Monoamine Release during Unihemispheric Sleep and Unihemispheric Waking in the Fur Seal

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Study Objectives: Our understanding of the role of neurotransmitters in the control of the electroencephalogram (EEG) has been entirely based on studies of animals with bilateral sleep. The study of animals with unihemispheric sleep presents the opportunity of separating the neurochemical substrates of waking and sleep EEG from the systemic, bilateral correlates of sleep and waking states.

Methods: The release of histamine (HI), norepinephrine (NE), and serotonin (5HT) in cortical and subcortical areas (hypothalamus, thalamus and caudate nucleus) was measured in unrestained northern fur seals (Callorhinus ursinus) using in vivo microdialysis, in combination with, polygraphic recording of EEG, electrooculogram, and neck electromyogram.

Results: The pattern of cortical and subcortical HI, NE, and 5HT release in fur seals is similar during bilaterally symmetrical states: highest in active waking, reduced in quiet waking and bilateral slow wave sleep, and lowest in rapid eye movement (REM) sleep. Cortical and subcortical HI, NE, and 5HT release in seals is elevated during certain waking stimuli and behaviors, such as being sprayed with water and feeding. However, in contrast to acetylcholine (ACh), which we have previously studied, the release of HI, NE, and 5HT during unihemispheric sleep is not lateralized in the fur seal.

Conclusions: Among the studied neurotransmitters most strongly implicated in waking control, only ACh release is asymmetric in unihemispheric sleep and waking, being greatly increased on the activated side of the brain.

Commentary: A commentary on this article appears in this issue on page 491.

Keywords: unihemispheric sleep, norepinephrine, histamine, serotonin, acetylcholine, fur seal, marine mammals


INTRODUCTION

Cetaceans (whales and dolphins) never show bilateral slow waves as seen in most land mammals. Instead they display only unihemispheric sleep (USWS), with the other hemisphere awake (UW). Cetaceans sleep with one eye closed while the other eye is open. Cetaceans also do not appear to exhibit rapid eye movement (REM) sleep.1,2 Fur seals (pinnipeds of the family Otariidae) are semiaquatic mammals. They sleep both on land and in water (Figure 1A). Fur seals have bilateral slow wave sleep (BSWS) and REM sleep as seen in land mammals and USWS/UW as seen in Cetaceans. When sleeping in water, the fur seal sleeps at the surface on the side holding two hind flippers and one fore flipper above the animal in air to reduce heat loss. The fore flipper in water is active and helps to maintain the animal’s posture. The fur seal also positions the head and nostrils above the surface to allow regular breathing. During USWS the hemisphere, which is contralateral to the active flipper, is always in a “waking” (or “activated”) state, characterized by the low voltage EEG pattern typical of waking in all mammals. When sleeping on land the fur seal is motionless lying or sitting on the ground. Both on land and in water, during USWS fur seals often open one eye (contralateral to the waking hemisphere) while the other eye contralateral to the “sleeping hemisphere (the hemisphere with a higher voltage EEG slow wave activity) remains tightly closed.3-5 It has been suggested that maintenance of waking in one hemisphere while in USWS enables vision, movement and allows breathing, while minimizing respiratory aspiration of water in fur seals and cetaceans. It also facilitates thermoregulation, protection of neonates, and predator avoidance.1 Some avian species also display short episodes of EEG asymmetry.6,7

Our knowledge on the neurotransmitters involved in the promotion and maintenance of waking and sleep states has been derived from species with bilaterally symmetrical EEG states, including humans, monkeys, cats, rats, and mice. However, such studies do not indicate whether these transmitters are released in relation to the waking state itself, with its tonic activation of the EEG, or whether the relation is to autonomic control, to motor activity or to emotions occurring within the waking state. Studies of USWS in aquatic mammals allow us to determine which of the many physiological and neurochemical changes seen bilaterally in terrestrial mammals are linked to the EEG-defined state, and which may be related to the behavioral quiescence, cardiorespiratory changes, and sensory input reduction that typically accompany sleep. Our understanding of the mechanisms of USWS in cetaceans is poor due to the “neurotransmitters” having roles, unrelated to EEG arousal. Examining the fur seal, an animal that can sleep with one side of the brain at a time, we found that the release of norepinephrine, histamine and serotonin are minimal and bilaterally symmetric when one side of the brain is asleep and the other awake. Of the transmitters most strongly implicated in waking control, only acetylcholine was elevated on the waking side. This finding provides a fundamental insight into the neurochemistry of waking and sleep.
to the obvious difficulty of experimental studies on dolphins and whales. Considering the similarity between the pattern of sleep in cetaceans and otariids, the fur seal appears to be the ideal species to investigate mechanisms of USWS using the tools of experimental physiological research.

In prior studies we found that cortical acetylcholine (ACh) release in fur seals was maximal during active waking (AW), minimal during BSWS, and intermediate both during quiet waking (QW) and REM sleep. This pattern is similar to that described in land mammals. During USWS, cortical ACh release was strongly lateralized in fur seals, with greater levels in the hemisphere displaying “waking” (lower voltage EEG activity). In a separate study we showed that cortical serotonin (5HT) in fur seals release was maximal during AW, progressively decreased during transition to QW and further during BSWS, and that the release was minimal during REM sleep. In contrast to ACh, cortical 5HT release was not lateralized during USWS in the fur seal. The current study was undertaken to measure cortical and subcortical release of monoamines that have been implicated in the control of waking—histamine (HI), norepinephrine (NE), and 5HT.

