. Note that %Fos/nNOS completely separated all high sleep pressure groups from the respective low sleep pressure groups. Horizontal lines indicate group medians. *$p < 0.05$, *U*-test. (f–h) Effect sizes for the difference between low and high sleep pressure groups using six different measures. Data for each of the six parameters listed on the abscissa were transformed to obtain normal distributions. For each variable, Hedges' g ± 95% confidence interval was calculated as an effect size for the difference between the two groups following VEH (f), ALM (g), and ZOL (h) treatment. Following each drug treatment, the strongest effect was seen for %Fos/nNOS. ***** indicates significantly ($p < 0.05$) smaller g than that for %Fos/nNOS after Holm–Sidak correction for multiple comparisons.

sleep pressure groups for each of the parameters measured in the present study (see Methods). For each drug treatment, Hedges' g was greatest for %Fos/nNOS (Figure 5f-h). Following VEH, %Fos/nNOS separated the sleep pressure groups significantly better than bout duration, NRD, or NRDE (Figure 5f) and better than all parameters following ALM (Figure 5g).

DISCUSSION

These results demonstrate that the extent of activation of cortical nNOS/NK1 neurons is determined by prior sleep history. When sleep pressure is assumed to be low, cortical nNOS neurons are largely inactive (Figure 5a–e) even in the presence of high amounts of NREM sleep as illustrated by the ZOL group in Figure 2e.

%Fos/nNOS Depends on Sleep/Wake History

Using hypnotic treatment, we were able to dissociate time spent asleep during the 90 min before killing from the prior

sleep/wake history. Following ZOL, neither time spent in wake, NREM, nor REM differed between rats that were sleep-deprived and rats that were left undisturbed for the preceding 6 h. Nonetheless, in ZOL-treated rats, the proportion of Fos^+ cortical nNOS neurons was significantly greater in the high sleep pressure than the low sleep pressure group. We conclude that cortical nNOS neurons are not activated simply by the occurrence of NREM sleep, rather, the %Fos/nNOS depends on the magnitude of sleep pressure that has accumulated during the time preceding sleep onset. Together with our previous studies (Gerashchenko *et al*, 2008; Morairty *et al*, 2013), these results demonstrate that cortical nNOS neurons are responsive to homeostatic sleep drive.

We found that the proportion of Fos^+ cortical nNOS neurons was a better indicator of prior sleep/wake history than total time spent in NREM, average NREM bout duration, NREM delta power, or the average slope of NREM slow waves (Figure 5f–h). Following ZOL, %Fos/nNOS was the only measure that significantly distinguished between the low and high sleep pressure groups. This observation makes it unlikely that Fos expression in cortical nNOS neurons is downstream of any of these variables (e.g., driven by slow wave activity), although experimental confirmation will depend on the ability to selectively manipulate cortical nNOS/NK1 neurons.

In agreement with our previous findings (Morairty *et al*, 2014), the hypnotic efficacy of ALM and ZOL was comparable at the doses used in the present study. Nonetheless, the same dose of ALM produced different amounts of sleep in the high sleep pressure and low sleep pressure groups. This result is consistent with the view that ALM removes a wake-inducing input—Hcrt tone—whereas ZOL actively inhibits neuronal activation. Nonspecific neuronal inhibition might bias the system towards sleep, whereas Hcrt antagonism might just impair the ability to stay awake in the presence of endogenous sleep pressure. A more detailed comparison will be needed to test if this is indeed a qualitative difference between the drugs or an effect of non-equivalent doses. Nonetheless, despite increased NREM sleep, ALM did not increase levels of Fos in cortical nNOS neurons in the low sleep pressure condition, which is consistent with the results obtained from the ZOL experiment.

nNOS/NK1 Neurons and NREM Delta Power

The finding that, following ZOL, NRD did not differ between sleep-deprived and undisturbed rats despite the pronounced difference in %Fos/nNOS between groups was surprising, as we have previously found that activation of these neurons may facilitate NRD (Morairty *et al*, 2013). Therefore, we had expected that increased Fos expression in cortical nNOS neurons would coincide with increased NRD in conditions when total NREM time did not differ. Given the pronounced effects of ZOL on the EEG, it is conceivable that ZOL masked the effects of cortical nNOS neuron activation on the NREM EEG. The low frequencies of the NREM power spectra were conspicuously increased following ZOL irrespective of sleep pressure (Figure 3d). As cortical nNOS neurons are GABAergic (Kubota *et al*, 2011), the $GABA_A$ modulator ZOL might act directly on the downstream targets of these neurons. This interpretation is in agreement with the finding that the sleep pressure-dependent modulation of the NREM EEG was not impaired by the Hcrt antagonist ALM, as sleep-deprived rats showed significantly elevated NRD. Based on Hedges' g, the difference in NRD between high and low sleep pressure conditions was not smaller following ALM than VEH treatment (Figure 5).

Although the downstream targets of sleep-active cortical nNOS neurons have not yet been identified, these neurons are present in all cortical areas (Vincent and Kimura, 1992), form long-range cortico-cortical projections (Tomioka *et al*, 2005), and appear to be the origin of a dense nNOS-positive fiber network (Vincent and Kimura, 1992; Yousef *et al*, 2004) that is suited for producing a near simultaneous NO signal throughout a large cortical volume (Philippides *et al*, 2005). Thus, it seems likely that cortical nNOS neurons may exert a widespread effect on the cortex during sleep. This inference is supported by our recent finding that nNOS KO mice show deficits in the regulation of delta power and consolidation of NREM sleep (Morairty *et al*, 2013). However, direct and specific experimental manipulation of these neurons will be necessary to determine the specific effects on cortical activity patterns.

Regulation of nNOS/NK1 Neurons

Although NREM sleep appears to be a permissive state for the activation of cortical nNOS neurons, we demonstrated here that the magnitude of activation of these cells during NREM depends on prior sleep/wake history. The mechanism by which prior wake time is linked to the activation of cortical nNOS neurons is of great interest, as it could provide insight into how the accumulation of sleep pressure is tracked by the brain. A better understanding of this mechanism could prove relevant for facilitating restorative sleep or combating pathological sleepiness. The integration of time spent awake might occur at the level of the nNOS/NK1 neurons themselves. Locally accumulating sleep factors, such as adenosine and cytokines, might activate these neurons (Kilduff *et al*, 2011). Another such factor could be SP. mRNA levels of the gene coding for SP are increased in the cortex by SD (Martinowich *et al*, 2011). Cortical nNOS neurons co-express the SP receptor NK1 and are strongly and directly activated *in vitro* by SP (Dittrich *et al*, 2012).

Fos expression in cortical nNOS/NK1 neurons remains minimal as long as rats are kept awake, irrespective of accrued sleep pressure (Morairty *et al*, 2013). Therefore, if integration of sleep pressure indeed occurs at the level of cortical nNOS neurons, a wake-related inhibitory input must be assumed that prevents activation of these cells before sleep onset. This view is congruent with the model we have presented previously (Kilduff *et al*, 2011).

Alternatively, the integration of sleep pressure might occur upstream of cortical nNOS neurons. In this scenario, cortical nNOS neurons would receive activating input only during NREM, the magnitude of which depending on the sleep/wake history. In order to identify the mechanisms by which sleep pressure is linked to activation of cortical nNOS neurons, it will be critical to characterize the anatomical and neurochemical inputs to these cells.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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The Dual Hypocretin Receptor Antagonist Almorexant is Permissive for Activation of Wake-Promoting Systems

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The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) may promote sleep through selective disfacilitation of wake-promoting systems, whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through general inhibition of neural activity. Previous studies have indicated that HcrtR antagonists cause less-functional impairment than BzRAs. To gain insight into the mechanisms underlying these differential profiles, we compared the effects of ALM and ZOL on functional activation of wake-promoting systems at doses equipotent for sleep induction. Sprague-Dawley rats, implanted for EEG/EMG recording, were orally administered vehicle (VEH), 100 mg/kg ALM, or 100 mg/kg ZOL during their active phase and either left undisturbed or kept awake for 90 min after which their brains were collected. ZOL-treated rats required more stimulation to maintain wakefulness than VEH- or ALM-treated rats. We measured Fos co-expression with markers for wake-promoting cell groups in the lateral hypothalamus (Hcrt), tuberomammillary nuclei (histamine; HA), basal forebrain (acetylcholine; ACh), dorsal raphe (serotonin; 5HT), and singly labeled Fos^+ cells in the locus coeruleus (LC). Following SD, Fos co-expression in Hcrt, HA, and ACh neurons (but not in 5HT neurons) was consistently elevated in VEH- and ALM-treated rats, whereas Fos expression in these neuronal groups was unaffected by SD in ZOL-treated rats. Surprisingly, Fos expression in the LC was elevated in ZOL- but not in VEH- or ALM-treated SD animals. These results indicate that Hcrt signaling is unnecessary for the activation of Hcrt, HA, or ACh wake-active neurons, which may underlie the milder cognitive impairment produced by HcrtR antagonists compared to ZOL.

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INTRODUCTION

Hypocretin (Hcrt, also known as orexin) neurons located in the tuberal hypothalamus (de Lecea *et al*, 1998; Sakurai *et al*, 1998) are wake-promoting neurons that project widely throughout the brain to subcortical wake-promoting regions including the tuberomammillary nuclei (TMN), basal forebrain (BF), dorsal raphe (DR), and locus coeruleus (LC) (Peyron *et al*, 1998). Hcrt neurons activate these brain regions (Carter *et al*, 2012; Eggermann *et al*, 2001; Eriksson *et al*, 2001; Liu *et al*, 2002; Schone *et al*, 2012) through the co-release of Hcrt, glutamate (GLU) (Schone *et al*, 2012), and dynorphin (Eriksson *et al*, 2004; Li *et al*, 2014; Muschamp *et al*, 2014). Hcrt neurons are active primarily during wakefulness (Lee *et al*, 2005), and extracellular Hcrt levels are highest during awakening and periods of heightened emotionality (Blouin *et al*, 2013), consistent with a role in the regulation of arousal. Degeneration of Hcrt neurons underlies the sleep disorder narcolepsy, underscoring the importance of this system for the regulation of sleep and wakefulness (Hara *et al*, 2001; Thannickal *et al*, 2000). Hcrt neurons receive afferents from several brain regions involved in homeostatic processes (Sakurai *et al*, 2005; Yoshida *et al*, 2006), indicating that these cells may integrate arousal with other physiological functions.

Insomnia affects between 10 and 30% of the population (Mai and Buysse, 2008; Roth, 2007) and can cause degradation in cognitive performance (Lamond *et al*, 2007). Benzodiazepine receptor agonist (BzRA) hypnotics such as zolpidem (ZOL) that are currently used to treat insomnia modulate the $GABA_A$ receptor and induce sleep through a general inhibition of neural activity. Although BzRAs are effective for the induction of sleep, they can have detrimental effects on cognitive performance (Huang *et al*, 2010; Uslaner *et al*, 2013; Wesensten *et al*, 1996), resulting in the need for hypnotics with an improved cognitive profile. Because of the involvement of the Hcrt system in sleep and arousal, Hcrt receptor (HcrtR) antagonists have been extensively investigated for the treatment of insomnia (Brisbare-Roch *et al*, 2007; Dugovic *et al*, 2009; Morairty *et al*, 2014; Roecker and Coleman, 2008; Uslaner *et al*, 2013; Winrow *et al*, 2011) and are thought to promote sleep through selective disfacilitation of wake-promoting systems.

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Because HcrtR antagonists may act via disfacilitation rather than generalized inhibition, they are predicted to cause less functional impairment than BzRAs (Morairty *et al*, 2014; Steiner *et al*, 2011), a hypothesis that has been supported by recent behavioral studies (Morairty *et al*, 2014; Tannenbaum *et al*, 2014; Uslaner *et al*, 2013).

Although HcrtR antagonists demonstrate a favorable neurocognitive profile compared with BzRAs, the neural mechanisms underlying the differential functional impairment of these drugs is not well understood. To gain insight into this phenomenon, we compared the effects of ALM and ZOL on the functional activation of the currently known wake-promoting systems to which Hcrt neurons project. We hypothesized that HcrtR blockade with ALM would inhibit activation of wake-promoting neurons to a lesser extent than ZOL treatment at equivalent sleep-promoting doses. As a test of this hypothesis, we compared Fos expression in five wake-promoting neuronal groups in the presence of these two drugs both in undisturbed conditions and during prolonged wakefulness.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines. Male Sprague-Dawley rats ($n = 46$; Harlan Laboratories) were housed under constant temperature (22 ± 2 °C, 50 ± 25% relative humidity) on a 12 h light–dark cycle with food and water *ad libitum*. Rats were distributed among the six experimental groups described below. EEG/EMG recordings from eight rats were not included in the final sleep data owing to poor signal quality but were included in the Fos analysis.

Surgical Procedures

To monitor EEG/EMG activity, rats were implanted with sterile telemetry transmitters (F40-EET; Data Sciences, St Paul, MN) as described previously (Morairty *et al*, 2008, 2013) and detailed in Supplementary Materials.

Drugs

Almorexant (ALM; (2*R*)-2-[(1*S*)-6,7-Dimethoxy-1-[2-(4-trifluoromethyl-phenyl)-ethyl]-3,4-dihydro-1H-isoquinolin-2-yl]-*N*-methyl-2-phenyl-acetamide) was synthesized at SRI International (Menlo Park, CA) according to the literature (Koberstein *et al*, 2003, 2005). ZOL was supplied by Actelion Pharmaceuticals (South San Francisco, CA) or purchased from IS Chemical Company (Shanghai, China).

Experimental Protocol

Rats were orally dosed with vehicle (VEH) (1.25% hydroxypropyl methyl cellulose, 0.1% dioctyl sodium sulfosuccinate, and 0.25% methylcellulose in water), ALM (100 mg/kg), or ZOL (100 mg/kg) at zeitgeber time 18 h (ZT18, Figure 1a), where ZT0 = lights on and ZT12 = lights off. Doses were chosen on the basis of their similar sleep-promoting efficacy, taking into account that ZOL is approximately threefold more potent when administered intraperitoneally compared with oral administration (Vanover *et al*, 1999), whereas ALM is equipotent through both routes of administration (Morairty *et al*, 2014). Following dosing, rats were either left undisturbed or kept awake (ie, sleep deprived; SD) for 90 min, after which rats were euthanized, perfused, and their brains collected for analysis. Thus, there were a total of six experimental groups: three drug treatments (VEH, ALM, and ZOL) under both undisturbed and SD conditions.

Sleep Deprivation Procedures

Subsets of rats were kept awake from ZT18–19.5 using procedures similar to those previously published (Dittrich *et al*, 2015; Morairty *et al*, 2014). Rats were continuously observed under dim red light during concurrent EEG/EMG recording and their cages were tapped when they were inactive and appeared to be entering a sleep state. As rats became more difficult to keep awake, progressively stronger stimulations were employed such as more forceful cage tapping, introducing novel objects into the cage, and stroking fur or vibrissae with an artist's brush.

