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Stress-free automatic sleep deprivation using air puffs



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HIGHLIGHTS

- Gentle handling sleep deprivation is time- and personnel-intensive.
- Manual sleep scoring adds unwanted variability to studies.
- We developed an automated air-puff sleep deprivation procedure for rats.
- AP is no more stressful than gentle handling sleep deprivation.
- AP is a viable, automated alternative to current sleep deprivation techniques.

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ABSTRACT

Background: Sleep deprivation via gentle handling is time-consuming and personnel-intensive.

New method: We present here an automated sleep deprivation system via air puffs. Implanted EMG and EEG electrodes were used to assess sleep/waking states in six male Sprague-Dawley rats. Blood samples were collected from an implanted intravenous catheter every 4 h during the 12-h light cycle on baseline, 8 h of sleep deprivation via air puffs, and 8 h of sleep deprivation by gentle handling days.

Results: The automated system was capable of scoring sleep and waking states as accurately as our offline version (~90% for sleep) and with sufficient speed to trigger a feedback response within an acceptable amount of time (1.76 s). Manual state scoring confirmed normal sleep on the baseline day and sleep deprivation on the two manipulation days (68% decrease in non-REM, 63% decrease in REM, and 74% increase in waking). No significant differences in levels of ACTH and corticosterone (stress hormones indicative of HPA axis activity) were found at any time point between baseline sleep and sleep deprivation via air puffs.

Comparison with existing method: There were no significant differences in ACTH or corticosterone concentrations between sleep deprivation by air puffs and gentle handling over the 8-h period.

Conclusions: Our system accurately detects sleep and delivers air puffs to acutely deprive rats of sleep with sufficient temporal resolution during the critical 4–5 h post learning sleep-dependent memory consolidation period. The system is stress-free and a viable alternative to existing sleep deprivation techniques.

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1. Introduction

Sleep plays a crucial role in the memory consolidation of learning tasks (Ferrara et al., 2008; Poe et al., 2010; Smith, 1995). In fact, it has been shown that sleep must occur within a critical 4 to 5 h

window after the completion of the task for hippocampal memory consolidation to take place (Bjornerem et al., 2005; Hagewoud et al., 2010; Prince et al., 2014). Given this precarious sleep-dependent period for hippocampal plasticity and memory, acute use of sleep deprivation techniques can enable the elucidation of memory consolidation mechanisms during specific sleep stages.

A variety of techniques have been developed and validated to deprive laboratory animals of total or partial sleep using electromyography (EMG) and cortical electro-encephalography (EEG)

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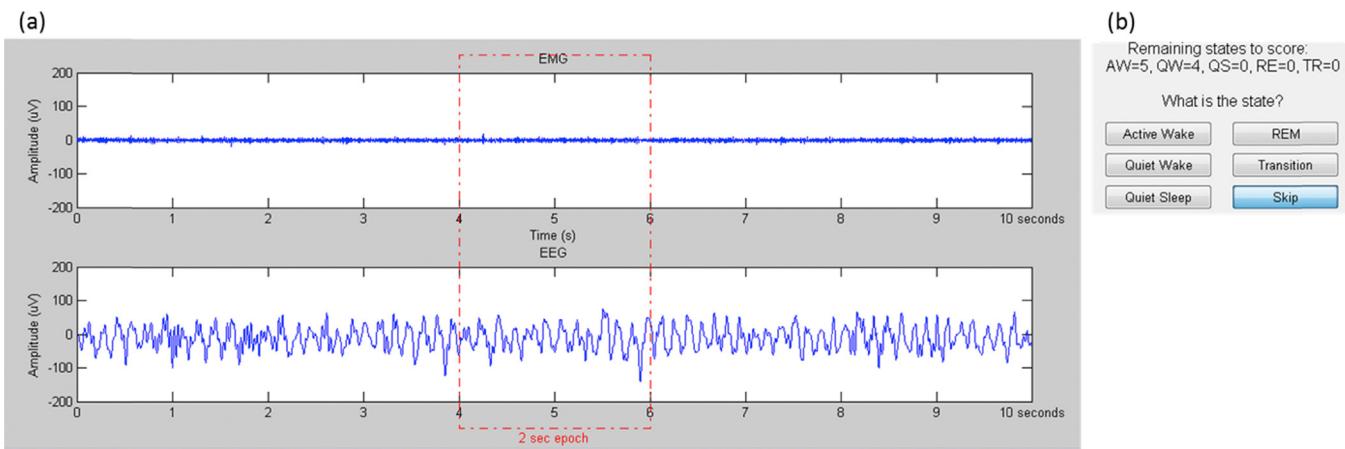