**METHODS**

**Animals**

All procedures were approved by the University of California Los Angeles and the Veterans Affairs Greater Los Angeles Healthcare System Committees. All studies were conducted...
in accordance with the National Institute of Health Guide for the Care and Use of Experimental Animals. Experiments were performed at the Utrish Marine Station of the Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences on the Black Sea, Russia. Data were collected from 2 groups of juvenile northern fur seals (Callorhinus ursinus L.), a total of 8 animals (5 males and 3 females, 18–25 kg, 2–3 years). Fur seals were captured on the Commander Islands (the Western Pacific, Russia) one year before the study and were well adapted to captivity. Five days before surgery, each fur seal to be studied was moved to an empty indoor laboratory pool and placed into the microdialysis recording chamber (1.1 × 0.8 × 0.8 m) for 4–6 h each day for 4 days. Fur seals were fed 1.5 kg fish twice per day (between 8:00 and 09:00 and 18:00 to 19:00) and sprayed with water for 10 min after each feeding. Seals typically approached the water stream and appeared to enjoy the spray, which is an accepted husbandry practice for these marine mammals. During the daytime (8:00 to 20:00), the enclosure was illuminated by artificial light (400–500 lux at floor level); at night the level of illumination was reduced to ≤ 50 lux. Room temperature during recording ranged from 15–25°C, following the adjacent Black Sea environment.

**Surgical Procedures**

Surgical procedures have been previously described in detail. Briefly, in addition to EEG, EMG (electromyogram), and EOG (electrooculogram) electrodes, 1 to 4 pairs of guide cannulas sealed with stylets (CMA Guide Cannulas, CMA Microdialysis AB, Solna, Sweden) were implanted in symmetrical locations in each hemisphere or in subcortical areas (Figure 2). The length of the cortical guide cannulas was 12 mm and the length of subcortical cannulas was 40, 45, or 50 mm. After implantation, the animal was returned to the indoor enclosure and allowed a minimum of 5 days to recover before insertion of the microdialysis probes through the guide cannulas.

**Microdialysis Procedure**

On the morning of the experiment, the fur seal was lightly anesthetized with isoflurane to replace the stylets with microdialysis probes. The procedure, lasting on average 1.0 h, was conducted at the 0.5% level of isoflurane following a 5–10 min 1.5–2.0% induction dose. Cortical probes had a 4 mm long semipermeable polarylthethersulfone membrane (0.5 mm diameter, 20 kDa cut-off; CMA-12 Elite Microdialysis Probe; CMA Microdialysis AB). All probes were 4 mm longer than the guide cannulas so that the membrane was fully projected out of the guide cannula. Shielded Teflon tubing (1.5 m, TJT-10-150HS; Eicom Corporation, Kyoto, Japan) encased within an additional protective sleeve was connected to the inlet and outlet of each probe. While under anesthesia, the fur seal was connected to the polygraph via a low noise cable. After the implantation procedure, the seal was placed in the recording chamber where it remained unrestrained for the duration of the experiment. Fur seals quickly recovered from anesthesia. They did not show any signs of behavioral impairment by 1 h after the cessation of anesthesia. This short anesthetic procedure did not have significant effect on the parameters of sleep during the recording period, including EEG slow wave power in the range of 1.2–4.0 Hz (slow wave activity, SWA) in the right and left hemispheres and the degree of EEG asymmetry (Figure S1, supplemental material).
The tubing from each probe inlet was connected to a syringe pump (ESP-64; Eicom) and the microdialysis probe was perfused with ACSF (Perfusion Fluid CNS: 147 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl2, and 0.85 mM MgCl2; CMA Microdialysis AB) at a rate of 1.0 µL/min for NE assays and 1.5 µL/min for HI. The tubing from each probe outlet was connected to a fraction collector (EFC-82; Eicom). After a 4-h stabilization period, samples were collected every 10 min over the course of 42 h, on average. Prior to sample collection, 5.0 µL of anti-oxidant solution (20 mM phosphate buffer, pH 3.5, containing 25 mM EDTA-2Na) was added to each sample vial for NE and 7.5 µL of EDTA-2Na (50 mg/L; Dojindo Laboratories), and 5% methanol, was added to each sample vial for HI and 7.5 µL of antioxidant solution (20 mM phosphate buffer, pH 3.5, containing 100 µM EDTA-2Na) for HI. During collection, samples (15 µL or 22.5 µL for NA and HA, respectively) were kept at 4°C (EFR-82 Cooling Unit; Eicom) and each hour they were transferred to a −80°C freezer and stored until analyzed. Probe placements within cortex and subcortical sites were verified histologically.12,13