Identification of Sleep/Wake States

At least 3 weeks post surgery, EEG and EMG were recorded as previously described (Morairty *et al*, 2014) using DQ ART 4.1 software (Data Sciences). After completion of data collection, expert scorers blinded to drug treatment and sleep/wake conditions determined states of sleep and wakefulness in 10 s epochs using Neuroscore software (Data Sciences). Epochs were assigned to waking (W), rapid eye movement (REM) sleep, or non-rapid eye movement (NREM) sleep on the basis of EEG and EMG as described previously (Dittrich *et al*, 2015; Morairty *et al*, 2014). NREM latency was defined as the time to the first three consecutive 10 s epochs of NREM sleep.

Perfusion, Fixation, and Brain Sectioning

Rats were deeply anesthetized with an overdose of euthanasia solution (150 mg/kg SomnaSol, Butler-Schein, Dublin, OH) and transcardially perfused with heparinized phosphate-buffered saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were incubated overnight in PFA before immersion in 30% sucrose. Sections were cut at 40 μm and stored in a cryoprotectant solution at −20 °C.

Immunohistochemistry

Sections were first incubated with rabbit anti-Fos antibody and developed to create a black nuclear reaction product. The same sections were then incubated with either goat anti-choline acetyltransferase (ChAT) to detect cholinergic (ACh) neurons, goat anti-orexin-B for Hcrt neurons, rabbit anti-adenosine deaminase for histaminergic (HA) neurons, or rabbit anti-serotonin (5HT) for serotonergic neurons, and then developed to form a brown (ChAT, ADA, 5HT) or purple-red (Hcrt) cytoplasmic reaction product. Detailed

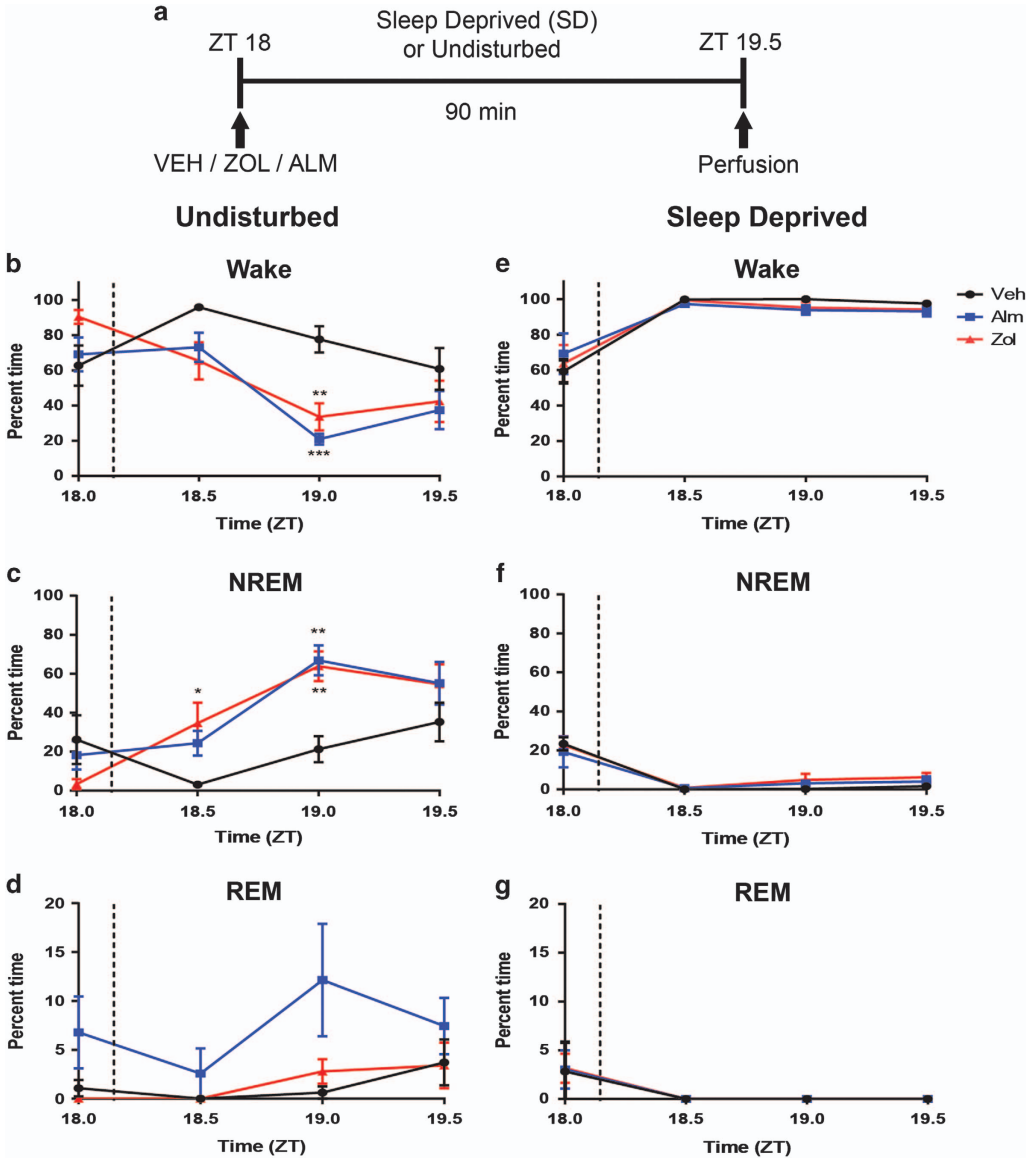

Figure 1 Experimental paradigm and sleep/wake data. (a) At ZT18, rats were administered VEH, ALM, or ZOL and either sleep deprived or left undisturbed for 90 min. (b–d) Time spent (b) awake, (c) in NREM sleep, and (d) REM sleep following dosing in undisturbed rats. Time periods represent 30 min bins ending at the indicated time; the dashed lines show the approximate dosing time. Time spent (e) awake, (f) in NREM sleep, and (g) REM sleep in rats kept awake following dosing. $N = 5–7$ animals/group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with VEH treatment.

immunohistochemistry procedures and antibody information are described in Supplementary Materials.

Cell Counts

An experimenter blinded to the drug treatments and sleep/wake conditions counted single- and double-immunoreactive cells on a Leica DM 5000B microscope (Leica Microsystems, Buffalo Grove, IL) with a Microfire S99808 camera (Optronics, Goleta, CA) using StereoInvestigator software (MBF Biosciences, Williston, VT). Counting areas in each brain region were defined in a manner similar to those previously described (Deurveilher *et al*, 2006, 2013) with minor modifications as outlined in Supplementary Materials. A rat brain atlas (Paxinos and Watson, 2005) was used to define all brain regions.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (San Diego, CA). Differences in the proportion of time spent in Wake, NREM, and REM were analyzed in 30 min time bins by two-way ANOVA on factors 'drug treatment' (between subjects) and 'time' (within subjects) followed by Tukey's multiple comparison test where appropriate. Sleep latency was analyzed by one-way ANOVA. Differences in Fos co-labeling were analyzed by two-way ANOVA on factors 'drug treatment' (between subjects) and 'sleep/wake condition' (SD or undisturbed, between subjects) followed by Tukey's multiple comparison test if two-way ANOVA indicated an interaction between the factors. Statistical significance was established as $p < 0.05$.

RESULTS

Pharmacologically-Induced Sleep

The doses of ALM and ZOL used in the present study (100 mg/kg, p.o) were chosen because they have roughly equivalent sleep induction efficacy in rats and, for ALM, result in high occupancy of both Hcrt receptors (Morairty *et al*, 2012, 2014). Both ALM and ZOL significantly decreased the latency to first NREM sleep bout relative to VEH (18.6 ± 2.6 min for ALM, 16.1 ± 3.9 min for ZOL *vs* 36.7 ± 7.6 min for VEH; $p < 0.05$ for both comparisons with VEH) in rats left undisturbed after dosing. Significant interactions between time and drug treatment were found in undisturbed animals for wake ($F(6,42) = 4.34$; $p = 0.002$) and NREM ($F(6,42) = 3.86$; $p = 0.004$) time during the 90 min period post dosing. Both ALM- and ZOL-treated rats exhibited less time awake and more time in NREM than VEH-treated rats; no differences were found between ALM- and ZOL-treated rats (Figure 1b and c). For REM sleep, a main effect was found for drug treatment ($F(2,14) = 6.074$; $p = 0.01$) that was largely due to ALM treatment (Figure 1d).

SD was highly effective for all groups, as rats were awake $>95\%$ of the time during SD regardless of drug treatment (Figure 1e). No significant differences were found in wake, NREM (Figure 1f), or REM (Figure 1g) time during SD for either ALM or ZOL compared with VEH. However, frequent and vigorous manipulations during SD were required to maintain wakefulness in ZOL-treated rats, whereas ALM-treated rats required only mild interventions to maintain wakefulness.

Fos Immunoreactivity in Hypocretin Neurons

Figure 2a–d and Supplementary Figure S1 present representative sections from the LH in which the sections were stained for both Fos and Hcrt. Across the entire Hcrt field, there was an interaction between drug treatment and sleep/wake condition ($F(2,40) = 4.945$; $p = 0.012$). Fos expression in Hcrt neurons was elevated during SD compared with undisturbed controls under both VEH ($p = 0.007$) and ALM ($p < 0.0001$) but not ZOL treatment. Furthermore, ALM-treated SD rats co-expressed significantly greater levels of Fos than did ZOL-treated SD rats ($p = 0.002$; Figure 2e), with VEH-treated rats trending similarly.

Given the differences in Fos expression in the entire Hcrt neuron population, we sought to determine whether regional heterogeneity occurred and counted Hcrt cells within the medial, perifornical, and lateral hypothalamus as described above. In the medial portion of the Hcrt field (Figure 2f), a main effect for sleep/wake condition ($F(1,40) = 17.76$; $p < 0.0001$) but not drug treatment was found.

In the perifornical area (Figure 2g), there was a significant interaction between drug treatment and sleep/wake condition ($F(2,40) = 6.137$; $p = 0.0047$). SD significantly elevated Fos expression in Hcrt neurons under VEH ($p = 0.014$) and ALM ($p = 0.0001$) but not ZOL treatments. Similarly, both VEH- ($p = 0.035$) and ALM-treated ($p = 0.0002$) SD rats exhibited significantly greater levels of Fos co-expression than did ZOL-treated SD rats.

In the LH (Figure 2h), we identified a significant interaction between drug treatment and sleep/wake condition ($F(2,40) = 12.11$; $p < 0.0001$). Fos co-expression was elevated by SD relative to undisturbed conditions only in ALM-treated rats ($p < 0.0001$); VEH-treated rats exhibited a similar trend. ALM treatment resulted in greater Fos expression compared with both VEH- ($p = 0.02$) and ZOL-treated ($p < 0.0001$) SD animals.

These results indicate that VEH and ALM are permissive for forced wakefulness-induced activation of Hcrt neurons, whereas this phenomenon is suppressed by ZOL. These effects were most robust in the perifornical area and attenuated in the medial Hcrt field.

Fos Immunoreactivity in HA Neurons

Figure 3a–d and Supplementary Figure S2 present representative sections from the TMN that were stained for Fos and ADA. A significant interaction between drug treatment and sleep/wake condition ($F(2,40) = 3.57$; $p < 0.038$) was found in a combined analysis of the HA field. Fos expression in HA neurons was increased during SD relative to undisturbed conditions for both VEH- ($p = 0.02$) and ALM- ($p < 0.0004$) but not ZOL-treated rats (Figure 3e). Both VEH- ($p = 0.0004$) and ALM-treated ($p = 0.0011$) SD rats exhibited significantly elevated Fos co-expression compared with ZOL-treated SD animals.

On the basis of the overall differences in Fos expression in HA neurons, we examined the three major subregions of the TMN: the dTMN, vTMN, and cTMN. For the dTMN, there was a significant interaction between drug treatment and sleep/wake condition ($F(2,40) = 5.53$; $p = 0.008$). VEH- ($p = 0.03$) and ALM- ($p < 0.0001$) but not ZOL-treated rats exhibited enhanced Fos co-expression during SD compared with undisturbed conditions. Similarly, Fos expression was significantly higher in SD rats treated with VEH ($p = 0.0004$) or ALM ($p = 0.0002$) compared with ZOL (Figure 3f).

Less-pronounced effects were observed in the vTMN (Figure 3g), as main effects for drug treatment ($F(2,40) = 8.74$; $p = 0.0007$) and sleep/wake condition ($F(1,40) = 17.16$; $p = 0.0002$) were found, but there was no significant interaction between the factors.

Clear differences were observed for the cTMN (Figure 3h), as a significant interaction was found between drug treatment and sleep/wake condition ($F(2,40) = 6.66$; $p = 0.003$). VEH- ($p = 0.001$) and ALM- ($p < 0.0001$) but not ZOL-treated rats exhibited increased Fos levels during SD compared with undisturbed conditions; both VEH- ($p < 0.0001$) and ALM-treated ($p < 0.0001$) SD rats exhibited greater Fos co-expression than ZOL-treated SD rats.

These results indicate that, as in Hcrt neurons, VEH and ALM permit SD-induced activation of HA neurons, whereas this effect was suppressed by ZOL. This phenomenon was attenuated in the vTMN.

