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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

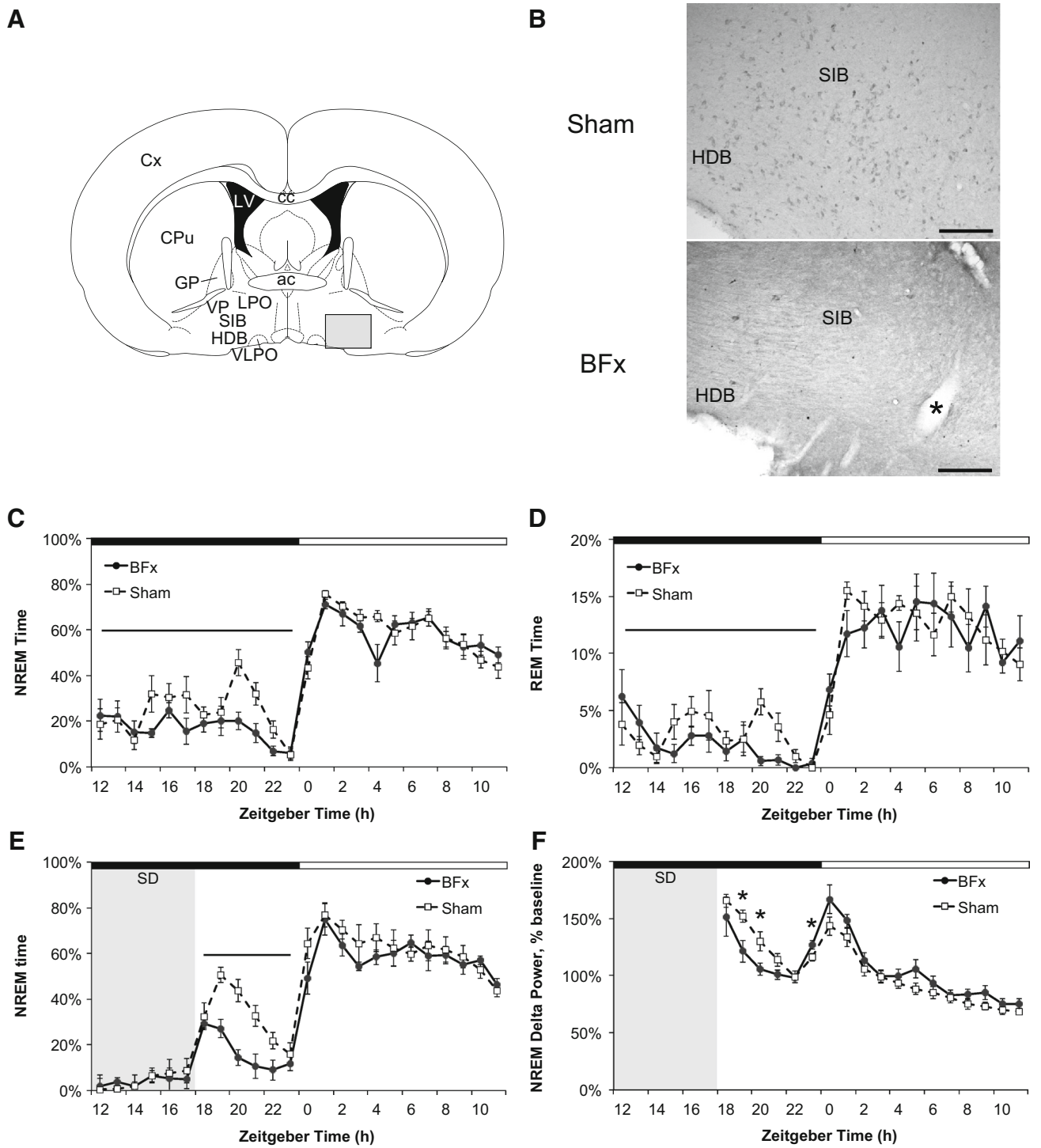


Fig. 1 BF lesions decrease sleep time in the dark phase. **a** Schematic of rat brain (Paxinos and Watson 2007) showing the BF target area (*gray box*). **b** ChAT immunoreactivity (-ir) in the BF of rats given bilateral microinjections of sterile saline (*upper panel*) and 192-IgG-saporin (*lower panel*). 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

