Published in final edited form as:

Neuroscience. 2008 January 2; 151(1): 1–11. doi:10.1016/j.neuroscience.2007.09.051.

Administration of the protein synthesis inhibitor, anisomycin, has distinct sleep-promoting effects in lateral preoptic and perifornical hypothalamic sites in rats

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Abstract

Although a robust relationship between sleep and increased brain protein synthesis is well-documented, there have been few reports of the effects of local application of a protein synthesis inhibitor (PSI) on sleep. In this study, we compared the effects of local microdialytic administration of the protein synthesis inhibitor, anisomycin (ANI) into the lateral preoptic area (LPOA), a sleep promoting area vs. the perifornical/lateral hypothalamus (PF / LH), a wake and rapid eye movement (REM) sleep-promoting area. ANI administered to the LPOA at night resulted in an increase in stage 2 of rat non-REM sleep, whereas ANI delivered into the PF/LH during the daytime increased REM sleep. ANI microdialysis into hippocampus did not affect sleep or waking. These differential effects of local protein synthesis inhibition on sleep support a hypothesis that mechanisms controlling protein synthesis are critically involved in the regulation of both NREM sleep and REM sleep.

Keywords

microdialysis; protein synthesis inhibitor; sleep/wake; site specificity

Several findings support a hypothesis that one function of sleep is to facilitate brain protein synthesis (Oswald, 1978, Adam and Oswald, 1983, Horne, 1983). A relationship between sleep and increased brain protein synthesis is well-documented. Protein synthesis rates as measured by L-[1-¹⁴C] leucine uptake were positively correlated with slow wave sleep (SWS) in discrete brain regions of rat (Ramm and Smith, 1990), monkey (Nakanishi et al., 1997) and near-term ovine fetus (Czikk et al., 2003). A higher cerebral protein synthesis rate was associated with a higher amount of SWS (Ramm and Smith, 1990). Sleep is associated with increased expression of mRNAs of two key proteins facilitating protein synthesis through translational control (Cirelli et al., 2004, Cirelli, 2005). SWS at sleep onset facilitates secretion of growth hormone (GH) which promotes protein synthesis (Van Cauter and Copinschi, 2000). Sleep deprivation reduces the protein content in dorsal raphe neurons (Panov and Malikov, 1981, Panov, 1982), and the levels of many proteins in mouse cerebral cortex (Ding et al., 2004) and rat basal

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Section Editor: Gregory J. Quirk, Department of Physiology, Ponce School of Medicine (Ponce, PUERTO RICO)

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forebrain (Basheer et al., 2005). We have found that 48 h sleep deprivation reduces protein levels of brain-derived neurotrophic factor in hippocampus (Guzman-Marín et al., 2006).

A related hypothesis is that mechanisms underlying regulation of protein synthesis plays a role in the regulation of sleep. Consistent with this are the following findings: (1) GH administration increases REM sleep in rats (Drucker-Colin et al., 1975) and (2) exogenous application of growth hormone releasing hormone (GHRH), the hypothalamic neurohormone stimulating GH secretion, promotes non-rapid eye movement (NREM) sleep in various species, while suppression of endogenous GHRH results in simultaneous inhibition of NREM (Obal and Krueger, 2004). Several early studies employed the use of protein synthesis inhibitors (PSIs) to examine this question. In general, PSI administration increased NREM sleep and reduced REM sleep. However, the specific results varied depending on the species, the inhibitor used, and the mode of administration. In mice, sub-cutaneously administered cycloheximide (Cyc) significantly increased NREM while decreasing REM and wake (Pegram et al., 1973).

Intracerebroventricular (i.c.v.) administration of Cyc in cats produced an initial and transient (one day) decrease in waking and increase of slow wave sleep (SWS) followed by prolonged (7-10 days) elevation of REM sleep (Stern et al., 1972). In rats, Cyc given i.c.v. suppressed REM sleep; intraperitoneally it reduced both REM and deep NREM sleep (Uezu et al., 1981). Intraperitoneal (i.p.) chloramphenicol in rats decreased SWS and REM sleep and reduced brain glucose utilization (Moulin-Sallanon et al., 2005). Anisomycin (ANI) administration decreased REM sleep in rats (Rojas-Ramirez et al., 1977). REM sleep frequency and the neuronal discharge of midbrain reticular formation were reduced by i.p. and oral administration in cats of anisomycin or chloramphenicol (Drucker-Colin et al., 1979); similar results were obtained with the local delivery of chloramphenicol into brain stem reticular formation. These findings do not permit a clear conclusion about the role of protein synthesis in sleep regulation.