Fos Immunoreactivity in Cholinergic Neurons

Representative sections from ACh neurons in the BF are shown in Figure 4a–d and Supplementary Figure S3. Consistent with previous reports on the effects of forced wakefulness in the BF (McKenna *et al*, 2009), relatively modest absolute levels of Fos/ChAT co-expression were

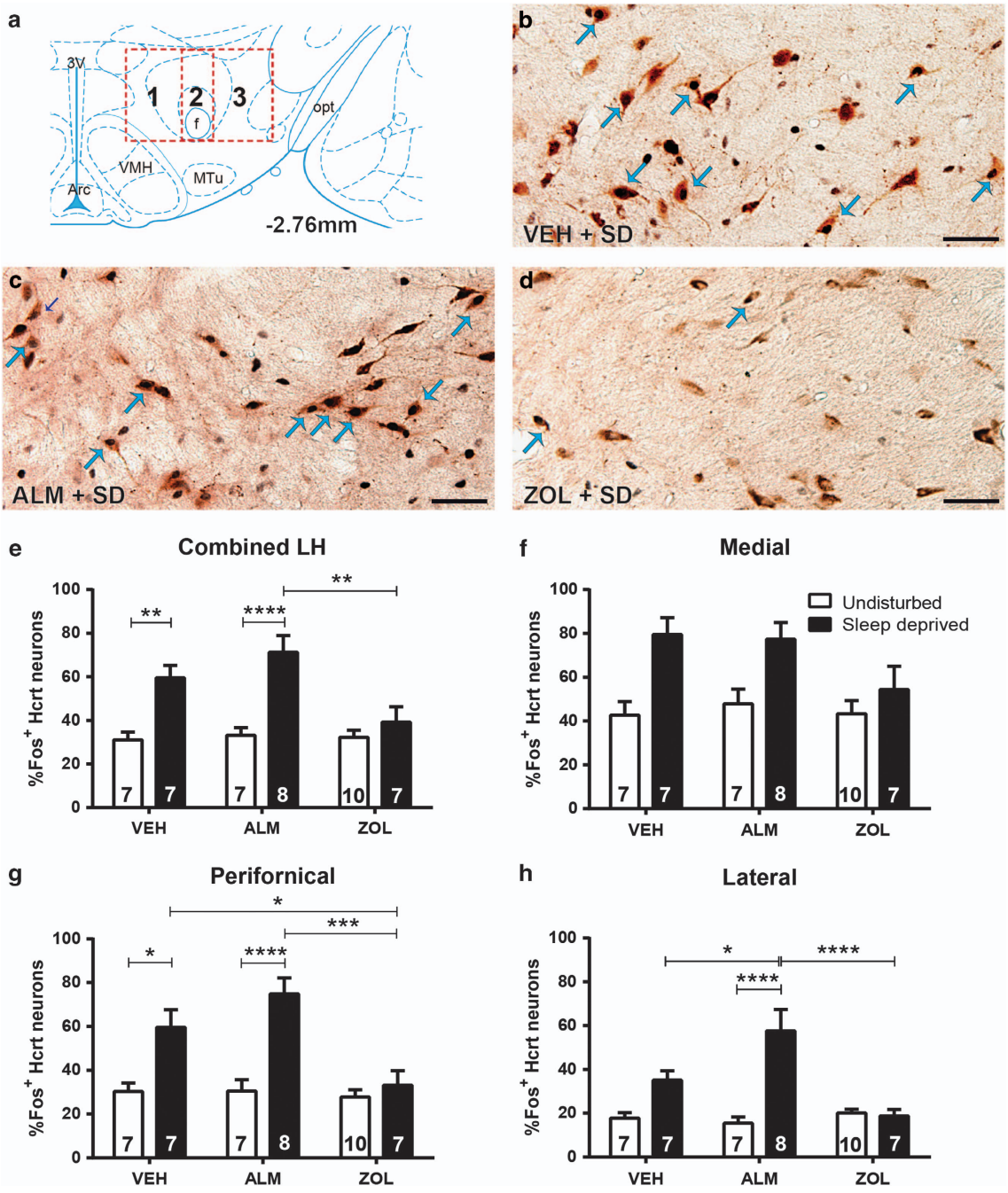

Figure 2 Fos labeling of Hcrt neurons in the LH. (a) Boxes delineate the regions scored within the Hcrt field; 1, 2, and 3 in the boxes, respectively, correspond to the medial, perifornical, and lateral hypothalamic regions. (b–d) Fos-positive nuclei are small black dots and Hcrt neurons have reddish-brown cytoplasmic reaction products. Blue arrows indicate representative co-labeled neurons. Scale bar = 50 μM. Representative image of Hcrt and Fos expression in a sleep-deprived rat treated with (b) VEH, (c) ALM, or (d) ZOL. (e) Combined analysis of all Hcrt neurons examined throughout the Hcrt field, (f) in the medial field, (g) perifornical area, and (h) lateral portions of the LH. Numbers in bars indicate *N* per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared with the indicated treatment group.

found compared with levels in the LH and TMN. However, drug treatment and sleep/wake condition had significant effects on Fos co-expression across the BF, as a significant interaction ($F(2,40)=6.747$; $p=0.003$) between these factors was found. SD increased Fos co-expression compared with undisturbed conditions for both VEH ($p=0.044$) and ALM ($p=0.0012$) but not ZOL-treated animals. Both VEH ($p=0.0024$) and ALM ($p<0.0001$) led to significantly higher Fos co-expression compared with ZOL treatment in SD animals (Figure 4e).

We next investigated Fos co-expression in subregions of the BF to understand the regional specificity of this effect. In the VDB (Figure 4f), sleep/wake condition was the only factor to exhibit a main effect ($F(1,40)=22.19$; $p=0.0001$),

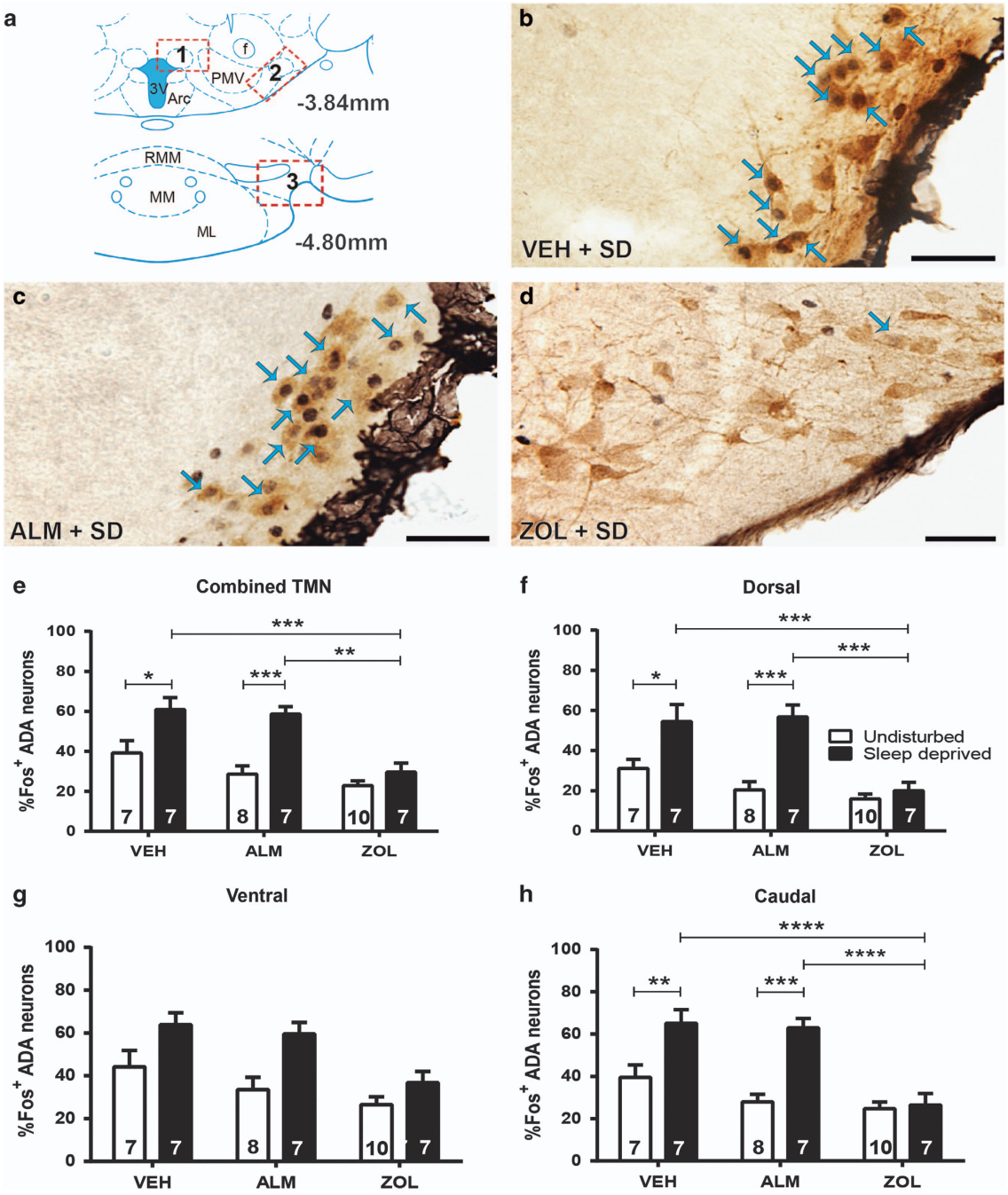

Figure 3 Fos labeling of HA neurons in the TMN. (a) Boxes delineate the TMN regions scored; 1, 2, and 3 correspond to the dTMN, vTMN, and cTMN, respectively. (b–d) Fos-positive nuclei are small black dots and HA-expressing neurons have brown cytoplasmic reaction products. Blue arrows indicate examples of co-labeled neurons. Scale bar represents 50 μM. Representative image of HA neurons and Fos in a sleep-deprived rat treated with (b) VEH, (c) ALM, or (d) ZOL. (e) Combined analysis of all scored HA neurons throughout the HA field, (f) in the dTMN, (g) vTMN, and (h) cTMN. Numbers in bars indicate *N* per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared with the indicated treatment group.

indicating that the overall effect is less prominent in this subregion.

The effects of drug treatment and sleep/wake condition were more profound in the HDB where a clear interaction between the two factors ($F(2,40)=7.566$; $p=0.002$) was found. SD significantly elevated Fos/ChAT co-expression compared with undisturbed condition for ALM-treated rats ($p<0.0001$). ALM-treated SD rats exhibited greater Fos co-expression than both VEH- ($p=0.04$) and ZOL-treated ($p<0.0001$) SD rats (Figure 4g).

A significant interaction between drug treatment and sleep/wake condition ($F(2,40)=5.03$; $p=0.01$) occurred in the MCPO (Figure 4e). SD increased Fos expression compared to undisturbed conditions in ALM-treated rats

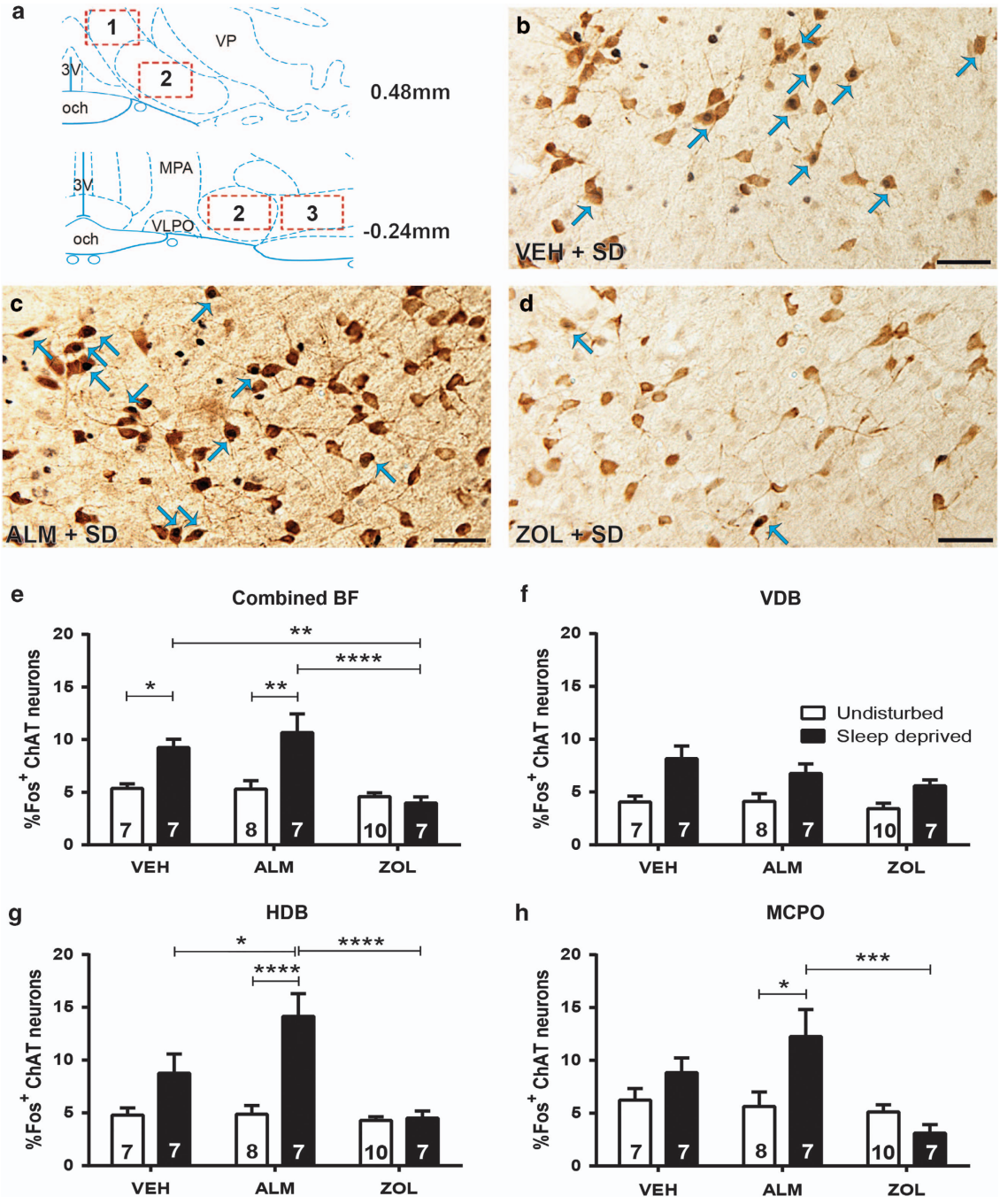

Figure 4 Fos co-labeling with ACh neurons in the BF. (a) Boxes delineate the BF regions that were scored; 1, 2, and 3 correspond to the VDB, HDB, and MCPO, respectively. The HDB was counted at multiple rostral-caudal levels. (b–d): Fos-positive nuclei are small black dots and ACh-expressing neurons have brown cytoplasmic reaction products. Blue arrows indicate examples of co-labeled neurons. Scale bar represents 50 μM. Representative image of HA and Fos expression in sleep-deprived rat treated with (b) VEH, (c) ALM, or (d) ZOL. (e) Combined analysis of all ACh neurons examined for co-labeling throughout the ACh field, (f) in the VDB, (g) HDB, and (h) MCPO. Numbers in bars indicate *N* per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared with the indicated treatment group.

($p=0.02$), which exhibited significantly greater Fos expression than ZOL-treated SD rats ($p=0.0008$).

Taken together, these results indicate that ALM is permissive for forced wakefulness-induced activation of BF cholinergic neurons, whereas ZOL inhibits their activation. This effect was evident in the HDB and, to a lesser extent, the MCPO.

Fos Immunoreactivity in Serotonergic Neurons

5HT-expressing neurons were analyzed for Fos co-labeling following VEH, ALM, or ZOL treatment under SD and undisturbed conditions (Figure 5a, c–e and i and Supplementary Figure S4A–C). No significant effects of drug treatment or sleep/wake condition on Fos co-expression were found.