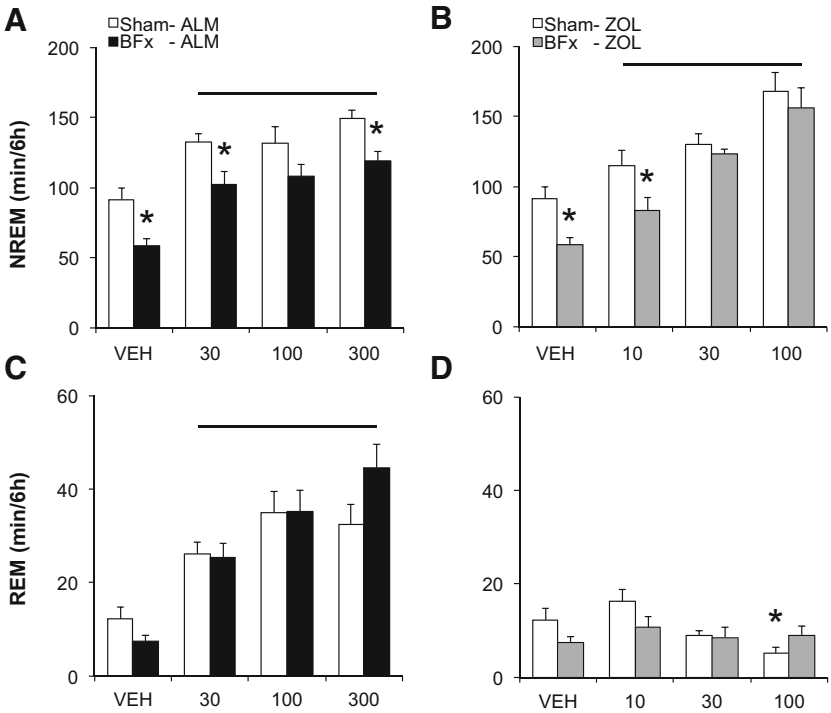


Fig. 2 BF lesions attenuate efficacy of ALM, but not ZOL. **a**, **b** NREM sleep time in BFx and Sham rats following VEH, ALM or ZOL at doses (mg/kg) indicated on x-axes. ALM and ZOL data were graphed separately for clarity; VEH data are repeated for comparison. **a** ALM increased NREM sleep time compared 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