There have been very few studies of the effects on sleep of local application of PSI. We compared the effects of local administration of a PSI in a sleep-promoting versus a wake-promoting area. We hypothesized that such comparison would allow us to differentiate between specific sleep-promoting actions and more unspecific actions. Therefore, we compared the effects on sleep of local microdialytic administration of the protein synthesis inhibitor, anisomycin (ANI), into the lateral preoptic (LPOA), a sleep-promoting site and perifornical/lateral hypothalamic (PF/LH) area, a wake/REM promoting region. These two regions are known to contain clusters of sleep promoting and wake / REM sleep promoting neurons respectively (Szymusiak et al., 1998, Alam et al., 2002, Verret et al., 2003, Mileykovskiy et al., 2005). We have also administered the ANI into hippocampus (HIPPO), a region not directly linked to sleep regulation to study the site-specificity of the local protein synthesis inhibition.

Experimental Procedures

Animals

Experiments were performed on male Sprague-Dawley rats (Harlan, IN, 250–300 g at the time of surgery), three groups consisting of six rats each, maintained on a 12h:12h light-dark cycle (lights on at 0700 AM and lights off at 1900 PM) with *ad libitum* availability of food and water. All experiments were conducted in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals," and procedures were reviewed and approved by the Internal Animal Care and Use Committee of the Veterans Affairs, Greater Los Angeles Healthcare System.

Surgery

Under aseptic conditions and anesthesia induced by intraperitoneal administration of a mixture of ketamine HCl (80 mg/kg) and xylazine (8 mg /kg), rats were surgically prepared for polygraphic recording of sleep and waking states as previously described (Methippara et al., 2005). Briefly, four gold plated stainless steel machine screws with soldered leads were threaded through holes made over the parietal and frontal cortices for EEG recording; three Teflon coated stranded stainless steel wires were hooked into the dorsal neck muscles for EMG recording. A screw electrode threaded onto the nasal bone was used as the ground. All electrodes were soldered to a standard connector. A 23 G cannula as a guide for the microdialysis probe was stereotactically implanted unilaterally 1–2 mm above either the LPOA (AP = - 0.4 mm; DV = 8.8 mm; L = 1.3 mm from bregma), PF/LH (AP = - 3.1 mm; DV = 8.6 mm; L = 1.4 mm from bregma) or HIPPO (AP = - 4.16 mm; DV = 3.2 mm; L = 2.6 mm from bregma) (Paxinos and Watson, 1998). The guide cannula was kept patent by inserting a removable obturator stylet. The plug with the soldered electrodes and the guide cannula were affixed on the skull with dental acrylic. Post-operatively, the analgesic Buprenex (0.02 mg/Kg; i.m.) was administered twice daily for two days. A topical antibiotic was applied around the incision twice daily for 5 days.

Sleep Recording and Histology

After surgery, animals were housed individually in Plexiglas cages placed in an electrically shielded, sound -attenuated, temperature controlled recording chamber (temperature: $25 \pm 2^\circ$ C). During recovery from surgery rats were acclimatized to the recording environment. A week after the surgery and at least 18 hrs before the recording session, the rat was connected to the recording cable, the obturator stylet through the guide cannula was replaced with a microdialysis probe (outer diameter 0.22 mm; semipermeable membrane tip length, 1mm; molecular cut off 50kD; EiCom Corp., Japan) which was fixed in place with dental acrylic. The inlet and outlet ports of the microdialysis probe were connected to low dead volume Teflon tubing (100 cms, EiCom Corp., Japan). The probe was perfused for 2 hrs with artificial cerebrospinal fluid (ACSF) containing Na^+ 145, K^+ 2.7, Mg^{2+} 1.0, Ca^{2+} 1.2, Cl^- 1.5 and Na_2HPO_4 2 (mM ; pH 7.2) at the flow rate of 2 $\mu\text{L}/\text{min}$ by a peristaltic pump kept outside the recording chamber. On the day after the probe insertion, either ACSF as a control treatment or ANI was microdialyzed in a random order for two hrs followed by 18 hrs of ACSF. ANI was administered at either ZT3 or at ZT15 in the two groups of rats (LPOA and PF / LH) and at ZT15 alone in the third group (HIPPO); ACSF control studies were performed in the same rats at ZT 3 or ZT 15 on another day.