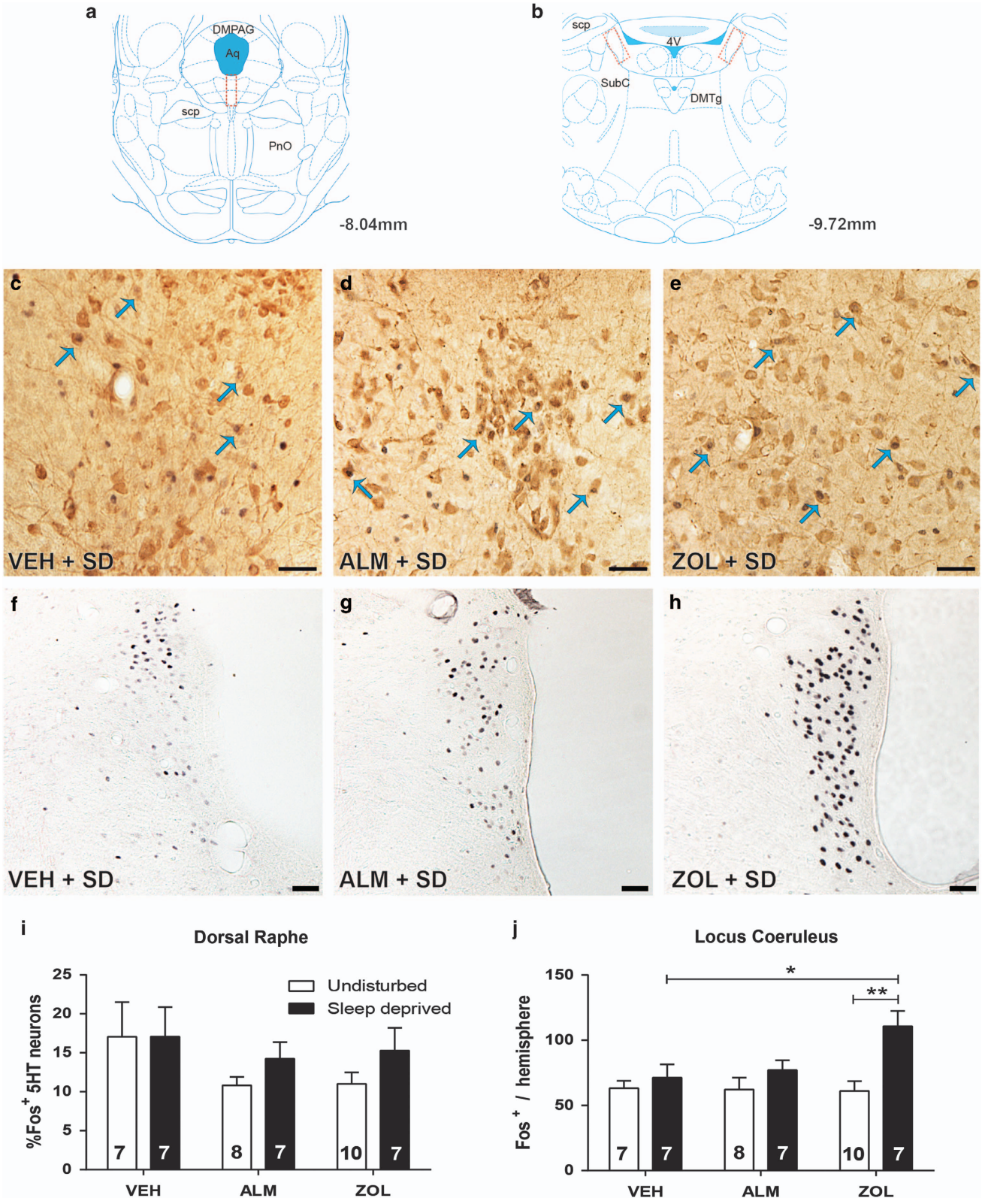

Figure 5 Fos co-labeling with 5HT neurons in the DR and single-labeled Fos cells in the LC. (a–b) Boxes delineate the regions scored in the (a) DR and (b) LC. In (c–h) Fos-positive nuclei are small black dots and 5HT-expressing neurons have brown cytoplasmic reaction products. Blue arrows indicate examples of co-labeled neurons. Scale bar represents 50 μM. Representative image of 5HT and Fos expression in a sleep-deprived rat treated with (c) VEH, (d) ALM, or (e) ZOL. (f) Fos expression in the LC of a sleep-deprived rat treated with VEH, (g) ALM, or (h) ZOL. (i) Combined analysis of all scored 5HT neurons in the DR. (j) Analysis of Fos labeling in the LC for each treatment condition. Numbers in bars indicate *N* per group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the indicated treatment group.

Fos Immunoreactivity in LC Neurons

Analysis of single-labeled Fos expression in the LC (Figure 5b, f–h and j, and Supplementary Figure S4D–F) identified a significant interaction between sleep/wake condition and drug treatment ($F(2,40) = 3.27$; $p = 0.049$). In contrast to other brain regions, SD increased Fos expression in ZOL-treated ($p = 0.0022$) but not in VEH- or ALM-treated rats.

DISCUSSION

These results establish that ALM is permissive for the activation of Hcrt neurons in the LH, HA neurons in the TMN, and ACh neurons in the BF during periods of forced vigilance, whereas an equipotent dose of ZOL inhibits activation of these cell groups. These observations are consistent with different downstream sleep-promoting mechanisms for the two drugs, with ZOL promoting sleep through pan-neuronal inhibition and ALM through disfacilitation mediated by HcrtR blockade. We conclude that ALM does not inhibit the recruitment of wake-promoting Hcrt, HA, or ACh neurons during periods of increased vigilance. These results also indicate that HcrtRs are not essential for short-term activation of Hcrt, HA, or ACh neuronal populations. Surprisingly, our results also demonstrate that ZOL-treated animals exhibit elevated Fos expression in the LC following SD.

All-known wake-promoting cell groups express HcrtRs and are innervated by Hcrt neurons (Bayer *et al*, 2001; Eggermann *et al*, 2001; Eriksson *et al*, 2001; Li *et al*, 2002; Marcus *et al*, 2001; Peyron *et al*, 1998; Yamanaka *et al*, 2002). If ALM were to promote sleep through inhibition of downstream targets, it would be expected to interfere with the activation of wake-promoting cell groups. The fact that three major wake-active cell groups in the LH, TMN, and BF all demonstrated elevated Fos expression during periods of forced vigilance in the presence of ALM indicates that ALM and, by extension, at least partial HcrtR blockade is permissive for the functional activation of these groups. As many of these groups exhibit subregion-specific connectivity patterns (Ericson *et al*, 1987; Harris and Aston-Jones, 2006; Jones, 2003; Lee *et al*, 2008; Yoshida *et al*, 2006), we examined Fos expression in the major subregions of the LH, TMN, and BF. We found some degree of variation in Fos expression patterns between the subregions, suggesting this may influence response to HcrtR antagonists. Hcrt neurons in the perifornical area regulate stress and arousal responses (Harris and Aston-Jones, 2006), which may partially explain their greater sensitivity to SD in VEH-treated rats compared with the lateral Hcrt field. Variation in Fos co-expression patterns were found between the BF subregions, but similar patterns were found between the HDB and MCPO, which exhibit differential projection patterns (Jones, 2003), suggesting that most outputs of the BF may be affected similarly by ALM. The projections of HA subregions are not topographically organized (Schwartz *et al*, 1991), so the significance of their subregional heterogeneity is unclear.

Our results from the DR and LC are inconclusive in that forced vigilance did not elevate Fos expression under most conditions nor was there any indication that ALM or ZOL inhibited their activity, ie, reduced Fos expression relative to VEH. Although it was unexpected that the LC and DR did not increase Fos expression in response to SD in VEH-treated animals, the mixed downstream effects of NE and generally inhibitory effects of 5HT on other wake-promoting cell groups (Brown *et al*, 2012; Li *et al*, 2002; Li and van den Pol, 2005) contrast with the excitatory effects of Hcrt, HA, and ACh on these groups (Saper *et al*, 2001), suggesting that the LC and DR may be differentially regulated. The elevated Fos expression observed in the LC following forced wakefulness in ZOL but not VEH or ALM treatment groups is also surprising considering the inhibitory effect of ZOL in other regions, but this may be a result of the greatly increased level of stimulation required to keep ZOL-treated rats awake during forced wakefulness coupled with the high sensitivity of Fos expression in the LC to stress (Sved *et al*, 2002). However, it is also possible that ZOL facilitates activation of the LC.

These results indicate that ALM disfacilitates Hcrt-induced excitation of wake-promoting regions, but does not block activation of these cell groups by other neurotransmitters during periods of forced vigilance. Hcrt neurons also co-release GLU (Henny *et al*, 2010; Schone *et al*, 2012, 2014); thus, Hcrt neurons may release GLU in terminal fields within the LH, TMN, and BF in response to stressors requiring increased vigilance in the presence of ALM. GLU release from Hcrt neurons is sufficient to enhance firing of HA neurons in the TMN *in vitro* (Schone *et al*, 2012), suggesting that Hcrt neurons can engage fast glutamatergic regulation of downstream targets in the absence of HcrtR activation. However, it is also possible that increased Fos co-labeling is independent of Hcrt neuronal activity and is driven by other arousal systems. This combination of glutamatergic input from Hcrt neurons and excitatory transmission originating from other wake-promoting regions may explain why Hcrt, HA, and ACh neurons can be activated in the presence of ALM.

Another clue as to the mechanisms underlying how Hcrt antagonists promote sleep comes from our recent study, demonstrating that ALM increases adenosine (ADO) levels in both the BF and cortex (Vazquez-DeRose *et al*, 2014), and that some of its sleep-promoting effects are dependent on the intact functionality of BF cholinergic neurons. Interestingly, microinjection of ALM directly into the BF both promoted sleep and increased ADO concentrations in the cortex, further supporting a role for the BF as a component of the circuitry underlying ALM's sleep-promoting effects. Microinjection of ALM into other wake-promoting regions was not performed in this study, so their relative contribution to ALM's effects are unknown. However, these results are consistent with those of the current study and suggest that ALM may function both by promoting the release of a sleep-promoting neurotransmitter and by disfacilitating the wake-promoting effects of Hcrt.

Limitations in the study design should be considered when interpreting the current results. First, the rats were dosed at ZT18, a time with low natural sleep pressure when wake-promoting systems are highly active. It is possible that different results would be obtained if rats were dosed during the light phase when homeostatic and circadian influences may contribute to a higher activation threshold for these neuronal groups. Second, the SD protocol used in this study lasted only 90 min and thus was unlikely to markedly

increase sleep pressure, suggesting that novelty and handling stress also contributed to the elevated Fos co-expression observed in most wake-active regions. The intensity of SD required to maintain wakefulness was not quantified, making it difficult to determine the degree to which differences in handling stress between the treatment groups may have influenced results. Third, although our results indicate that neither ALM nor ZOL inhibit Fos co-expression in wake-promoting neurons of undisturbed animals, the poor temporal resolution of Fos must be considered as it is possible that 90 min post dosing may not allow sufficient time for baseline Fos level to be fully degraded, which could mask inhibitory drug effects.

Interestingly, we found that ALM-treated SD rats exhibited significantly greater Fos co-expression than did VEH-treated rats in the lateral Hcrt field and the HDB. The underlying cause of this is unclear, as our central hypothesis suggests that two groups should exhibit similar levels of Fos co-expression. One possible explanation is that these regions may be particularly stress-sensitive and the stimulation required to keep ALM-treated rats awake during SD provoked a stress response, resulting in greater Fos activation. However, although LH Hcrt neurons are known to be stress-sensitive (Espana *et al*, 2003), evidence for HDB stress-responsiveness is limited. Another possibility is that ALM may facilitate greater Fos co-expression in these regions through the mechanisms that are not yet understood.

ZOL and similar drugs like eszopiclone are widely prescribed and generally considered effective at inducing sleep (Greenblatt and Roth, 2012), but their use is associated with a high incidence of adverse effects such as driving impairment (Gunja, 2013; Verster *et al*, 2006), memory impairment (Balkin *et al*, 1992; Mintzer and Griffiths, 1999; Wesensten *et al*, 1995, 1996), complex sleep behaviors (Chen *et al*, 2014; Dolder and Nelson, 2008), and psychomotor deficits (Storm *et al*, 2007; Wesensten *et al*, 2005), highlighting the need for hypnotics that induce less-functional impairment. ALM and other HcrtR antagonists effectively induce sleep (Brisbare-Roch *et al*, 2007; Cox *et al*, 2010) but cause less impairment in memory tasks (Dietrich and Jenck, 2010; Morairty *et al*, 2014) or motor function in rodents (Ramirez *et al*, 2013; Steiner *et al*, 2011), dogs (Tannenbaum *et al*, 2014), and non-human primates (Uslaner *et al*, 2013) than do traditional hypnotics. Although some degree of impairment may occur at high doses in humans (Hoever *et al*, 2010, 2012; Jacobson *et al*, 2014), HcrtR antagonists are expected to exhibit a favorable safety profile compared with ZOL and other hypnotics, though years of post-market surveillance will be needed to confirm this hypothesis.

Our finding that the functional activation of Hcrt, HA, and ACh wake-promoting neurons is unaffected by ALM but inhibited by ZOL provides a possible explanation for reports describing a reduced impairment profile for Hcrt antagonists compared with ZOL. The ability of these neurons to be recruited in response to stimuli requiring alertness in the presence of ALM indicates that these arousal systems can function normally in the presence of the drug. The current results strongly suggest that ALM causes less functional impairment than ZOL at least in part because it does not impair activation of wake-promoting systems in response to salient stimuli.

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Locus Coeruleus and Tuberomammillary Nuclei Ablations Attenuate Hypocretin/orexin Antagonist-mediated REM Sleep

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Abstract

Hypocretin 1 and 2 (Hcrts, also known as orexin A and B), excitatory neuropeptides located in the lateral hypothalamus, play a central role in the control of sleep and waking. Hcrt inputs to both the locus coeruleus norepinephrine (LC NE) system and the histaminergic tuberomammillary nuclei (TMN HA) of the posterior hypothalamus are important pathways for Hcrt-induced wakefulness. The dual

Hcrt/orexin receptor antagonist almorexant (ALM) decreases wakefulness and increases NREM and REM sleep time. We hypothesized that ALM induces sleep by selectively disfacilitating subcortical wake-promoting populations; consequently, ALM should have reduced efficacy in LC-lesioned (LCx) and TMN-lesioned (TMNx) rats. We selectively ablated the LC NE neurons or the TMN HA neurons using cell-type specific saporin conjugates, and subsequently evaluated sleep following treatment with ALM and the $GABA_A$ receptor modulator zolpidem (ZOL). Both LCx and TMNx selectively attenuated promotion of REM sleep by ALM, but did not affect ALM-mediated increases in NREM sleep. LCx, but not TMNx, also blocked the ALM-mediated decrease in NREM sleep latency. However, neither lesion altered ZOL efficacy on any measure of sleep-wake regulation. These findings support the hypothesis that ALM promotes sleep via selective disfacilitation of subcortical arousal systems as previously proposed. Along with published studies, these results suggest that Hcrt neurotransmission influences distinct aspects of NREM and REM sleep at different locations in the sleep-wake regulatory network.

Introduction

Hypocretin-1 and -2 (Hcrts, also known as orexin-A and -B), excitatory neuropeptides synthesized in neurons located in the tuberal hypothalamus, are involved in metabolism, feeding, reward, addiction, and sleep/wake control (Ohno and Sakurai, 2008). Hcrt neurons are wake-active (Estabrooke et al., 2001; Lee et al., 2005), with some activity reported during REM sleep (Kiyashchenko et al., 2002; Mileykovskiy et al., 2005; Takahashi et al., 2008). Hcrt administration (Bourgin et al., 2000; Morairty et al., 2011) or optogenetic stimulation of Hcrt neurons (Adamantidis et al., 2007; Carter et al., 2010) is wake-promoting. Deficient Hcrt signaling underlies narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Thannickal et al., 2000), a sleep disorder characterized by fragmented sleep, degraded sleep-wake rhythms, and profound dysregulation of REM sleep. Hcrt signaling thus plays a critical role in the organization and consolidation of sleep-wake states.