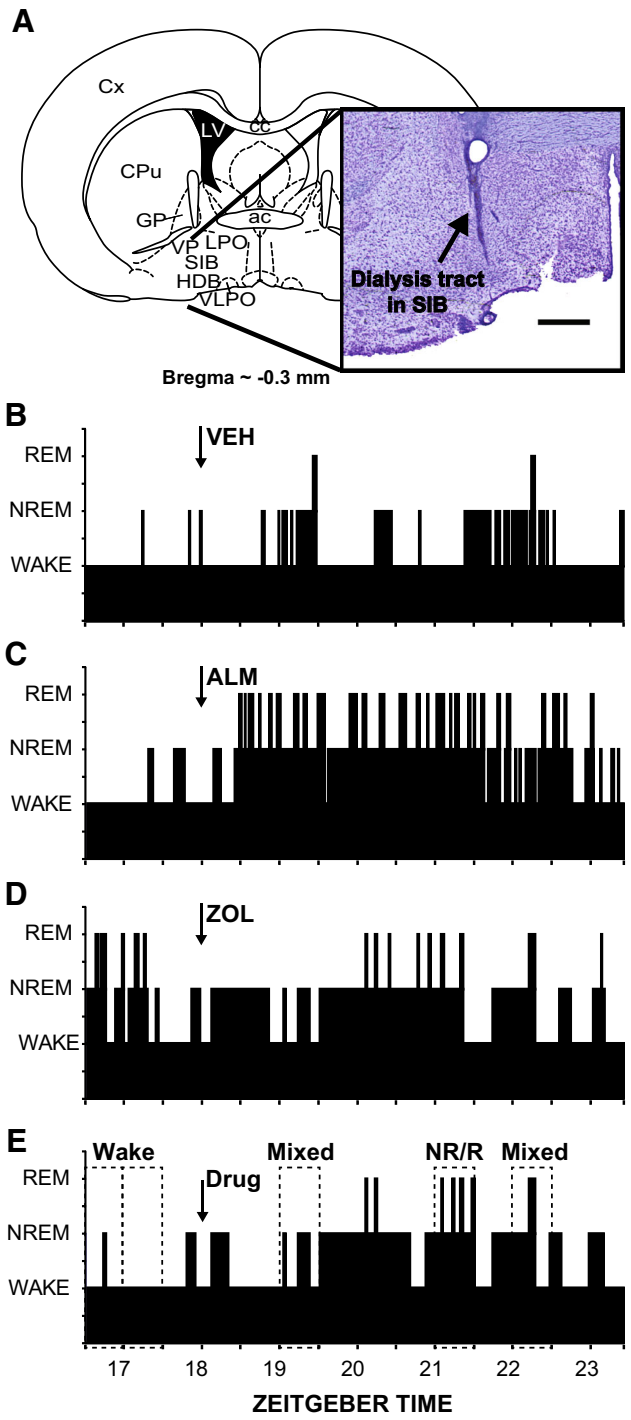


Fig. 3 Dialysis probe placement in the BF and sleep–wake architecture following drug delivery. **a** Representative probe tract shown in brain with arrow indicating dialysis probe was located within the substantia innominata. Hypnograms in **b** through **d** present the effects of orally delivered VEH (**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 ($P < 0.05$).

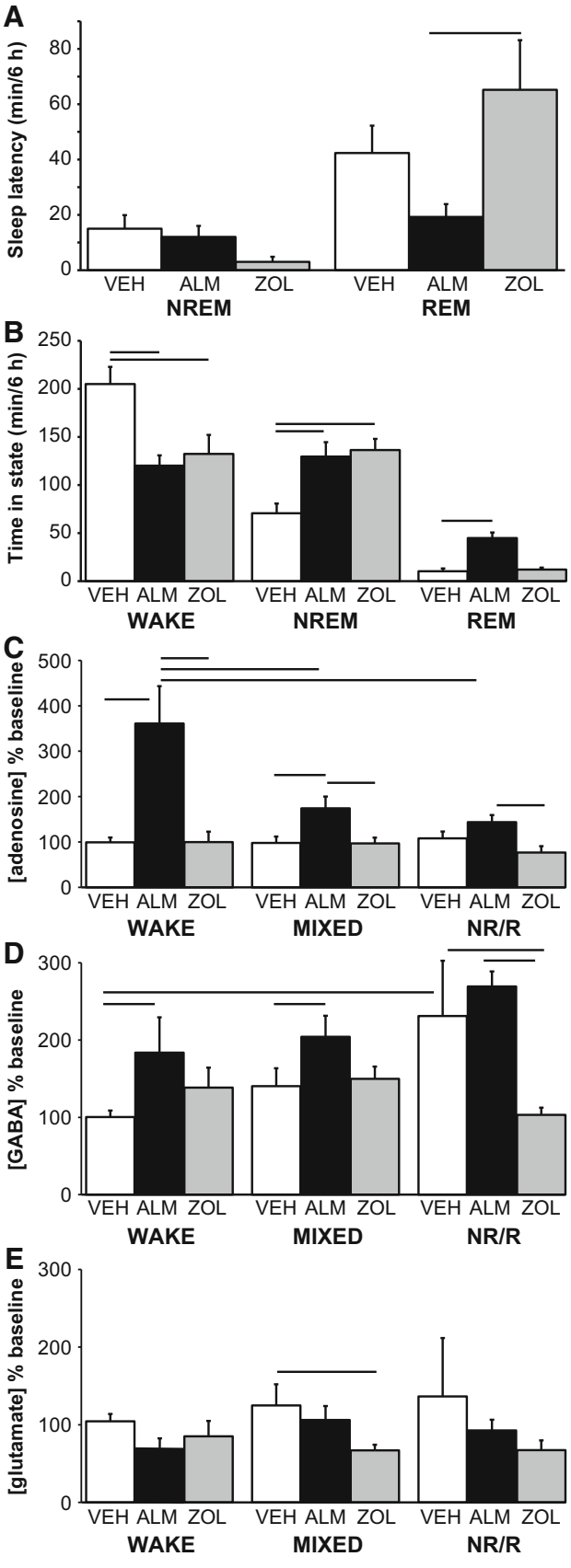
GLU levels in the BF were significantly reduced by ZOL during MIXED states compared to VEH ($F_{2,85} = 3.7$; $P = 0.02$; Fig. 4e). There were no other effects of any drug on GLU.