Although several earlier studies had used ANI doses exceeding 300 mM (Barrientos et al., 2002, Luft et al., 2004), more than 80 % protein synthesis inhibition was induced by intracerebral microinjections of both 377 mM (Luft et al., 2004) or 40 nM of ANI (Barea-Rodriguez et al., 2000), indicating a possible plateau of the drug's action in sub - mM range of ANI concentration. In addition, any dose of ANI higher than 20 mM was found to induce lesions in cerebellum (Bracha et al., 1998). Therefore, in this study, two ANI doses, 500nM and 5 μM , were used taking into account the typical 10/1 concentration gradient between inside and outside of the microdialysis probes. Rats with microdialysis probes in the LPOA were microdialysed with both the doses of ANI (500 nM and 5 μM) while the rats with probes implanted in the PF/LH or HIPPO were administered with only 5 μM ANI. All rats in the LPOA group ($n = 6$) thus received 2 concentrations each of ANI at ZT 3 and ZT 15 for a total of four ANI perfusions, whereas rats in the PF/LH group ($n = 6$) received two ANI treatments (ZT 3 and ZT 15) at one dose, and rats in the HIPPO group ($n = 6$) received only one ANI treatment at ZT 15. Each rat received only one treatment (either ACSF or one dose of ANI) during 24 hrs. ANI was initially dissolved in dimethyl sulfoxide (DMSO) to make a 75 mM stock solution which was diluted with ACSF to aliquots of the final concentrations; the aliquots

were kept in - 20 ° C until use. The amounts of DMSO in the final dilutions were 6.6 nL (0.006 % DMSO) and 66 nL (0.06 % DMSO) in 1 ml of 500 nM and 5 μ M of ANI respectively. Since sleep was unaffected by peripheral administration of 5 or 10 % DMSO (Cavas et al., 2005) or intra-preoptic microinjection of 25% DMSO (Ramesh and Kumar, 1998), microdialysis with DMSO alone was not undertaken in this study.

Sleep-wake parameters based on the EEG and EMG signals were continuously recorded during the microdialysis as follows. EEG signals were amplified and band pass filtered at 0.3 -100 Hz, and nuchal EMG was recorded after band pass filtering at 10 -300 Hz (Model 78 D, Grass Instruments, Quincy, MA). All signals were analog-to-digital converted using a 1401 Plus data acquisition interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. Polygraphic data were digitized at a sampling rate of 128 Hz for EEG and 256 Hz for EMG.

After recordings were completed, rats were intraperitoneally injected with heparin (500 U), followed by sodium pentobarbital anesthesia (100 mg/kg). The animals were transcardially perfused with 50 ml of PBS (pH 7.4) followed by 300 ml of 4% paraformaldehyde. The brains were removed and kept for a day in 10% sucrose in PBS and transferred to 30% sucrose until they settled at the bottom. The brains were cut on a freezing microtome through the coronal plane in 40 μ M thick serial sections and stained for Nissl (cresyl violet) for verifying the implantation sites of the microdialysis probes.

Data Analysis

As previously described (Methippara et al., 2005), based on the EEG and EMG patterns, polysomnographic data were scored manually at 10 s intervals into five sleep-wake stages: active waking (AW), quiet waking (QW), slow-wave sleep 1 (SWS1), slow-wave sleep 2 (SWS2) and rapid eye movement (REM) sleep. The amount of each of the five sleep-wake stages represented as the percentage of the recording duration, the total number of episodes and the mean episode duration of each of the stages were determined for the first six hours of microdialysis. In addition, REM latency was determined as the time taken for the appearance of the first REM episode of duration of 10 sec or more after the microdialysate reached the animal. All sleep data were averaged in bin sizes of 2 or 4 hrs.

Since the increase in EEG delta power during recovery sleep is a strong indicator of the homeostatic regulation of sleep (Franken et al., 1991), the EEG was subjected to fast Fourier transformation analysis using a Spike 2 script. Spectral power in the delta (0.75– 4.0 Hz) band of the NREM sleep and the theta range (4.5 – 8.0 Hz) of REM sleep was calculated as follows. NREM segments of at least 90 sec duration occurring after wake episodes of 60 sec or more duration were identified. Delta power was calculated from segments of 30 sec or more beginning 30 sec after the onset of the NREM. Delta power from several such NREM segments was averaged during the initial 4 hr of treatment (2hr ANI + 2hr ACSF). REM episodes of 60 sec or more in duration were identified and theta power was averaged from the middle third portion of these episodes. The spectral data during the drug perfusion were calculated as the percentage of the corresponding data obtained during the ACSF perfusion.