Hcrt neurons project to several wake-promoting brain populations, including the locus coeruleus (LC) (Peyron et al., 1998; Chemelli et al., 1999; Horvath et al., 1999). LC activation desynchronizes cortical activity and precedes transitions to waking, exhibiting a strongly wake-active, REM-silent firing profile (Aston-Jones and Bloom, 1981; Berridge and Foote, 1991; Takahashi et al., 2010). Optogenetic inhibition or activation of LC norepinephrine (NE) neurons increases or decreases the likelihood of sleep, respectively (Carter et al., 2010). Disruption of NE signaling via cell-type specific LC lesions or knockout (KO) is reported to increase NREM sleep (González et al., 1998; Blanco-Centurion et al., 2004; Ouyang et al., 2004) or block wakefulness following arousing stimuli (Hunsley and Palmiter, 2004; Gompf et al., 2010), consistent with a role

in maintenance of wakefulness. The LC expresses Hcrt receptor 1 (HcrtR1) (Marcus et al., 2001) and Hcrt-1/orexin-A infusion into the LC increases LC neuron firing and promotes wakefulness (Hagan et al., 1999; Bourgin et al., 2000) in a HcrtR1-dependent manner (Soffin et al., 2002; Choudhary et al., 2014). Conversely, optogenetic LC inactivation blocks transitions to wakefulness following Hcrt neuron activation (Carter et al., 2012), indicating that the LC is important for Hcrt-induced wakefulness.

Hcrt neurons also strongly innervate histaminergic (HA) cells in the tuberomammillary nuclei (TMN) of the posterior hypothalamus (Peyron et al., 1998; Chemelli et al., 1999). TMN HA neurons express HcrtR2 (Marcus et al., 2001) and are excited by the Hcrt peptides (Eriksson et al., 2001). HA is wake-promoting (Chu et al., 2004; Ramesh et al., 2004) and HA neurons, like LC NE neurons, exhibit a wake-active, REM-off firing pattern (Takahashi et al., 2006). TMN HA lesions have relatively mild effects on sleep-wake states (Gerashchenko et al., 2004). However, histidine decarboxylase (HDC) KO mice exhibit decreased wakefulness at lights-off, increased REM sleep time during the light phase, and short sleep latency in a novel environment (Parmentier et al., 2002; Anaclet et al., 2009). Wake promotion by Hcrt-1/orexin A is mediated in part through histaminergic neurotransmission (Huang et al., 2001). Thus, Hcrt inputs to the LC NE system and the TMN HA system are important pathways for Hcrt-induced wakefulness.

The dual Hcrt/orexin receptor antagonist (DORA) almorexant (ALM) blocks the excitatory effects of the Hcrt peptides at HcrtR1 and HcrtR2, decreasing wakefulness and increasing NREM and REM sleep time (Brisbare-Roch et al., 2007; Morairty et al., 2012). In contrast, zolpidem (ZOL; trade name Ambien®) induces somnolence by

activating $GABA_A$ receptors, thereby causing widespread neuronal inhibition (Dang et al., 2011). Previously, we showed that ALM, but not ZOL, requires an intact BF for maximal hypnotic efficacy and that in intact rats, ALM, but not ZOL, induces neurochemical events associated with the transition to normal sleep (Vazquez-DeRose et al., 2014). These findings support the hypothesis that ALM induces sleep by selectively disfacilitating subcortical wake-promoting populations, whereas ZOL acts via generalized inhibition throughout the brain. Here, we tested this hypothesis by selectively ablating the LC NE neurons or the TMN HA neurons using cell-type specific saporin conjugates, and subsequently evaluating the efficacy of ALM and ZOL in lesioned and intact rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (*n* = 25; 200-250 g; Harlan Laboratories) were housed in light-tight, sound-attenuated environmental chambers under constant temperature (22±2^{o}C, 50±25% relative humidity) on a 12 h:12 h dark/light cycle with food and water *ad libitum*. All dosing procedures were carried out under dim red light (<2 lux). All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at SRI International. Every effort was made to minimize animal discomfort throughout the experimental protocols.

Chemicals. ALM was synthesized by the Medicinal Chemistry Laboratory at SRI International according to previously published methods (Koberstein et al., 2003; 2005). ZOL was purchased from IS Chemical Company (Shanghai, China). All drugs that were delivered orally were suspended and sonicated for 1 h in 1.25% hydroxypropyl methyl cellulose (HPMC) with 0.1% dioctyl sodium sulfosuccinate (DOSS; 2.24 mM) in sterile water (hereafter referred to as 'VEH' for Vehicle). All drug solutions were made on the day of the experiment and serially diluted to their final concentrations.

Saporin lesions. Under isoflurane anesthesia, rats were placed into a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) and the skull was exposed. For LC lesions, rats were injected i.c.v. with 10 µL of anti-DBH saporin (n=8; DBH-SAP; 0.3 µg/µL; Advanced Targeting Systems, San Diego, CA) (Wrenn et al., 1996) or sterile saline (n=7; hereafter referred to as "Sham" rats) via a 26ga stainless steel injection cannula connected to a 10 µL Nanofil Hamilton syringe and a digitally-controlled microinjector (World Precision Instruments, Sarasota FL) at -0.8 mm AP and +1.5 mm ML relative to

bregma, and 3.3 mm below dura. Injections lasted ~10 min; the cannula was left in place for 5 min after the injection. For TMN lesions, rats were injected bilaterally with 250-350 nL of Hcrt2-saporin (n=13; Hcrt2-SAP; 0.228 µg/µL; Advanced Targeting Systems, San Diego, CA) (Gerashchenko et al., 2001; 2004) or sterile saline (n=7) via glass micropipettes (inner tip diameter ~30-50 µm) using a Picospritzer (Parker Hannifin, Cleveland, OH) at -4.2 mm or -4.35 mm AP and ±0.8 mm ML relative to bregma, and 9.3 mm below dura. Injectate volume was measured via precalibrated marks on the barrel of the pipette. Injections lasted 5 min/side; the pipette was left in place for 5 min after the injection. Following SAP injections, rats were instrumented for EEG/EMG telemetry.

Telemetry surgery. All rats were surgically implanted with a sterile abdominal transmitter (F40-EET, DSI, St Paul, MN) for continuous telemetric recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_b), and locomotor activity (LMA) as described previously (Morairty et al., 2008; 2012). Briefly, the wires from the transmitter were subcutaneously channeled rostrally to the head. Two biopotential leads (used as EEG electrodes) were inserted into drilled holes over the skull and affixed with dental acrylic. Two additional biopotential leads (EMG electrodes) were sutured into the neck musculature and closed with non-absorbable suture. Animals were singly-housed after surgery and allowed to recover in their home cage for at least 3 weeks before recording.

Assessment of hypnotic efficacy in saporin-lesioned rats. Rats were kept in their home cages for the duration of the study in ventilated, light-tight and sound-attenuated chambers in 12:12 LD. Prior to initiation of sleep recordings, animals were acclimated

to handling and oral gavage with VEH for approximately one week, then left undisturbed for 2 d after acclimation was complete. Rats were administered ALM (30, 100 and 300 mg/kg), ZOL (10, 30 and 100 mg/kg), or VEH p.o. starting at lights-out (ZT 12) in balanced order with at least 3 d between treatments in a cross-over study design; previous work from our lab has shown that this dosing regimen allows sufficient time for washout between doses (Morairty et al., 2012). EEG was then recorded for 24 h following dosing.

To confirm the extent of lesions, rats were deeply anesthetized and transcardially perfused with heparinized 0.1M phosphate-buffered saline followed by 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde, and then transferred to 30% sucrose until sectioning. Brains were sectioned at 40 µm on a freezing microtome. Free-floating sections containing the LC (Bregma -9.16mm to -10.30mm) were incubated with 1% H_2O_2 for 15 min to quench endogenous peroxidase activity, followed by (i) 1 h in blocking buffer containing 3% normal donkey serum, (ii) overnight in mouse anti-DBH (1:100,000, MAB308, EMD Millipore), (iii) 2 h in biotinylated donkey anti-mouse IgG (1:500; Jackson Immunoresearch), and (iv) 2 h in avidin–biotin complex (ABC; Vector Laboratories). DBH was visualized by reacting sections in 0.05% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 to form a brown reaction product. Sections were then mounted, dehydrated and coverslipped. To visualize HA neurons, sections containing the TMN (Bregma -3.80mm to -4.80mm) were processed using a similar protocol that was modified as follows: (ii) sections were incubated overnight in rabbit anti-ADA (1:20,000, ab176, EMD Millipore), followed by (iii) 2h in biotinylated donkey anti-rabbit IgG (1:500; Jackson Immunoresearch).

The extent of the LC was delineated by the 4th ventricle and other landmarks. The exact number of DBH-positive LC neurons could not be accurately counted in Sham rats because of the high density of these cells (Figure 1); accordingly, Sham rats were only scored for the presence of DBH-positive cells. In DBH-SAP injected rats, all residual DBH-positive cells in the LC region were counted. To evaluate the extent of TMN HA neuronal loss, ADA-positive neurons were counted in the dorsal (dTMN), ventral (vTMN), and caudal TMN (cTMN) subregions as identified previously (Ko et al., 2003; Parks et al., 2015). All neurons expressing ADA in each subregion were scored.

EEG and EMG analyses and sleep/wake determinations. EEG and EMG were recorded via telemetry on a PC running Dataquest ART 3.1 (DSI). All recordings were manually scored offline by a trained expert in 10 s epochs as Wake, NREM, or REM sleep using NeuroScore 2.1 (DSI). Any epochs that contained recording artifacts were tagged and excluded from spectral analyses. Individual state data were quantified as time spent in each state per 1 h or 6 h. Latency to NREM and REM onset for each animal was calculated from the time of drug injection. Bouts were defined as a minimum of 3 consecutive epochs of wake or NREM, and 2 consecutive epochs of REM sleep.

Statistical analyses. Latency to NREM and REM sleep, total time in each state (Wake, NREM, REM), REM:NREM ratio, average bout duration and total number of bouts for the 6 h following dosing were analyzed by a two-way mixed model analysis of variance (ANOVA) comparing lesion condition (between-subjects) and drug treatment (within-subjects). Bout architecture was further analyzed using a three-way mixed model ANOVA comparing the effects of lesion condition (between-subjects), drug treatment

(within-subjects) and bout duration (within-subjects) on bout number. To assess fragmentation of arousal states, we included all sleep/wake bouts without a minimum bout length requirement. Significant main effects and interactions ($P < 0.05$) were subsequently analyzed with Bonferroni *post hoc* tests. In some cases, near-significant trends in the omnibus ANOVA were followed up with planned comparisons (*F* test), examining the effects of lesion at each drug dose; these planned comparisons are specified in the results. All statistical analyses were run using Statistica (Statsoft, Omaha NE).

Results

LC lesion evaluation

Fig. 1A shows the LC area targeted by the DBH-SAP lesions (red), as well as the approximate location of the nearby A5 NE neurons (yellow). In Sham-injected rats, the LC was clearly delineated by densely-packed DBH-positive cells and fibers (Fig. 1B). The darkly-stained neuropil and proximity of DBH-positive cells to each other made it difficult to accurately count individual cells. In DBH-SAP-injected rats, only a few scattered DBH-positive neurons were visible in the LC (Fig. 1C); bilateral counts in the LC revealed 15.75 ± 4.2 DBH-positive cells in SAP-treated rats, ranging from 2-38 neurons remaining in individual animals. In contrast, the more ventral A5 neurons were largely or entirely spared following i.c.v. DBH-SAP injections (Sham, Fig. 1D; DBH-SAP, Fig. 1E) as previously reported (Wrenn et al., 1996). All DBH-SAP-injected rats were thus considered to have complete LC lesions.

LCx attenuates sleep induction by ALM

Both ALM and ZOL (all doses) shortened the latency to NREM sleep compared to VEH in Sham rats, whereas only ZOL (10, 100 mg/kg) was effective in LCx rats (interaction: $F_{6,78} = 2.553$, $P = 0.026$; Fig. 2A-B). Accordingly, LCx attenuated the ALM-induced decrease in NREM latency. While LC lesions shortened NREM sleep latency following VEH from ~60 min to ~40 min, the decrease was not significant compared to VEH-treated Sham rats likely due to the large variance in NREM latency among the Sham rats. Importantly, ZOL further decreased NREM latency from this baseline in LCx rats (Fig. 2B), indicating that the lack of efficacy following ALM was not due to a lesion-

induced “floor” effect. REM sleep latency was significantly increased by ZOL at 100 mg/kg independently of lesion status (main effect of drug: $F_{6,78}$ = 12.58, P < 0.001; Fig. 2C-D).

LCx attenuates REM sleep increases following ALM

Both ALM (all doses) and ZOL (30, 100 mg/kg) decreased wake time from ZT 12 to ZT 18 independent of lesion status (main effect of drug: $F_{6,78}$ = 21.532, P < 0.001; Fig. 3A-B). NREM sleep time was increased by LC lesions (main effect of lesion: $F_{1,13}$ = 5.722, P = 0.033) and by all doses of ALM and ZOL (main effect of drug: Fig. 3C-D; $F_{6,78}$ = 18.821, P < 0.001), with no drug-lesion interaction. By contrast, ALM (100, 300 mg/kg) increased REM sleep time compared to VEH in both Sham and LCx rats, but this increase was attenuated in LCx compared to Sham rats at the ALM 300 mg/kg dose (interaction: $F_{6,78}$ = 4.439, P < 0.001; Fig. 3E-F). Similarly, ALM (100, 300 mg/kg) increased the ratio of REM to NREM sleep (REM:NREM) compared to VEH in Shams, but not LCx rats (interaction: $F_{6,78}$ = 5.010, P < 0.001; Fig. 3G), such that REM:NREM was significantly attenuated in LCx rats compared to Shams following ALM (100, 300 mg/kg). While ZOL did not significantly affect REM sleep time (Fig. 3F), ZOL decreased REM:NREM in both Sham (30, 100 mg/kg) and LCx rats (100 mg/kg) (Fig. 3H). Thus, LCx blocked ALM-induced, but not ZOL-induced shortening of NREM sleep latency, attenuated the ALM-mediated increase of REM sleep time, and increased NREM sleep time independent of drug effects.

LCx alters sleep-wake architecture

LC lesions decreased the total number of wake bouts compared to Shams (main effect: $F_{1,13}$ = 5.891, P = 0.030), and ALM (all doses) increased the total number of

wake bouts whereas ZOL did not (main effect of drug: $F_{6,78}$ = 29.152, P < 0.001; Fig. 4A-B). There was a borderline interaction effect between these factors ($F_{6,78}$ = 2.136; P = 0.058); planned comparisons revealed that LCx rats had fewer wake bouts than Shams following VEH and ALM (all doses) but not after ZOL (Fig. 4A-B).