**Assay of Histamine**

Cortical samples were assayed for HI using high-performance liquid chromatography (HPLC) coupled with post-column derivatization and fluorescence detection. Samples maintained at 4°C were injected into the HPLC system using an autosampler (832 Temperature Regulator, 402 Syringe Pump, 231 XL Sample Injector; Gilson). The mobile phase, consisting of 0.1 M phosphate buffer (pH 6.0) containing sodium-1-octanesulfonate (400 mg/L; Nacalai Tesque), EDTA-2Na (50 mg/L; Dojindo Laboratories), and 5% methanol, was filtered through a guard (pre) column (PC-03 with CA-ODS packing material; Eicom) and delivered at a rate of 230 µL/min. NE was separated on a reversed phased column (CA-5ODS column (2.1 ID × 150 mm)) maintained at 25°C. The amount of NE was electrochemically detected by a graphite working electrode (WE-3G; Eicom) set to +450 mV against an Ag/AgCl reference electrode (RE-500). The signal from the detector was recorded using a data acquisition system (EPC-280; Eicom) and analyzed using PowerChrom software (eDAQ). Before each experiment, the HPLC system was tested for linearity and sensitivity using 6 concentrations of HI ranging from 5 fmol (detection limit for HI with a 3:1 signal-to-noise ratio) to 1.5 pmol per injection (16 µL). Every 8 h during sample analysis, the HPLC system was calibrated using an external standard containing a known concentration of HI. Quantification of HI in each dialysate was determined by comparing the sample peak height to the calibration curve generated from the HI standards. Representative chromatograms of HI peaks obtained from samples collected across the sleep-wake cycle are presented in Figure 3A.

**Assay of Norepinephrine**

Cortical and subcortical samples were assayed for NE using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (HTEC-500; Eicom). Samples maintained at 4°C were injected into the HPLC system using an autosampler (832 Temperature Regulator, 402 Syringe Pump, 231 XL Sample Injector; Gilson). The mobile phase, consisting of 0.1 M phosphate buffer (pH 6.0) containing sodium-1-octanesulfonate (400 mg/L; Nacalai Tesque), EDTA-2Na (50 mg/L; Dojindo Laboratories), and 5% methanol, was filtered through a guard (pre) column (PC-03 with CA-ODS packing material; Eicom) and delivered at a rate of 230 µL/min. NE was separated on a reversed phased column (CA-5ODS column (2.1 ID × 150 mm)) maintained at 25°C. The amount of NE was electrochemically detected by a graphite working electrode (WE-3G; Eicom) set to +450 mV against an Ag/AgCl reference electrode (RE-500). The signal from the detector was recorded and analyzed using PowerChrom software (eDAQ). Before each experiment, the HPLC system was tested for linearity and sensitivity using 10 concentrations of NE ranging from 50 fg (detection limit for NE with a 3:1 signal-to-noise ratio) to 10 pg per injection (12 µL). Every 8 h during sample analysis, the HPLC system was calibrated using an external standard containing a known concentration of NE. Quantification of NE in each dialysate was determined by comparing the sample peak height to the calibration curve generated from the NE standards. Representative chromatograms of NE peaks obtained from samples collected across the sleep-wake cycle are presented in Figure 3B.

**Assay of Serotonin**

Subcortical samples were assayed for 5HT using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (HTEC-500; Eicom) as described in our prior study of cortical release.11

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Figure 3—Chromatograms of histamine (HI) and norepinephrine (NE) in fur seals. (A,B) Typical chromatograms displaying the level of HI and NE in dialysates collected during bilaterally symmetrical EEG states in fur seals. AW, active waking; QW, quiet waking; BSWS, bilateral slow wave sleep; REM, rapid eye movement sleep. HI eluted at a retention time of ~16 min and NA eluted at a retention time of ~8.9 min.
Electroencephalogram Recording and Data Analysis
Electrophysiological parameters were recorded using a 16-channel amplifier (Medicor, Hungary or A-M Systems 3500, USA), an ADC-convertor (Power 1401 and Spike 2, CED, Great Britain), and then analyzed as described in detail previously. Briefly, for each hemisphere, EEG was visually scored in 20-s epochs as (1) desynchronization (i.e., low amplitude, high frequency waves, stage 1 EEG), (2) low-voltage slow waves (0.5–4.0 Hz) and spindles (stage 2 EEG), or (3) high-voltage slow waves (stage 3 EEG). Waking was characterized by sustained muscle tone and bursts of muscle activity and by stage 1 EEG in both hemispheres. AW was scored when the animal was moving around the recording cage or grooming. Two additional active behaviors were analyzed separately: AW-feeding was scored when the seal was being fed and AW-hosing when the animal was being hosed down with water. QW was scored when none of the above behaviors was occurring in the waking animal, typically when the seal was lying down or sitting either motionless or with occasional shifts in body position during which time its eyes could be open or closed. REM sleep was scored when a desynchronized EEG typical of waking (i.e., stage 1 EEG) in both hemispheres was accompanied by a significant reduction in EMG tone or full atonia, muscle jerks, facial tichnesses, and rapid eye movements. BSWS was scored when stage 2 or stage 3 EEG appeared simultaneously in both hemispheres along with lower or similar amplitude EMG activity as seen during QW. Left or right USWS was scored when stage 3 EEG occurred in one hemisphere and stage 2 or stage 1 EEG occurred in the other hemisphere, or when stage 2 EEG occurred in one hemisphere and stage 1 EEG occurred in the other hemisphere. When in slow wave sleep (SWS) fur seals were usually lying on the chamber floor on their sides or on the bellies. Occasionally slow waves started with the seal in the sitting position. However, both high voltage BSWS and USWS always developed in these experiments while the animals were lying down. REM sleep was always recorded when the animals were lying with their heads resting on the floor.