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.

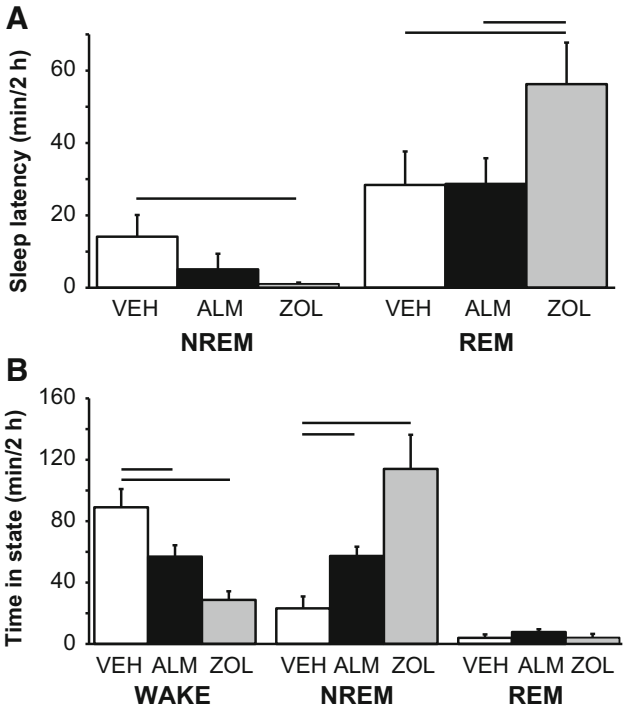


Fig. 5 Effects of oral drug administration on sleep–wakefulness during the 2-h RS opportunity after cessation of SD. **a** Latency to NREM and REM sleep after 6 h of SD following ALM (100 mg/kg) or ZOL (100 mg/kg). *Horizontal lines* indicate that ZOL significantly decreased the latency to the onset 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