A similar effect was observed for the number of bouts of NREM sleep (interaction: $F_{6,78}$ = 2.514, P = 0.028, Fig. 4C-D); ALM (all doses) significantly increased NREM bout number compared to VEH in all rats, but bout numbers were consistently lower in LCx compared to Sham rats at all doses (Fig. 4C). By contrast, ZOL tended to equalize NREM bout number between lesion conditions, especially at the highest dose (100 mg/kg; Fig. 4D).

ANOVA for the number of REM bouts revealed a significant drug x lesion interaction ($F_{6,78}$ = 4.519; P < 0.001; Fig. 4E-F). ALM increased the number of REM bouts compared to VEH in both Sham (all doses) and LCx rats (100, 300 mg/kg); however, LCx attenuated the ALM-induced increase at 300 mg/kg, with a borderline effect at the 100 mg/kg dose (Bonferroni, p=0.055). ZOL did not affect REM bout number in either Sham or LCx rats.

The mean duration of NREM bouts was independently increased by LC lesions ($F_{1,13}$= 8.848; P = 0.011) and by ZOL (100 mg/kg) ($F_{6,78}$ = 6.734; P < 0.001), with no interaction between the factors. There were no other effects on the mean duration of NREM, REM or wake bouts.

We next asked whether changes in bout number were associated with changes in bout duration (Fig. 5). ALM preferentially increased the number of short (<0.5 min) wake bouts in both LCx and Sham rats ($F_{30,390}$= 3.723, P < 0.001; Fig, 5A, D), but LCx

rats had fewer short wake bouts compared to Shams following VEH and ALM. Similarly, ALM also increased the number of short NREM bouts in both LCx and Sham rats ($F_{30,390}$= 4.700, P < 0.001; Fig, 5B, E), but LCx rats had fewer NREM bouts under 1 min than Shams following VEH and ALM. In other words, ALM increased the number of short sleep-wake bouts, whereas LC lesions decreased the number of short bouts. By contrast, LC lesions had very little effect on wake and NREM bout architecture following ZOL. ZOL appeared to increase the number of long NREM bouts (>4 min) in both Sham (Fig. 5B) and LCx (Fig. 5E) rats. Although the *post hoc* comparisons were not statistically significant, the additional long bouts likely account for the significant increase in mean NREM bout duration under ZOL treatment.

There were significant drug x lesion ($F_{6,78}$ = 4.491; P < 0.001) and bout x lesion interactions ($F_{5,65}$ = 7.041; P < 0.001) that affected REM bout composition, but there was no three-way interaction between drug, bout and lesion ($F_{30,390}$= 1.440, P =0.066; Fig. 5C, F). As described above for total bout number, LCx attenuated the increase in REM bout number following ALM (100, 300 mg/kg); Fig. 5C and 5F show that the changes in REM bout number were distributed across short and long REM bouts.

TMN lesion evaluation

Fig. 6A-C shows the posterior hypothalamic region targeted by the Hcrt2-SAP lesions, with the dorsal, ventral and caudal TMN subgroups highlighted. ADA-immunostaining clearly visualized the HA-positive neurons in Sham-injected rats (Fig. 6D). There were 1375 ± 78 ADA-positive cells in the TMN of Sham rats (combined count of dorsal, ventral and caudal TMN). Hcrt2-SAP injections decreased ADA-immunostaining in the TMN (Fig. 6E); of 13 Hcrt2-SAP injected rats, 6 exhibited

substantial bilateral reductions in ADA-positive cell number (<50% of the Sham group mean). These 6 rats were used as the TMNx group. This TMNx group exhibited 445 ± 73 ADA-positive TMN cells, with individual lesions ranging from 216 - 612 ADA-positive cells (Fig. 6F; individual counts from each TMNx rat superimposed on the group mean). The remaining rats exhibited little to no ADA cell loss (>75% of Sham group mean), and were excluded from further analysis.

TMNx attenuates REM sleep promotion by ALM

As in the LCx study described above, there was a significant main effect of drug treatment on NREM sleep latency ($F_{6,66}$= 11.243; P < 0.001) such that ZOL but not ALM significantly shortened the latency to NREM sleep. There was also a significant main effect of drug treatment on REM sleep latency ($F_{6,66}$= 5.390; P < 0.001); while *post hoc* tests showed no significant changes compared to VEH, ALM tended to decrease REM sleep latency, while ZOL tended to increase it. Neither NREM nor REM sleep latency was affected by TMN lesion.

Consistent with the LCx study, both ALM and ZOL decreased wake time (main effect of drug: $F_{6,66}$= 29.346, P < 0.001; Fig. 7A-B) and increased NREM sleep time (main effect of drug: $F_{6,66}$= 27.612, P < 0.001; Fig. 7C-D), but with no main or interaction effect of TMN lesion. By contrast, ALM (100 and 300 mg/kg) increased REM sleep time compared to VEH in both Sham and TMNx rats (drug x lesion interaction: $F_{6,66}$= 2.436, P = 0.035; Fig. 7E-F). Pairwise comparisons of TMNx and Sham rats in each drug treatment condition revealed that TMNx rats had less total REM sleep time following ALM (30 and 300 mg/kg) compared to Shams, whereas there were no differences in REM sleep time between Sham and TMNx rats following ZOL (Fig. 7E-F). REM:NREM

was significantly increased by ALM (100 and 300 mg/kg) and decreased by ZOL (100 mg/kg) (main effect of drug: $F_{6,66}$= 28.419, P < 0.001; Fig 7G-H). However, these drug effects were qualified by a borderline interaction effect ($F_{6,66}$= 2.131; P = 0.061), such that TMNx decreased REM:NREM compared to Shams following ALM (300 mg/kg) (pairwise comparison p< 0.05). Thus, TMNx affected ALM-induced REM sleep increases in a similar manner to that seen following LCx.

TMNx blocks increases in REM bout number following ALM

ALM (all doses) increased the total number of wake and NREM bouts compared to VEH (main effect of drug: Wake, $F_{6,66}$= 48.670, P < 0.001; NREM, $F_{6,66}$= 46.346, P < 0.001) without an effect of TMN lesion (Fig. 8A, C). ZOL did not affect the total number of wake or NREM bouts (Fig 8B, D). Further analysis of bout duration histograms showed that TMNx preferentially increased the number of short (<0.5 min) NREM bouts (bout x lesion interaction: $F_{5,55}$= 3.401; P = 0.010) with no additional influence of drug treatment (data not shown).

While ALM (100, 300 mg/kg) increased REM bout number in TMNx and Sham rats, TMNx significantly attenuated this increase at the highest dose of ALM (interaction: $F_{6,66}$= 2.860, P = 0.015; Fig. 8E). ZOL did not affect the total number of REM bouts in either lesion group (Fig. 8F). There were no additional effects of lesion or drug treatment on total REM or wake bout numbers, nor on the distribution of bout numbers as a function of their duration (data not shown). Thus, TMNx attenuated the promotion of REM sleep by ALM primarily by decreasing the number of REM episodes, while increasing the number of short NREM sleep bouts independently of drug treatment.

Discussion

The Hcrt system promotes wakefulness in part through excitation of subcortical wake-active monoaminergic populations, including the noradrenergic LC and histaminergic TMN. In this study, neurotoxic lesions of the LC NE neurons or TMN HA neurons selectively attenuated promotion of REM sleep by the dual Hcrt receptor antagonist ALM, but did not affect ALM-mediated increases in NREM sleep. Furthermore, neither lesion altered the efficacy of the $GABA_A$ receptor agonist ZOL. These findings support the hypothesis that ALM promotes sleep via selective disfacilitation of subcortical arousal systems as previously proposed (Vazquez-DeRose et al., 2014). In addition, these results highlight the important role of Hcrt input to HA- and NEergic populations in regulating the expression of REM sleep.

Lesion efficacy

DBH-SAP infusions caused a near-complete loss of LC DBH-immunoreactive cells, with no signs of collateral or nonspecific damage. DBH-SAP is highly selective for LC NE neurons when delivered i.c.v. or directly into the LC (Wrenn et al., 1996), (Blanco-Centurion et al., 2004). Medullary and pontine NE populations receive Hcrtergic projections (Baldo et al., 2003) and have been suggested to play a role in the inhibition of REM sleep (Fenik et al., 2002; Rukhadze et al., 2008; Léger et al., 2009). While we cannot rule out the possibility of collateral damage to these non-LC NE groups, the nearby A5 noradrenergic neurons appeared intact in our LCx rats following 3µg DBH-SAP, consistent with previous work showing that higher doses are required to lesion these populations (Wrenn et al., 1996). Furthermore, LC lesions increased NREM sleep but not REM sleep following VEH. We therefore conclude that the observed effects on

ALM and ZOL efficacy are attributable to NE cell loss concentrated in the LC, and not to damage of neighboring NE-ergic or other monoaminergic cell groups.

In contrast to our LC lesions, our TMN-HA lesions were less complete (16%-45% of HA neurons remaining in TMNx rats). Consistent with our observations in VEH-dosed rats, Hcrt2-SAP TMN lesions have previously been found to have mild effects on basal sleep-wake parameters (Gerashchenko et al., 2004). Since Hcrt2-SAP destroys all neurons that express Hcrt receptors (Gerashchenko et al., 2004), we cannot rule out the possibility that the loss of non-HA neurons expressing Hcrt receptors may contribute to our observed results. Indeed, the posterior hypothalamus contains non-histaminergic (possibly GABAergic) REM-active neurons (Steininger et al., 1999; Sapin et al., 2010); destruction of such REM-on neurons could account for our observations of attenuated REM sleep in ALM-treated TMNx rats.

The LC is a critical site of action for sleep induction by ALM

LC lesions blocked the reduction in NREM sleep latency following ALM. Importantly, ZOL reduced NREM latency to a comparable extent in both lesioned and Sham rats, indicating that the lack of an effect by ALM was not due to a lesion-induced "floor" effect. The LC is therefore likely to be a critical site of action for ALM-mediated sleep induction. Increases in spontaneous LC firing rate anticipate transitions to wakefulness (Aston-Jones and Bloom, 1981; Takahashi et al., 2010); LC stimulation activates the cortex and hippocampus (Berridge and Foote, 1991) and promotes transitions from sleep to wakefulness (Carter et al., 2010). Ablation of NE neurons causes deficits in maintenance of arousal following stressful or novel behavioral manipulations (Hunsley and Palmiter, 2004; Ouyang et al., 2004; Gompf et al., 2010).

LC NE signaling is thus a key component of spontaneous arousal and wakefulness, particularly in association with attention.

LCx increased NREM sleep time and NREM bout duration while decreasing the number of short (<0.5 min) wake and NREM bouts, suggesting increased consolidation of NREM sleep. While some studies found no effect of LC NE ablation on basal NREM sleep time (Hunsley and Palmiter, 2003; Gompf et al., 2010), others observed increased NREM sleep in the dark phase or around the lights-off transition (González et al., 1998; Blanco-Centurion et al., 2004; Ouyang et al., 2004), similar to our results. ALM-induced sleep is typically fragmented compared to ZOL-induced sleep (Morairty et al., 2012). While ALM increased the number of wake and NREM bouts in LCx as well as Sham rats, the increased NREM bout duration in LCx rats was still evident at the highest dose of ALM. By contrast, ZOL increased NREM bout duration without affecting NREM or wake bout number in both LCx and Sham rats such that, at the highest dose, wake and NREM bout numbers were equivalent in LCx and Sham rats (Fig. 4B, D; Fig. 5A-B, D-E). LC ablation thus attenuated the typical fragmentation of bout architecture by ALM, whereas ZOL consolidated NREM sleep in both lesion groups. Stimulating Hcrt neurons (Adamantidis et al., 2007; Choudhary et al., 2014) or infusing Hcrt-1 centrally (Piper et al., 2000; Morairty et al., 2011) or directly into the LC (Bourgin et al., 2000) promotes waking, whereas local application of HcrtR1 antagonists or optogenetic LC inhibition blocks transitions to wakefulness (Carter et al., 2012), suggesting that Hcrt-mediated wakefulness in highly dependent on the LC NE system. Our results demonstrate that LC ablation impacts the sleep-induction profile of ALM and thus supports this concept.

By contrast, TMNx increased the total number of short NREM bouts but had no effects on either the initiation or duration of NREM sleep in any drug condition. Like the LC NE neurons, HA neurons exhibit a wake-active, REM-off firing pattern (Takahashi et al., 2006), elevated HA levels are correlated with wakefulness (Chu et al., 2004; Ramesh et al., 2004), and mice lacking histamine exhibit deficits in wakefulness at lights-off and in a novel environment (Parmentier et al., 2002) -- all of which suggest that HA is important for motivated arousal. Furthermore, Hcrt directly and indirectly excites TMN HA neurons (Eriksson et al., 2001; 2004; Schöne et al., 2014) and Hcrt infusion into the TMN promotes waking and induces cortical HA release (Huang et al., 2001). Although it was surprising that TMN lesions had no effects on NREM sleep latency or NREM sleep time in the vehicle or drug conditions, TMN HA neuron ablation with Hcrt2-SAP exhibited few effects on basal sleep in a previous study (Gerashchenko et al., 2004). While the residual HA neurons or compensation from other wake-promoting groups may have been sufficient to maintain normal function, we did observe substantial effects on REM sleep induction following ALM in TMNx rats (Figs. 7E, 7G, 8E), suggesting that lesions did impact HA-ergic sleep-wake regulation capacities.

Lesioning either LC or TMN attenuates ALM-induced REM sleep

Blockade of Hcrt signaling with ALM increased REM sleep in Sham rats, as previously reported in intact animals (Brisbare-Roch et al., 2007). We found that lesioning either the LC NE or TMN HA neurons selectively blocked the promotion of REM sleep by ALM, suggesting a specialized role for Hcrt signaling to these nuclei in regulating REM sleep.

Both the LC NE and HA TMN neurons exhibit wake-active, REM-off firing profiles (Takahashi et al., 2006; 2010). The LC inhibits nearby cholinergic brainstem 'REM-on' neurons (Hobson et al., 1975; McCarley and Hobson, 1975). Local Hcrt-1/orexin-A infusion activates the LC and suppresses REM sleep (Bourgin et al., 2000), whereas downregulation of LC HcrtR1 inhibits REM sleep (Chen et al., 2010; Choudhary et al., 2014). Similarly, HA inhibits the melanin-concentrating hormone (MCH) neurons (Parks et al., 2014) that have been implicated in REM sleep (Verret et al., 2003; Clément et al., 2012; Jego et al., 2013). Induction of REM sleep by ALM therefore depends on 'disfacilitation' of "REM-off" activity in the LC and the TMN, resulting in the downstream disinhibition of REM-active populations.

Both LC and TMN lesions powerfully attenuated the upregulation of REM sleep by ALM while having no effect on basal REM sleep in the early dark phase, as previously reported (González et al., 1998; Blanco-Centurion et al., 2004; Gerashchenko et al., 2004; Gompf et al., 2010). Thus, acute blockade of Hcrt input to the LC NE or TMN HA neurons via ALM increases REM sleep suggesting that, in the intact brain, Hcrtergic input to the LC and TMN is a critical component for the normal suppression of REM sleep in the early active phase. By contrast, compensatory responses following lesions preserve the basal expression of REM sleep while eliminating the ability of Hcrt receptor antagonism to increase it.