For each hemisphere, EEG spectral power in the frequency range of 1.2–4 Hz (SWA) was computed in consecutive 5-s epochs by fast Fourier transformation using Spike 2 software. Epochs containing artifacts were excluded from spectral analysis. The average SWA in each hemisphere was then calculated for each sample. To estimate the expression of interhemispheric EEG asymmetry during BSWS, left and right USWS, we used the asymmetry index (AI = (L – R) / (L + R), where L and R were standardized spectral powers in the range of 1.2–4.0 Hz in the left and right hemispheres (SWA), respectively; spectral power in each hemisphere was divided by the average power in the same hemisphere of the same frequency range during QW. Thus, the absolute value of AI is a numerical measure of the expression of EEG asymmetry, while the sign is an indication of laterализation of SWA (minus and plus indicates left or right dominance of EEG slow wave power, respectively). The percentage of time spent in each behavioral state was calculated for each sample.

Correlating Concentration of Neurotransmitters and Polygraphic Data
Dialysates were classified as AW-hosing, AW-feeding, AW, QW, BSWS, or REM sleep samples when a single behavioral state occupied ≥ 75% of the sampling interval. When SWS occupied ≥ 75% of the sampling interval but < 25% of it was BSWS, the sample was classified as either left of right USWS (or left and right UW). All remaining samples were classified as mixed state.

Neurotransmitter release was compared during (1) bilaterally symmetrical EEG states: AW, QW, BSWS, and REM sleep, (2) various waking behaviors (AW-hosing, AW-feeding, AW, and QW), and (3) asymmetrical EEG states (left and right USWS).

Mean levels of neurotransmitters (HI or NE) in the cortex during waking and sleep states were calculated for each cortical probe. The analyses revealed that during waking states cortical HI and NE levels were not significantly different between three cortical areas (frontal, parietal and occipital) in fur seals (see Results). For this reason, we have combined data across cortical areas. Statistical tests were then used to evaluate the difference between behavioral states for all cortical probes or separately for probes in the left and right hemispheres using mean values for each probe. When evaluating the effect of behavioral state, we used the probes for which microdialysis samples had been collected for each of the listed states (bilaterally symmetrical EEG states, waking behaviors and asymmetrical EEG states). The pattern of neurotransmitter release in all subcortical areas (the thalamus, hypothalamus, and caudate nucleus) was similar for each transmitter, as it was within each cortical hemisphere. For this reason, we have combined data for the corresponding subcortical sites (the thalamus, hypothalamus, or caudate nucleus). The concentration of neurotransmitter in each sample was normalized to the mean value during QW and BSWS (calculated for all samples of the given probe).

Statistical Analysis
All statistical analyses were performed using Sigma Plot 11. Data were assessed for statistical significance using one-way ANOVA followed by Tukey’s post hoc multiple-comparison tests, T-test or Pearson product moment correlation test. Values are given as mean ± SEM.

RESULTS
In agreement with prior studies, when sleeping on land (in experimental chambers) fur seals displayed both BSWS and SWS with interhemispheric EEG asymmetry. This type of sleep is usually called unihemispheric sleep, unihemispheric slow wave sleep (USWS), or asymmetrical SWS. USWS is seen in concert with UW in the contralateral hemisphere (Figure 1B, 1C; and Figure S2, supplemental material). In this study more sleep (both SWS and REM sleep) occurred in fur seals during the nighttime. However, both the amplitude of EEG slow waves (SWA) in the sleeping hemisphere and the expression of EEG asymmetry during USWS were comparable during the daytime and nighttime periods (Figure S1).
Localization of Microdialysis Probes

**Histamine Probes**
The HI probes were located within 3 cortical areas: frontal (n = 2), parietal (n = 8), and occipital (n = 5). Six hundred twenty-five samples collected from 15 cortical probes (7 left and 8 right; Figure 2A) in 5 fur seals (3 males and 2 females, seals numbers 1–5) met the criteria described for non-mixed state. These samples consisted of 29 AW-hosing, 33 AW-feeding, 111 AW, 197 QW, 156 BSWS, 23 left USWS, 47 right USWS, and 29 REM sleep samples.