Sleep data were analyzed for statistical significance of treatment effects using either paired *t* test or one way repeated measures analysis of variance (one way RM ANOVA) followed by Holm-Sidak post hoc test. All results are expressed as mean $\pm$ SEM.

Results

Histology of coronal brain sections showed that six rats in each group had microdialysis probes terminating in the LPOA, PF/LH or HIPPO (Fig 1). In the LPOA, the probe tips were between

stereotaxic planes 0.26 mm and 0.40 mm posterior to bregma. In the PF/LH, the probe tips were located between planes 2.80 mm and 3.30 mm posterior to bregma. In the hippocampus, probes were found between the planes 3.80 mm and 4.52 mm posterior to bregma (Paxinos and Watson, 1998).

In the LPOA, night time administration of 5 μ M ANI significantly increased SWS2 during the 2 hr period of administration and in the subsequent 10 hr of post-drug ACSF perfusion period, for a total of 12 hr. REM was significantly increased during the first 2 hrs, and AW was decreased for the initial 6 hrs and during hrs 9-10 (fig 2, 3). The increase in SWS2 was paralleled by increased delta power (Fig 4). The 500 nM concentration increased SWS2 for each 2 hr recording period after the initial 2 hrs for a total of 10 hrs, and significantly reduced AW for the initial 6 hrs, and for hrs 9-10 (Fig 3). The increase in SWS2 during the 2 hr of 5 μ M ANI microdialysis was significantly greater than that of 500 nM of ANI ($p < 0.008$). The facilitation of sleep within the first 4 hrs of microdialysis induced by 5 μ M ANI was due to the significant increase in the number of episodes of SWS 1 ($p < 0.012$), SWS 2 ($p < 0.001$) and REM ($p < 0.05$; Fig. 7) and not by the changes in the duration of sleep episodes (data not shown). In addition, night time 5 μ M of ANI significantly reduced ($p < 0.001$) the REM latency for 4 hrs (Fig. 8), although the EEG theta power during the REM remained unaffected (Fig. 4). The above effects were not observed when ANI was administered during the day (Fig. 6).

In the PF/LH, night time administration of ANI did not affect any of the sleep-wake stages (Fig. 6). On the other hand, microdialysis of 5 μ M of ANI during the day induced a significant increase ($p < 0.046$) in REM sleep and in the EEG theta power during the REM sleep (Fig 4) without affecting the amount of any other sleep - wake stages (Fig 5, 6), although ANI increased ($p < 0.006$) the number of AW episodes for the first 4 hrs (Fig 7). The promotion of REM sleep was due to the increase in the frequency of REM sleep episodes (Fig 7) and not due to changes in the duration of those episodes (data not shown). 5 μ M of ANI also significantly ($p < 0.001$) reduced the REM sleep latency (Fig 8). ANI administration into HIPPO did not significantly affect any of the sleep-wake stages for the entire duration of the recording (Fig 9).

In summary, a comparison of the sleep amounts for 4 hrs after the beginning of the ANI microdialysis showed that administration of 5 μ M ANI into the LPOA induced an increase in SWS2 along with an increase in EEG delta power during SWS 2, whereas in the PF / LH, ANI induced an increase in the amount of REM sleep and theta power in REM (Fig 4, 6). ANI delivery in the non-sleep related area of HIPPO did not have any effect on sleep (Fig 9).

Discussion

In this study, local induction of PSI by the administration of ANI resulted in differential effects on sleep, dependent on the site of administration. ANI administered to the LPOA resulted in an increase in SWS2 of NREM sleep, whereas in the PF/LH ANI administration increased REM sleep. This differential effect of local PSI on sleep has not been reported previously. The application of ANI induced an increase in the number of sleep episodes without affecting the duration of individual episodes. This finding suggests that a reduction in protein synthesis could be involved in initiating sleep. The administration of ANI accelerated the appearance of REM sleep in both LPOA and PF/LH.