Conclusions

DORAs, including ALM, promote sleep by blocking Hcrt signaling. In the present study, we showed that lesions of the wake-promoting LC NE or TMN HA cell groups compromised the hypnotic efficacy of ALM without affecting that of ZOL. We previously

showed that ALM, but not ZOL, requires an intact BF for maximum NREM-promoting efficacy, and that ALM elicits a neurochemical release profile more consistent with the transition to normal sleep than does ZOL (Vazquez-DeRose et al., 2014). Thus, Hcrt neurotransmission influences distinct aspects of NREM and REM sleep at different locations in the sleep-wake regulatory network. By selectively disfacilitating these subcortical wake-promoting populations, Hcrt antagonism effectively promotes sleep without negatively impacting cognitive performance (Morairty et al., 2014) and without globally blocking the capability for arousal (Parks et al., 2015).

Figure Legends

Figure 1. Characterization of DBH-SAP lesions. A, schematic showing location of LC NE neurons targeted by DBH-SAP infusions (red) and the more ventrally-located A5 noradrenergic neurons (yellow). B, DBH immunostaining of the LC in a Sham-injected rat shows densely-packed NE neurons, which were destroyed following DBH-SAP injections (C). By contrast, A5 neurons were intact in both Sham (D) and DBH-SAP-injected rats (E). 4v, 4th ventricle; A5, A5 NE group; LC, locus coeruleus; PRN, pontine reticular nucleus; scp, superior cerebellar peduncle, SOC, superior olivary complex; SubC, subcoeruleus; VIIn, facial nerve. Scale bar = 200 μm. Adapted from (Swanson, 2004).

Figure 2. Latency to NREM (A-B) and REM sleep (C-D) following ALM (A, C) and ZOL (B, D). Doses are in mg/kg. *, $p<0.05$ vs Vehicle; *', $p<0.06$ vs Vehicle.

Figure 3. Total Wake (A-B), NREM (C-D) and REM (E-F) sleep time, and the ratio of REM to NREM sleep (G-H) following ALM (A, C, E, G) and ZOL (B, D, F, H). Doses are in mg/kg. *, $p<0.05$ vs Vehicle; #, $p<0.05$ vs Sham.

Figure 4. Total number of Wake (A-B), NREM (C-D) and REM (E-F) bouts following ALM (A, C, E) and ZOL (B, D, F). Doses are in mg/kg. *, $p<0.05$ vs Vehicle; #, $p<0.05$ vs Sham; #', $p<0.06$ vs Sham; +, $p<0.05$, paired comparison F-test (Sham vs LCx).

Figure 5. Number of Wake (A, D), NREM (B, E) and REM (C, F) bouts as a function of bout duration in Sham (A-C) and LCx rats (D-F). Doses are in mg/kg. *, $p<0.05$ vs Vehicle; #, $p<0.05$ vs Sham.

Figure 6. Characterization of Hcrt2-SAP lesions. A-C, schematic showing location of TMN HA neurons targeted by Hcrt2-SAP infusions. D-E, HA-positive neurons in the DTMN and vTMN in a Sham-injected rat (D) and a Hcrt2-SAP injected rat (E). Photomicrographs depict the TMN at approximately the same rostrocaudal point as panel B. 3v, 3rd ventricle; cpd, cerebral peduncle; dTMN, dorsal tuberomammillary nuclei; f, fornix; LHA, lateral hypothalamic area; MM, medial mammillary nuclei; mt, mammillary tract; PH, posterior hypothalamic nucleus; vTMN, ventral tuberomammillary nuclei. Scale bar = 200 μm. Adapted from (Swanson, 2004).

Figure 7. Total Wake (A-B), NREM (C-D) and REM (E-F) sleep time, and the ratio of REM to NREM sleep (G-H) following ALM (A, C, E, G) and ZOL (B, D, F, H). Doses are in mg/kg. *, $p<0.05$ vs Vehicle; +, $p<0.05$, paired comparison F-test (Sham vs TMNx).

Figure 8. Total number of Wake (A-B), NREM (C-D) and REM (E-F) bouts following ALM (A, C, E) and ZOL (B, D, F). Doses are in mg/kg. *, $p<0.05$ vs Vehicle; #, $p<0.05$ vs Sham.

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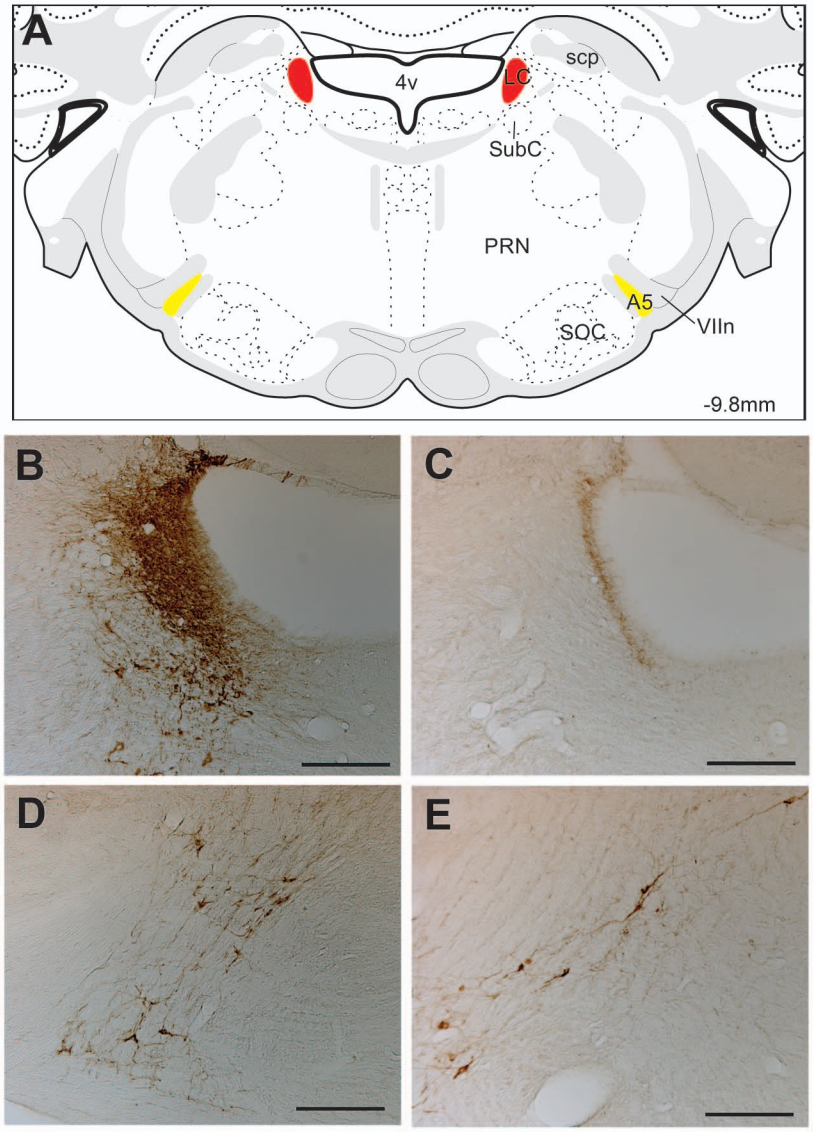
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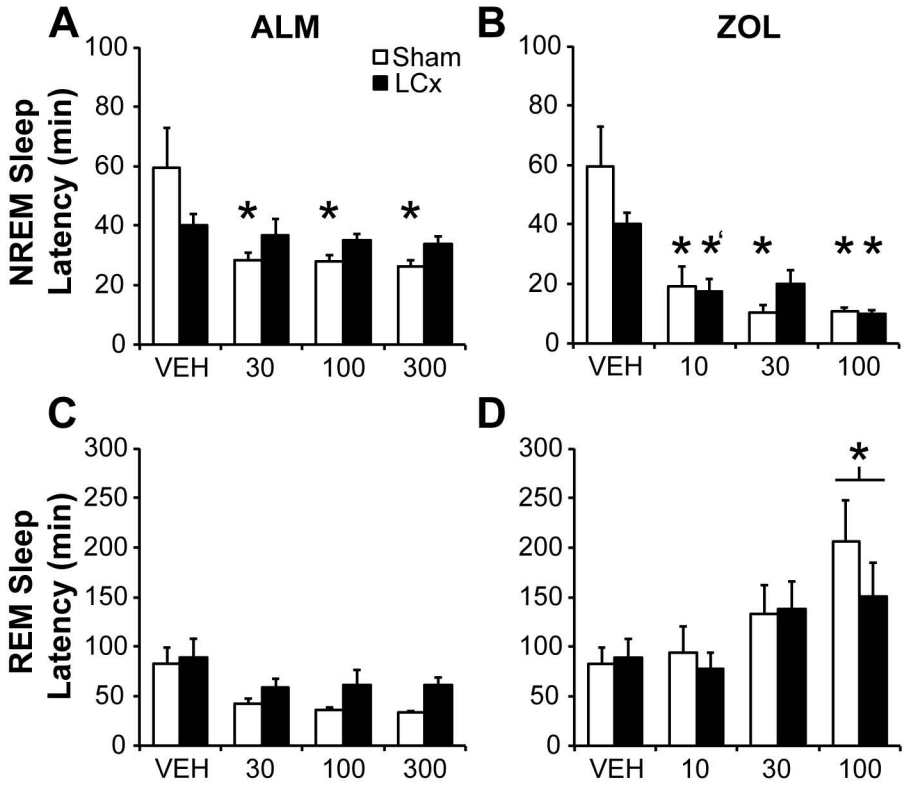
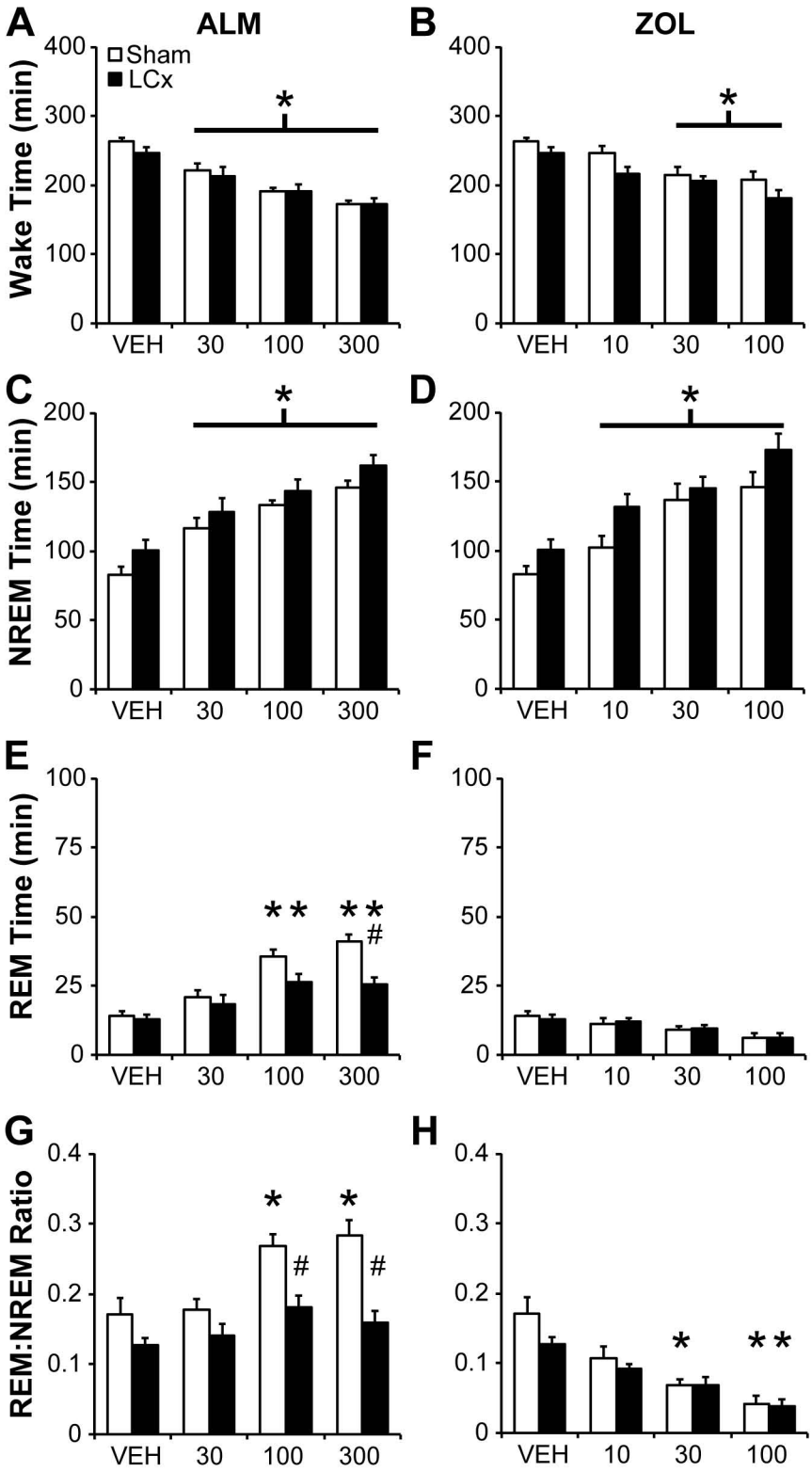

A
ALM
Sham
LCx
NREM Sleep Latency (min)
100
80
60
40
20
0
VEH
30
100
300
B
ZOL
10
C
REM Sleep Latency (min)
250
200
150
50
D