**Norepinephrine Probes**
Twenty-six microdialysis probes in a total of 4 seals (3 males and 1 female, seals numbers 3–6) used to collect for cortical NE were located within three cortical areas: frontal (n = 8), parietal (n = 10), and occipital (n = 8). One thousand forty-nine samples collected from a total of 26 cortical probes met the criteria described for non-mixed state (Figure 2B). These samples consisted of 50 episodes of AW-hosing, 43 AW-feeding, 205 AW, 357 QW, 248 BSWS, 49 left USWS, 32 right USWS, and 65 REM sleep. One pair of guide canulas were also implanted in one seal (male, seal number 3) in the posterior hypothalamus: one (right) probe was located in the area of n. paraventricularis/n. anterior and the second (left) probe was located in the vicinity of fornix/n. supraopticus (Figure 2C). Ninety samples collected from these 2 probes consisted of 2 episodes of AW-hosing, 4 AW-feeding, 9 AW, 16 QW, 33 BSWS, 8 left USWS, 4
right USWS, and 14 REM sleep samples.

**Serotonin Probes**

A total of 5 subcortical probes were implanted in 2 seals (one male and one female, seals number 7–8) targeting the thalamus and caudate nucleus (Figure 2D–2F). The first probe was located on the left side in the ventral part of medial thalamus (l-Th1, seal number 7, Figure 2D). The second probe was located on the right side in the ventral anterior nucleus (r-Th1, seal number 7, Figure 2E) and the third probe in the posterior thalamus/pulvinar (r-Th2, seal number 8, Figure 2F). Another 2 probes were located in the ventral and lateral part (adjacent to the external capsule) of the caudate nucleus (adjacent to the external capsule) (l-Nc, r-Nc, respectively; seal number 7, Figure 2D and 2E). Two hundred thirty-five samples were collected from the 3 thalamic probes. They consisted of 97 AW (including 5 AW-hosing and 10 AW-feeding), 68 QW, 28 BSWS, 20 left USWS, and 22 right USWS episodes. One hundred thirty-seven samples were collected from 2 caudate nucleus probes. They consisted of 22 AW, 49 QW, 23 BSWS, 19 left USWS, 22 right USWS, and 2 REM sleep episodes.

As revealed by ANOVA for each of 6 bilaterally symmetrical states (AW-feeding, AW-hosing, AW, QW, BSWS, and REM sleep) the release of both neurotransmitters did not depend on the location of probes within the cortex (in all cases P > 0.05). In all experiments used for the analysis at least a portion of 4 mm long active part of the membrane was located within the gray matter of the cerebral cortex.

**Histamine, Norepinephrine, and Serotonin Release Varies with Sleep-Waking State**

During bilaterally symmetrical EEG states (AW, QW, BSWS, and REM sleep), mean cortical HI release in the fur seal was state-dependent (one-way ANOVA with repeated measures; $F_{3,11} = 15.951; P < 0.001$). Mean HI levels were at 122 ± 3% of QW during AW, 72 ± 2% during BSWS, and 63 ± 4% of QW during REM sleep (Figure 4). The Tukey test indicated that the release of HI was significantly different when waking states were compared to both sleep states (AW vs. BSWS and AW vs. REM sleep, both P < 0.001; QW vs. BSWS P = 0.021 and QW vs. REM sleep P = 0.002). The release of HI did not significantly differ between AW and QW or between BSWS and REM sleep (P > 0.05).

During bilaterally symmetrical EEG states (AW, QW, BSWS, and REM sleep), mean cortical NE release in the fur seal was also state-dependent ($F_{3,23} = 21.68; P < 0.001$). Mean NE levels were at 132 ± 4% of QW levels during AW, at 73 ± 1% during BSWS, and 48 ± 2% during REM sleep. The release of NE was significantly different between all behavioral states (AW vs. QW, BSWS, and REM; QW vs. BSWS and REM sleep; BSWS vs. REM sleep; $P < 0.001$ for all states).

The pattern of release of cortical HI and NE across AW, QW and BSWS in fur seals was similar (Figure 4). However, during the transition from BSWS to REM there was a more pronounced decrease of NE (on average 34% relative to BSWS values) compared to HI (12% relative to BSWS values). Additional analysis revealed that mean REM to BSWS sleep release ratio of HI was 0.87 ± 0.05 (n = 12 probes, the decrease was significant with $P = 0.03$, paired t-test), while for NE and 5HT those values were smaller and both identical in magnitude: 0.66 ± 0.02 for NE (n = 24, $P < 0.0001$) and 0.65 ± 0.05 for 5HT (n = 9, $P = 0.0002$). ANOVA further demonstrated that the decrease of NE and 5HT during the transition from BSWS to REM was significantly more pronounced than the decrease of HI ($F_{2,42} = 11.370, P < 0.001$; $P < 0.001$ for HI vs. NE and $P = 0.002$ for HI vs. 5HT; $P = 0.998$ for 5HT vs. NE).