The increase in SWS2 induced by the ANI microdialysis in this study is consistent with the earlier report showing that peripheral PSI administration increased deep sleep (Uezu et al., 1981). In addition, the increase of REM sleep found in this study is consistent with a similar effect observed after i.c.v. or i.p administration of PSIs (Stern et al., 1972), and is also supported by the previous studies showing that PSI - induced alterations in REM sleep is due to the changes in frequency and not the duration of REM sleep episodes. However, this study differs

from the previous reports which showed a PSI induced decrease in REM sleep (Pegram et al., 1973, Drucker-Colin et al., 1979, Drucker-Colin et al., 1982). The difference could be due to the fact that PSI administration in these earlier studies were either systemic (Drucker-Colin et al., 1979, Uezu et al., 1981) or locally applied in the brain stem reticular formation (Drucker-Colin et al., 1982).

The sleep state facilitated by PSI in a particular site may depend on the predominant type of sleep normally regulated by the site, specifically both SWS and REM sleep by the LPOA (Szymusiak et al., 1998, Lu et al., 2002) and REM sleep by the PF/LH (Alam et al., 2002, Methippara et al., 2003, Verret et al., 2003). Both sites contain mixtures of wake-active and sleep-active neurons (Szymusiak et al., 1998, Estabrooke et al., 2001, Alam et al., 2002, Methippara et al., 2003). SWS - active neurons are more numerous in the LPOA, but they are intermixed with other neuronal types and probably constitute only about 20% of neurons in this region (Szymusiak et al., 1998). Although the PF/LH contains a population of wake promoting orexin (ORX) neurons (Eggermann et al., 2003, Lee et al., 2005), this region also contains a large population of MCH neurons which are specifically activated during sleep, particularly REM sleep (Modirrousta et al., 2005). The increase in REM sleep induced by ANI application at the PF/LH could potentially be mediated by the activation of MCH neurons present in the area. Effects of local administration of PSI into the brainstem reticular formation may depend on the critical state-related cell types at the injection sites, perhaps suppressing the activating process essential for REM sleep. However, the effects of PSI administration are likely to be complex and will require analysis at an electrophysiological and molecular level.

We found that ANI application to HIPPO had no effect on sleep-wake states. The HIPPO is not a primary sleep-regulatory site. We interpret our finding as showing that the effects of PSI on sleep are mediated specifically by sleep-regulatory sites. Although PSI application in HIPPO is likely to affect local protein synthesis and hippocampal function, and sleep may facilitate synthesis of plasticity-related proteins such as brain derived neurotrophic factor (BDNF) in HIPPO (Guzman-Marin et al., 2006), our findings suggest that these interactions are not critically related to the control of sleep-wake states.

Our findings suggest that the PSI effects are not readily explained by global neuronal inactivation or depolarization since previous reports of neuronal inhibition with GABA or adenosine agonists of LPOA (Lin et al., 1989; Methippara et al., 2005) or PF/LH (Thakkar et al., 2003) produced effects on sleep different from our current findings. It is more parsimonious to hypothesize that the suppression of protein synthesis generates a sleep need and activates sleep-promoting actions, with the predominant effects depending on the sleep state regulated by the region in question.

Although ANI was microdialysed both during day and night in the LPOA and PF / LH, sleep was significantly affected when it was administered into LPOA only at night and into the PF/ LH only during the day. ANI administration was ineffective at the other respective phases (day for LPOA group and night for PF / LH group) and in the non-sleep related area of hippocampus. The actions of SWS-promoting agents are often best demonstrated in the dark phase, since SWS is already at high levels in the light phase (Trachsel et al., 1986). On the other hand, the facilitation of REM may depend on a background of high sleep-propensity, since REM sleep timing and expression may be a function of NREM sleep expression (Benington and Heller, 1994, Benington and Heller, 1994).

Increased protein synthesis during SWS (see introduction) may be attributed to the increased availability of cellular 'energy charge' that favors anabolic restoration of components by protein synthesis (Horne, 1983). The increased anabolism during sleep is consistent with the previous findings of reduction in the circulating levels of anabolic hormones including GH

induced by sustained sleep deprivation (Everson and Crowley, 2004), and the evidence of a strong positive correlation between the deep sleep and protein synthesis in many brain regions (Ramm and Smith, 1990, Nakanishi et al., 1997). This is also consistent with the finding that sleep deprivation induces arrest of protein translation in mouse cortex (Naidoo et al., 2005).