A
ALM
B
ZOL
Sham
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Wake Time (min)
NREM Time (min)
REM Time (min)
REM:NREM Ratio
VEH
10
30
100
300
*
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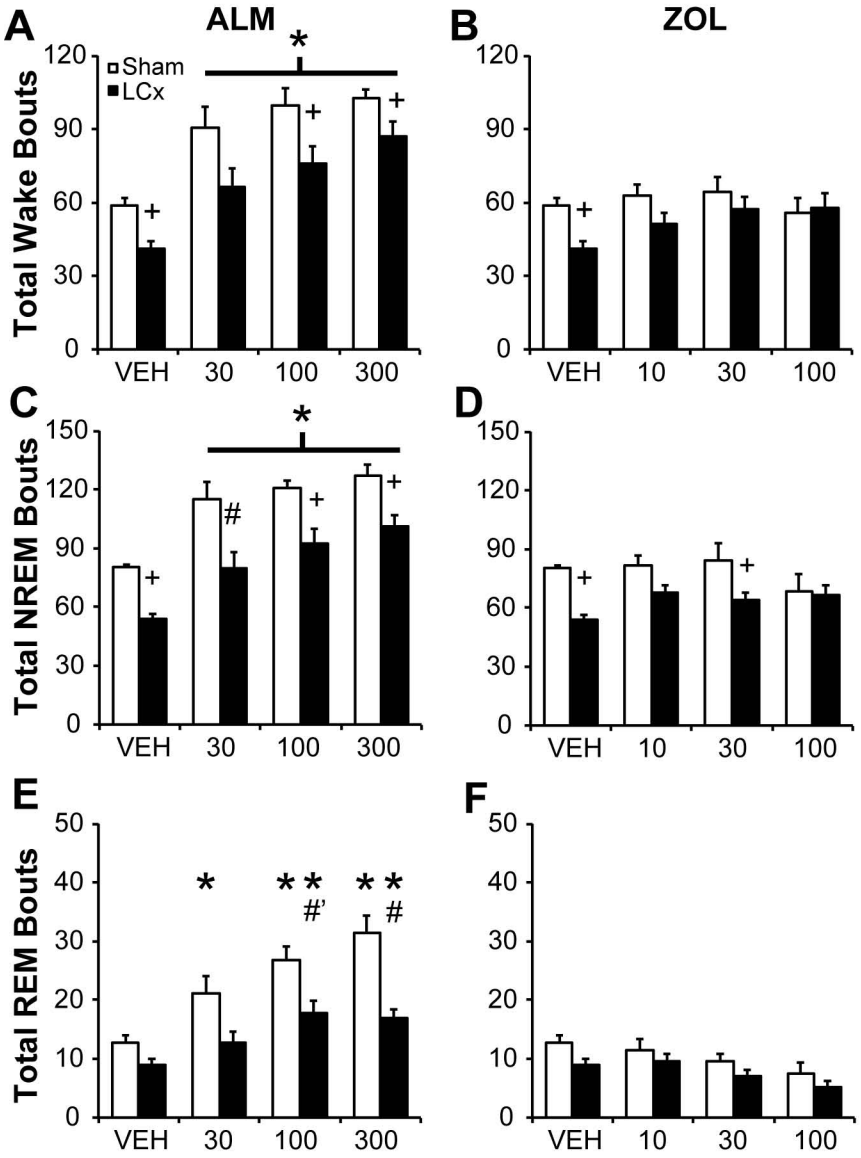
#


A
ALM
B
ZOL
Sham
LCx
Total Wake Bouts
120
90
60
30
0
VEH
30
100
300
10
*
+
C
D
Total NREM Bouts
150
120
90
60
30
0
#
E
F
Total REM Bouts
50
40
30
20
10
0
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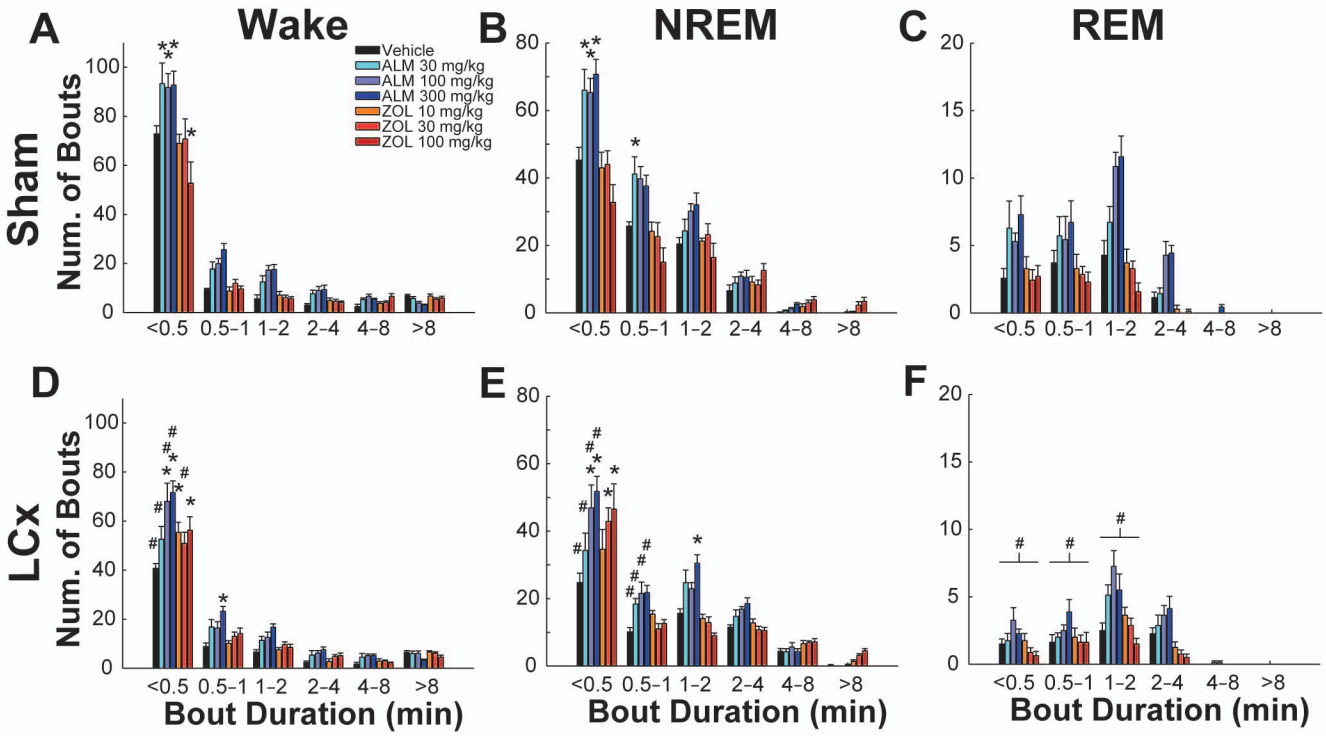
A
Wake
B
NREM
C
REM
Sham
LCx
Num. of Bouts
Vehicle
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ALM 100 mg/kg
ALM 300 mg/kg
ZOL 10 mg/kg
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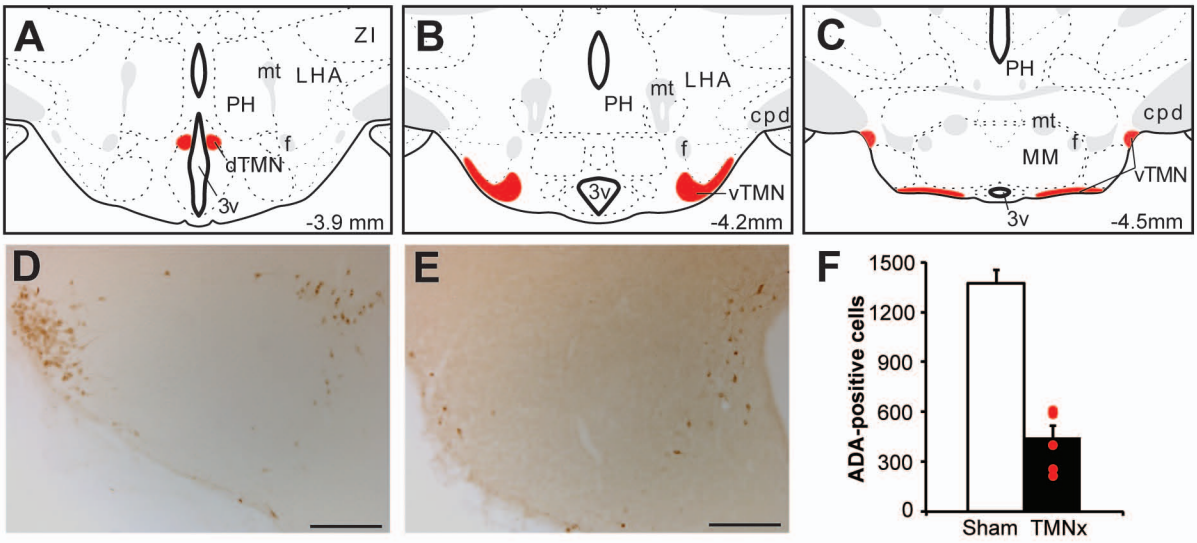
ZOL 100 mg/kg
<0.5 0.5-1 1-2 2-4 4-8 >8
Bout Duration (min)
D
E
F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LHA
PH
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dTMN
3v
-3.9 mm
B
PH
mt
LHA
cpd
f
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-4.2mm
C
PH
mt
cpd
f
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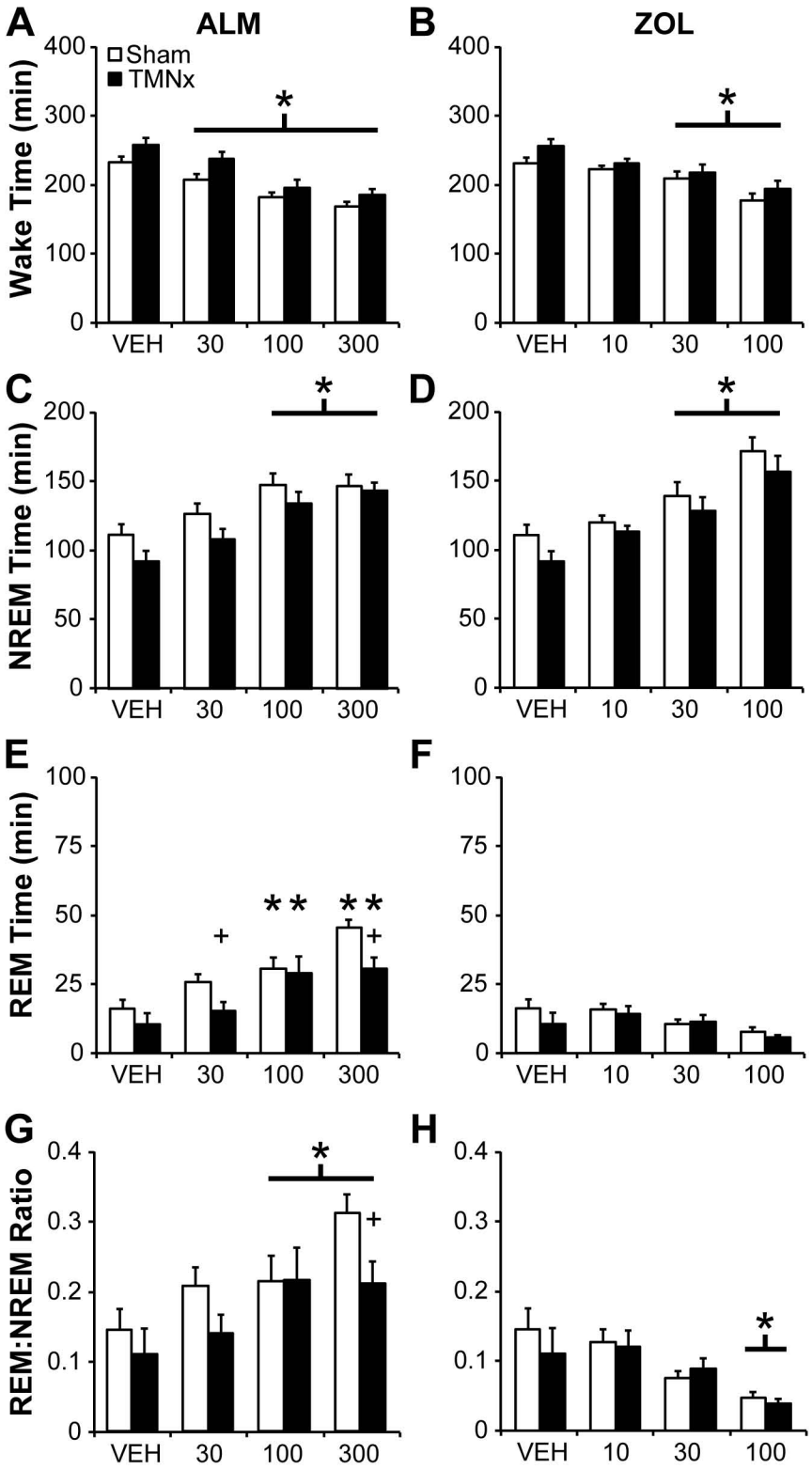
Sham
TMNx


A
ALM
B
ZOL
Sham
TMNx
Wake Time (min)
NREM Time (min)
REM Time (min)
REM:NREM Ratio
VEH
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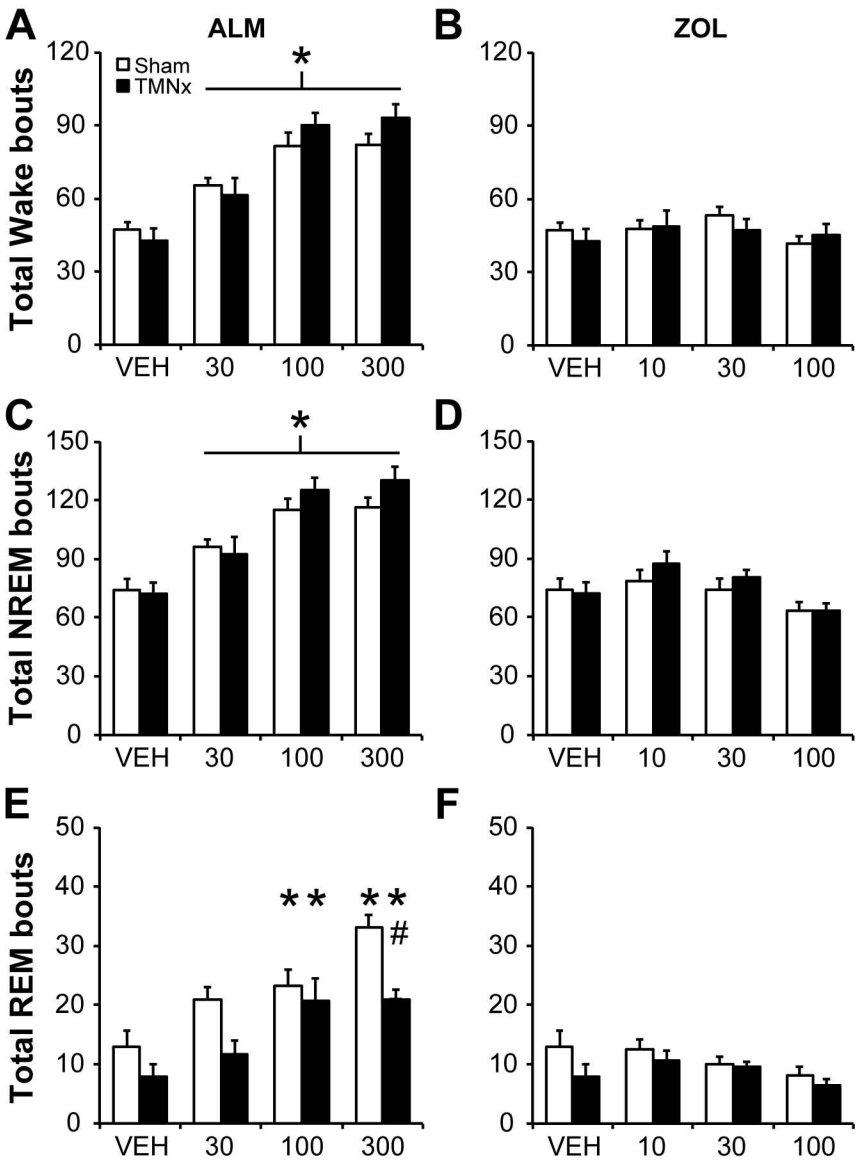
A
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B
ZOL
Sham
TMNx
Total Wake bouts
Total NREM bouts
Total REM bouts
VEH
10
30
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