The pattern of NE and 5HT release during transitions from AW to QW, and from BSWS to REM sleep was also evaluated in 7 subcortical locations (Figure 5A). As with cortical release, mean NE release in the hypothalamus and 5HT release both in the caudate nucleus and in the thalamus varied significantly between the active and quiet waking states with a more pronounced decrease of NE during certain waking activities in fur seals. Each column represents the mean ± SEM of the percent change in NE and 5HT relative to quiet wakefulness (QW). AW, active wakefulness; BSWS, bilateral slow wave sleep; USWS, unihemispheric slow wave sleep; in the hemisphere with a higher voltage EEG slow wave activity (S, “sleeping” hemisphere), or with a low voltage activity desynchronized activity (W, “waking” hemisphere); REM, rapid eye movement sleep; AW-hose, hosing seals down with water; AW-feed, feeding fish; AW, other active waking behaviors. The means were calculated for all combined samples collected from 2 hypothalamus probes (NE-Hy, a total of 90 samples, seal number 3), 3 thalamic probes (5HT-Th, 235 samples, seal numbers 7 and 8) and 2 caudate nucleus probes (5HT-Nc, 137 samples, seal number 7). The localizations of the probes are shown in Figure 2. *P < 0.05; ***P < 0.001. Nonsignificant correlations are not marked on these diagrams.
were state dependent (ANOVA, \(F_{3,61} = 18.061\), \(F_{3,80} = 19.851\), \(F_{2,154} = 25.009\), respectively; \(P < 0.001\) in all cases). For both neurotransmitters and at all sites the release was significantly higher during AW when compared to QW, BSWS, and REM (\(P < 0.05\)). The difference between other states was not significant (\(P > 0.05\)).

This shows that the pattern of HI, NE (as well as 5HT) release in the cortex, NE release in the hypothalamus, and 5HT release in the thalamus and in the caudate nucleus during the sleep-wake cycle in the fur seals was in general similar (highest in AW, reduced in QW and BSWS, and lowest in REM sleep). During REM sleep the levels of cortical NE and 5HT drop more substantially compared to BSWS than do levels of cortical HI. In addition, HI levels are less modulated within behavioral states being significantly different between waking (AW and QW) and sleep (SWS and REM sleep) states but not between AW and QW, or SWS and REM sleep.

**Histamine, Norepinephrine, and Serotonin Release Is Elevated during Certain Waking States**

The nature of waking behavior had a significant effect on mean cortical HI release (\(F_{3,32} = 11.693\), \(P < 0.001\)) and mean cortical NE release (\(F_{3,18} = 53.639\), \(P < 0.001\)) (Figure 6). The levels of both neurotransmitters were highest when the seals were being hosed down (AW-hosing), an activity they appeared to enjoy as evidenced by their approach to the water stream.

The post hoc Tukey test revealed that the difference in HI release between AW-hosing and AW (\(P = 0.015\)) and QW (\(P < 0.001\)) was significant as was the difference between AW-feeding and QW (both \(P < 0.001\)). The levels of HI did not differ significantly between other waking states (AW-hosing vs. AW-feeding, AW-feeding vs. AW, and AW vs. QW; all \(P > 0.05\)).

AW-hosing significantly elevated NE release when compared to AW-feeding, AW (without feeding or hosing) and QW (all \(P < 0.001\)). AW-feeding elevated NE release when compared to AW and QW (both \(P < 0.001\)). The levels of NE during AW were also significantly higher than during QW (\(P = 0.021\)).

As with the cortical release, the type of waking behavior also appears to have a large effect on mean NE release in the hypothalamus, and mean 5HT release both in the caudate nucleus and in the thalamus (ANOVA, \(F_{3,31} = 17.352\), \(F_{3,60} = 22.793\), \(F_{3,138} = 59.560\), respectively; \(P < 0.001\) in all cases). Hosing the seal down substantially elevated the levels of both neurotransmitters when compared to AW-feeding, AW and QW (\(P < 0.001\)) (all \(P < 0.001\)). AW-feeding also elevated the levels of 5HT in the thalamus compared to both AW and QW (\(P < 0.001\)). However, in the caudate nucleus the increase of 5HT during AW-feeding was significant only compared to QW (\(P < 0.05\)) but it was not significant compared to AW (\(P > 0.05\)). Feeding did not significantly elevate levels of NE in the hypothalamus compared to both AW and QW.

These findings show that the pattern of HI and NE release from the cortex locations, the level of NE release in the hypothalamus, and the level of 5HT release from thalamus and caudate nucleus in the fur seals were highly elevated during AW-hosing and at a lesser degree during AW-feeding states compared to non-feeding and non-hosing waking states. We can also see differences between the 3 monoamines: both cortical and subcortical 5HT and NE release were maximally elevated during AW-hosing behavior while cortical HI was equally elevated during both AW-hosing and AW-feeding behavior.

**Histamine, Norepinephrine, and Serotonin Release Are Not Lateralized during USWS**

All of the selected episodes of USWS/UW were characterized by a highly expressed EEG asymmetry as measured by the power spectra amplitude of the two brain hemispheres (Figures 1 and 4; Figure S2). As confirmed by statistical analysis (Table S1, supplemental material), SWA differed significantly between the

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**Figure 6**—Cortical histamine (HI), norepinephrine (NE) and serotonin (5HT) release during different waking behavior in fur seals. Cortical release is expressed as mean ± SEM of the change relative to QW. AW-hose, hosing seals down with water; AW-feed, feeding fish; AW, other active waking behaviors; QW, quiet waking). *P < 0.05; ***P < 0.001; ns, nonsignificant (P > 0.05).
two hemispheres for all selected episodes of left and right USWS/UW as first scored based on visual criteria. The absolute values of asymmetry index (a measure of the magnitude of EEG asymmetry) for the selected USWS episodes varied between 0.44 and 0.70. At the same time, SWA did not differ between the two hemispheres during sleep episodes scored as BSWS. The absolute asymmetry index for the selected BSWS episodes was smaller than 0.09.