ANI is known to inhibit protein synthesis by binding to the ribosomal peptidyl transferase site thereby preventing peptide bond formation (Rodriguez-Fonseca et al., 1995, Ioannou et al., 1998). This finding along with the conclusion from this study, that the direct application of ANI in hypothalamic sleep regulatory sites activates the predominant sleep-promoting action of the site, supports a hypothesis that mechanisms controlling protein synthesis are critically involved in the regulation of both NREM and REM sleep regulating neurons. Since local PSI in sleep-wake regulatory sites increase sleep, a need for protein synthesis may normally promote sleep, and sleep states may persist until completion of critical protein synthesis. Because ANI is a global protein synthesis inhibitor, this study cannot indicate which proteins are critical for the normal regulation of arousal states by the neurons of the respective regions. However, previous studies showed that sleep favors synthesis of specific functional classes of proteins, especially cytoskeletal and synaptic proteins (Basheer et al., 2005, Vazquez et al., 2005) as well as proteins acting as molecular chaperones involved in proper protein folding, especially several heat shock proteins (Shaw et al., 2002, Terao et al., 2003, Naidoo et al., 2005, Terao et al., 2006). Therefore, local PSI could preferentially affect these classes of proteins. ANI may also be inhibiting the local generation of specific proteins that were found previously to be endogenous wake-promoting or REM-inhibiting factors. These include NREM-inhibiting corticotrophin-releasing hormone, or REM-inhibiting cytokines such as IL- β (Garcia-Garcia and Drucker-Colin, 1999). The findings of this study together with the previous reports on the sleep deprivation-induced alterations in the molecular components of protein synthesis (Cirelli et al., 2004, Naidoo et al., 2005) lead to a hypothesis that increased brain protein synthesis is not only a correlate of sleep, but that the molecular mechanisms regulating protein synthesis may play a central role in the regulation of sleep.

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Fig. 1.

Brain sections showing the sites of tips of the microdialysis probes and the distribution of microdialysis perfusion sites in the stereotaxic planes of (A) LPOA (B) PF/ LHA and (C) HIPPO. Arrows and solid circles indicate the microdialysis probes' tips in the brain. 3V = third ventricle; ac = anterior commissure; fx = fornix; CA1, CA2, CA3 = Hippocampus; Ctx = cortex; LH = lateral hypothalamus; LPOA = lateral preoptic area; ox = optic chiasm.

Fig. 2. Hypnograms from a representative rat comparing the effects of night time (ZT 15) microdialysis of ACSF and ANI into the LPOA. Dark and light bars at the bottom represent ANI and ACSF perfusion respectively.

Fig. 3. Effects on sleep/wake stages of 2 hr microdialysis into LPOA of 500nM and 5 μ M of ANI at ZT 15 followed by 10 hr of ACSF perfusion. The light and dark bar at the bottom of the figure indicates L/D cycle. * and @ denote significant differences ($p \leq 0.05$) from the ACSF and # indicates difference from the lower ANI dose. Hatched bar represents the duration of ANI microdialysis and the thick black line denotes ACSF perfusion.

Fig. 4. Effects of 5 μ M of ANI administration into LPOA at ZT 15 and into PF/LH at ZT3 on the delta power of NREM EEG and the theta power of REM EEG. The data shows the percentage difference from the ACSF treatment (at 100%) represented by the black line.

Fig. 5. Hypnograms from a representative rat comparing the effects of day time (ZT 3) microdialysis of ACSF and ANI into the PF/LH. Dark and light bars at the bottom represent ANI and ACSF perfusion respectively.

Fig. 6. Comparison of effects on sleep/wake stages of microdialysis of 5 μM of ANI or ACSF into LPOA and PF/LH at ZT 3 (day) and ZT 15 (night). * indicates a significant difference ($p \leq 0.05$) from the ACSF.

Fig. 7. Effects on the number of sleep/wake episodes of 5 μ M of ANI microdialyzed at night (ZT 15) into LPOA and into the PF/LH in the daytime (ZT 3). * indicates a significant difference ($p \leq 0.05$) from the ACSF.

Fig. 8. Effects on REM latency in seconds of 5 μ M of ANI microdialysis into LPOA and PF/LH at both ZT 3 (open bars) and ZT15 (hatched bars). * indicates a significant difference ($p \leq 0.05$) from the ACSF. White and gray bars represent ACSF and ANI respectively.

Fig. 9. Effects on sleep/wake stages of 2 hr microdialysis into HIPPO of 5 μ M of ANI at ZT 15 followed by 10 hr of ACSF perfusion. The data are represented in 4 hr bins.