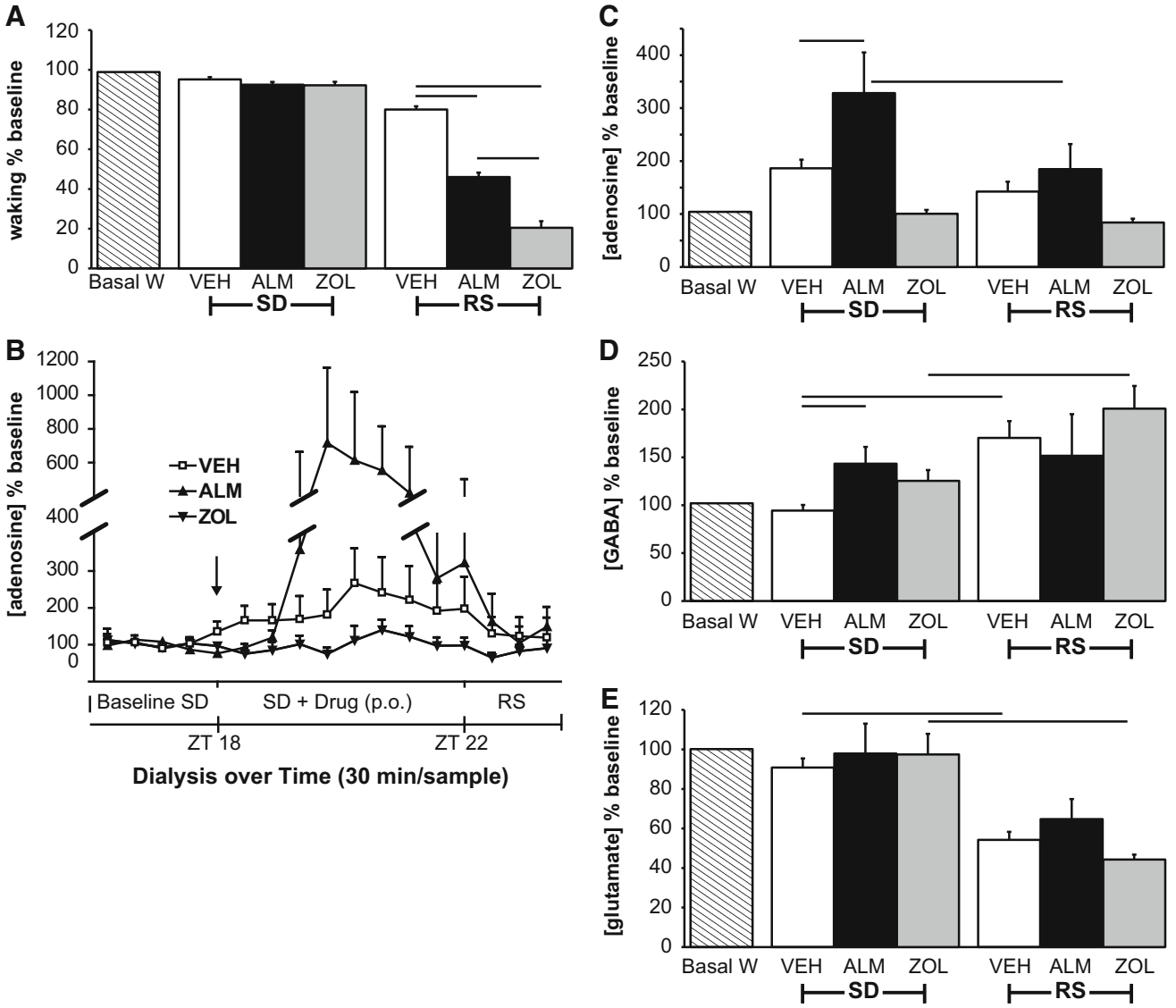


Fig. 6 BF ADO increases during SD and is affected by oral drug administration. **a** When rats are given ALM or ZOL, waking time during RS is significantly reduced relative to VEH ($P < 0.05$). **b** BF ADO levels rose during SD after VEH (*open squares*) administration relative to baseline levels of 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