As shown in Figure 7A, mean HI release during USWS in the hemisphere with the higher voltage EEG slow wave activity (“sleeping” hemisphere) was not different from that in the hemisphere with low voltage desynchronized activity (“waking” hemisphere, \( t_{10} = 1.105, P = 0.295 \), the paired t-test). As with HI, mean cortical NE release during USWS did not differ between the hemisphere with slow waves and the hemisphere with low voltage activity (\( t_{10} = 0.206, P = 0.841 \)). During USWS, average SWA and cortical HI or NE release were not correlated in 10-min epochs. In addition, there was no correlation between SWA and cortical HI or NE release in the contralateral hemisphere. The same conclusion was reached for cortical 5HT release when analyzing our previously collected data (Figure 8).\(^6\) In our prior publication, we showed that interhemispheric EEG asymmetry in the fur seal is expressed in the range of 1.2–4, 4–8, 8–12, and 12–16 Hz, and the degree of asymmetry in these 4 frequency ranges as measured by the asymmetry index is positively correlated.\(^5\) Based on the prior data we conclude that cortical monoamine release is not related to the pattern of EEG in the range of 1.2–16 Hz. As with HI, mean cortical NE release during USWS in the hemisphere with higher voltage EEG slow wave activity was not significant in the thalamus (\( t_{17} = 0.084, P = 0.933 \)) and in the caudate nucleus (\( t_{13} = 0.936, P = 0.339 \)). The same was true for mean NE release during USWS in the hypothalamus (\( t_{10} = 0.704, P = 0.437 \), Figure 7B).

In Figure 7 we compared cortical and subcortical release in the current study with cortical 5HT and cortical ACh levels in the fur seal from our previous studies.\(^8,11\) Similar to cortical 5HT, cortical HI, cortical and subcortical NE, and subcortical 5HT release in the fur seal, measured for the first time in the current study, were not lateralized during USWS and UW (Figure 7A, 7B). Cortical ACh release, however, was highly lateralized during USWS (Figure 7A). During USWS, cortical ACh levels in the hemisphere displaying slow waves did not differ from those observed during BSWS, whereas levels in the activated hemisphere were comparable to those observed during QW, a greater than 80% increase (Figure 7C). The pattern of cortical HI, NE, and 5HT release and subcortical 5HT and NE release clearly differs from that of ACh release observed under the same conditions (Figure 7C, 7D).

**DISCUSSION**

Here we show that over a period of 10 or more minutes USWS in the fur seal, NE and 5HT release are bilaterally symmetrical.
in the cortex and diencephalon, and HI release is bilaterally symmetrical in the cortex. Of the transmitters examined, only ACh release was asymmetrical in USWS, with significantly greater release in the activated hemisphere. We cannot rule out the possibility that over very short intervals of, for example, 1–3 seconds, symmetric or asymmetric release of the monoamines might occur. But clearly, although we readily detected changes in monoamine release during waking behaviors, the magnitude of any asymmetry during USWS was not significant for the monoamines, whereas it was for ACh.

Consistent with our finding, systemic blockade of ACh muscarinic receptors has long been known to produce bilateral EEG slowing, even during active behavior.9,14–16 We found that the pattern of NE, HI, and 5HT release across the sleep-wake cycle in each hemisphere of the fur seal is generally similar to that described in several species of terrestrial mammals.17–19 Both in bilaterally sleeping terrestrial mammals and unihemispherically sleeping fur seals the release of these monoamines in the brain declined progressively during transition from AW to SWS and then to REM sleep. This universal state dependent pattern of monoamine release correlates with the pattern of neuronal discharge of noradrenergic, histaminergic and serotoninergic neurons firing at highest rate during AW and declining to near complete cessation during REM sleep.20–23

Early concepts of waking being a result of sensory stimulation evolved to the Moruzzi and Magoun concept of a reticular arousal system that received input from sensory systems and was responsible for the generalized activation of the brain in response to sensory input and motivational states. The discovery of the monoaminergic and cholinergic systems within the reticular system, with what appeared to be diffuse, overlapping projections led to idea that they acted redundantly with other systems to produce waking, explaining the recovery of waking with damage to arousal systems.24

The arousal generated by the brainstem midbrain reticular formation can be attributed to the monoaminergic (noradrenergic locus coeruleus [LC], and the serotoninergic dorsal and median raphe nuclei), cholinergic (pedunculopontine and laterodorsal tegmental nuclei [PPT/LTP]), and glutamatergic components of this projection. The arousal pathway from the midbrain reticular formation splits at the level of diencephalon into two branches. The dorsal pathway ascends to the thalamus innervating the intralaminar and reticular nuclei which have been thought to play a critical role in regulating thalamo-cortical transmission and the EEG activity associated with sleep and wakefulness. The ventral pathway includes glutamatergic (parabrachial), NE, SHT, and dopaminergic (periaqueductal gray) axons. It runs through the lateral hypothalamus and basal forebrain to the cortex.25,26 The activity patterns of these basal forebrain (mostly ACh) neurons correlate with sleep-wake patterns and EEG waveforms and cortical arousal.27,28 The histaminergic
At the same time, as shown in the current study monoamine release during the transition from BSWS to REM sleep in fur seals (and 5HT) during the transition from BSWS to REM sleep in fur seals is smaller than in QW. Both stages are characterized by an activated EEG and movement, and the activity of the NE containing neurons of the locus coeruleus is correlated with vigilance and presentation of arousing stimuli (e.g., food). However, as with other arousal systems one cannot distinguish a possible relation to muscle tone and other variables correlated with alertness from a relation to EEG unless one observes the system under a condition in which these variables can be dissociated from each other. Cataplexy is such a situation, in which muscle tone is lost while EEG activation and alertness is maintained bilaterally. Under this condition, noradrenergic cells completely cease discharge in parallel with the loss of muscle tone, despite a very high degree of EEG activation. This is also consistent with our findings indicating a pronounced decrease of NE (and 5HT) during the transition from BSWS to REM sleep in fur seals.

Studies of HI neurons have shown that their activity increases during cataplexy relative to that during QW. Careful behavioral studies of HI and orexin (hypocretin) knockout mice have found distinct roles for these two “waking” neurotransmitters, consistent with the general conclusions of the present investigation. They find that HI, in contrast to orexin, promotes waking through enhanced locomotion. This work also raises the possibility of a differential role of orexin and HI in cognition. In prior work, we have reported that orexin release is linked to positive emotions in waking, rather than EEG activation in mice, rats, cats, dogs, and humans. During aversive states, including pain in humans, orexin cell activity, and release of the orexin peptide is minimal despite intense EEG activation.

As we showed in our prior study, ACh level in fur seals was also highly elevated in REM sleep at levels comparable to that in QW. Both stages are characterized by an activated EEG pattern. This suggests that ACh release is linked to cortical activation which is a common feature of waking and REM sleep. At the same time, as shown in the current study monoamine release is, in contrast, associated with level of behavioral arousal and motor activity within the waking state.

The current work extends our knowledge on the role of different neurotransmitters in initiation of waking and sleep. Our data show that several transmitters known to be maximally released in waking, and observed to be waking-active in the current study, are not strongly linked to the asymmetric EEG seen during USWS in the fur seal. We cannot rule out the possibility that other transmitters might also be involved in USWS and UW in fur seals and dolphins. Thus, it was shown that both cholinergic and non-cholinergic neurons in the rat basal forebrain (presumably mainly GABA and glutamate neurons) need to be lesioned to cause loss of wakefulness. Thus, the role of the glutamatergic and GABAergic system in maintenance of aspects of waking as well as bilateral and asymmetrical sleep states could be productively investigated.

**ABBREVIATIONS**

- 5HT, serotonin
- ACh, acetylcholine
- AI, asymmetry index
- AW, active wakefulness
- BSWS, bilateral slow wave sleep
- EEG, electroencephalogram
- EMG, electromyogram
- EOG, electrooculogram
- HI, histamine
- Hy, hypothalamus
- LC, locus coeruleus
- Nc, caudate nucleus
- NE, norepinephrine
- PPT/LTP, pedunculopontine and laterodorsal tegmental nuclei
- QW, quiet wakefulness
- REM sleep, rapid eye movement sleep
- SWA, slow wave activity
- SWS, slow wave sleep
- Th, thalamus
- USWS, unihemispheric slow wave sleep
- UW, unihemispheric wakefulness

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ACKNOWLEDGMENTS

The authors thank Dr. Paul Manger for help with localization of microdialysis probes in the fur seal brains and personnel of the Utrish Marine Station (Russia) for help with animal care. Author contributions: OIL, LMM, JLL and JMS designed research; JLL, POK, OIL, TK, AB, SMK, and JMS performed research; LMM and JHP contributed unpublished reagents/analytic tools; JLL, POK, OIL, TK, and JMS wrote the paper.

SUBMISSION & CORRESPONDENCE INFORMATION

Submitted for publication August, 2015
Submitted in final revised form October, 2015
Accepted for publication October, 2015
Address correspondence to: Dr. Jerome Siegel, Neurobiology Research Marine Station (Russia) for help with animal care. Author contributions: OIL, LMM, JLL and JMS designed research; JLL, POK, OIL, TK, AB, SMK, and JMS performed research; LMM and JHP contributed unpublished reagents/analytic tools; JLL, POK, OIL, TK, and JMS wrote the paper.

DISCLOSURE STATEMENT

This was not an industry supported study. This study was supported by grants from National Science Foundation (0919929), National Institute of Health MH064109, DA034748, Russian Fund for Basic Research (13-04-01470-A, 14-04-32075), Medical Research Service of the Dept. of Veterans Affairs, Utrish Dolphinarium Ltd., National Science and Engineering Council of Canada, and Canadian Institutes of Health Research. The authors have indicated no financial conflicts of interest. This study was performed at the University of California Los Angeles, Los Angeles, USA and Severtsov Institute of Ecology and Evolution, Moscow, Russia.