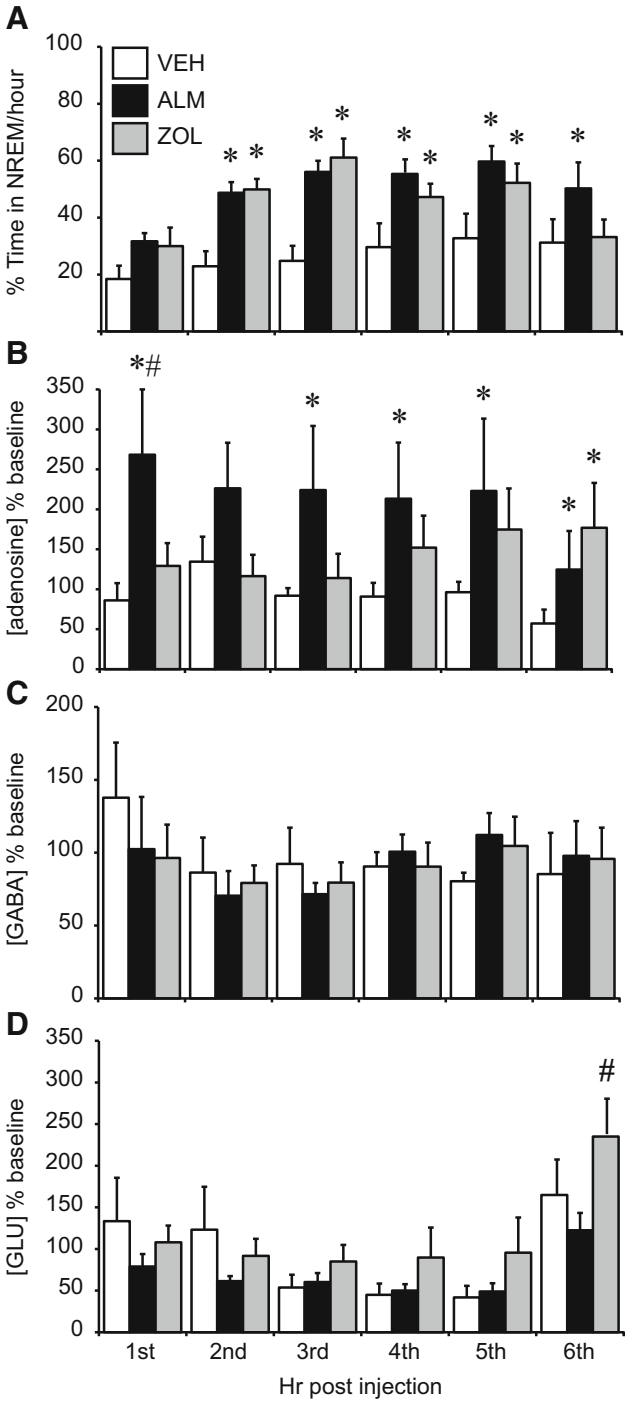


Fig. 7 BF microinjections induce sleep and facilitate cortical ADO release by ALM. **a** Percent time in NREM sleep (mean ± SEM) following BF microinjection of VEH, ALM or ZOL. *Asterisk* denotes significant drug effects ($P < 0.05$) relative to VEH. **b** BF administration of ALM significantly increased 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

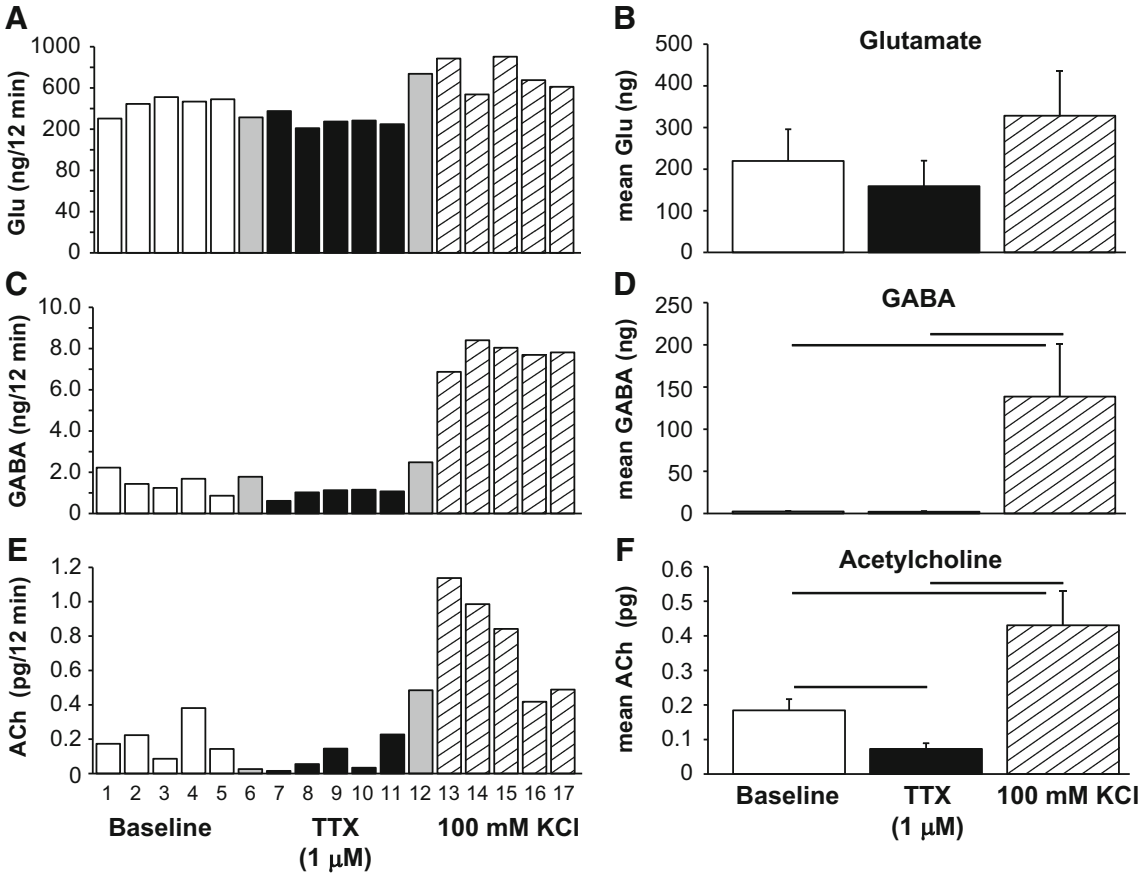


Fig. 8 BF neurotransmission in response to reverse dialysis of TTX under steady-state conditions. **a**, **c**, **e** Effects of TTX (1 μM; reverse dialysis) in the BF of an anesthetized rat. The *x*-axes show the temporal distribution of BF GLU (**a**), GABA (**c**), and ACh (**e**) levels collected 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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