Fig. 1. The manual scoring window is used to determine threshold values for real-time autoscorer. (a) The user scores a 2-s epoch (dashed outline) by (b) selecting one of six pre-defined sleep states until each state is scored 5 times. The minimum number of scored epochs can be modified based on the interests of individual laboratories. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

signals, including gentle handling (Franken et al., 1991; Tobler and Borbely, 1990), multiple platforms over water (Mendelson et al., 1974; Youngblood et al., 1997), and forced locomotion (Borbely and Neuhaus, 1979; Rechtschaffen et al., 1999). Manual sleep deprivation techniques can introduce unexpected confounds. For example, male experimenters have been shown to induce more stress (Sorge et al., 2014) and what procedures constitute gentle manipulation vary between experimenters. Manual scoring in sleep studies is also time-consuming, resulting in research delays and increased personnel costs. Furthermore, off-line scoring does not allow for real-time feedback control based on the sleep or waking state of the animal.

We have developed an on-line total sleep deprivation system based on our previously published off-line software, Auto-Scorer (Gross et al., 2009), for real-time automated detection of sleep states in animals outfitted with EMG and EEG electrodes and delivery of puffs of air to the animal's home cage to prevent further sleep in order to gain further understanding in sleep-dependent memory consolidation.

2. Materials and methods

The design and validation of both the software and hardware components of the system are described below, as well as a description of the animal experiments carried out to measure the real-time performance of the system. We also present the procedure used to characterize the stress response of rats to sleep deprivation via puffs of air.

2.1. Software design and testing

The software component of the air puff sleep deprivation system was adapted from our previously published, open source, logic-based, automated sleep scoring software, Auto-Scorer (Gross et al., 2009). The real-time software contains a manual scoring mode and an automated scoring mode that were developed in MATLAB, version 2012b (The MathWorks, Inc., Natick, MA). The sleep deprivation method relies on online automatic sleep scoring with thresholds initially set in manual scoring mode.

2.1.1. Manual scoring mode to determine thresholds

The manual scoring mode allows the user to determine the threshold values of neck EMG and EEG (delta, theta and sigma) power for each animal that are used in the logic of the automated scoring algorithm (refer to Gross et al., 2009 for further details).

The user scores a small number of epochs of each user-defined sleep or waking state (Fig. 1a and b). With the animal connected to the recording system, the most recent 10 s of the EEG and EMG signals are displayed in the graphical user interface (GUI) and the user marks which state the animal is in. Discussed in Gross et al. (2009) and by others Benington et al. (1994), Robert et al. (1999), Witting et al. (1996), an epoch size of 10 s is commonly used in manual scoring of rat sleep and wake states due to the trade-off between accuracy and manual labor. The user scores the state based on the middle 2-s window contained in a red rectangle. Once five epochs of each state are scored (countdown displayed in Fig. 1b), the software displays two 3-dimensional plots (Fig. 2a and b). The first plot includes power spectral densities (PSD) of the EEG signal in the delta, theta, and sigma frequency bands for each epoch. The PSD of the EMG, the delta/theta PSD ratio, and sigma × theta PSD of each epoch comprise the second plot. These graphs are inspected by the user to determine threshold settings for the logic-based automated scoring mode. Refer to Gross et al. (2009) for the protocol to select threshold values. Once the thresholds have been set, the GUI prompts the user to save the values (Fig. 2c) and then returns to the main menu.

2.1.2. Auto-scoring mode

The automated scoring mode of the software requires the user to enter the PSD threshold values for each animal determined from the Manual Scoring mode, and the length of time (in seconds) that the program should run. Given that the goal is to provide real-time feedback control based on the sleep or waking state of the animal, an epoch size of 2 s was selected to decrease the likelihood of allowing micro-sleeps to occur. A shorter delay for feedback to deliver puffs of air would awaken the animal from sleep faster. Automated scoring in 2-s epochs has been shown to improve agreement with manual scoring (Ruitg et al., 1989), so this shortening of the epoch size in our algorithm was expected to have a beneficial effect on the logic algorithm's automated scoring performance.

The algorithm's auto-scoring performance of 2-s epochs was initially evaluated offline using a data set of six sleeping rats. The states scored by the algorithm were compared to manually scored states from three experienced technicians, and the agreement of each state between users and the algorithm was calculated.

2.2. Hardware design

The air puff sleep deprivation system (Fig. 3) includes Neuralynx data acquisition hardware (digital amplifier and Cheetah EDT

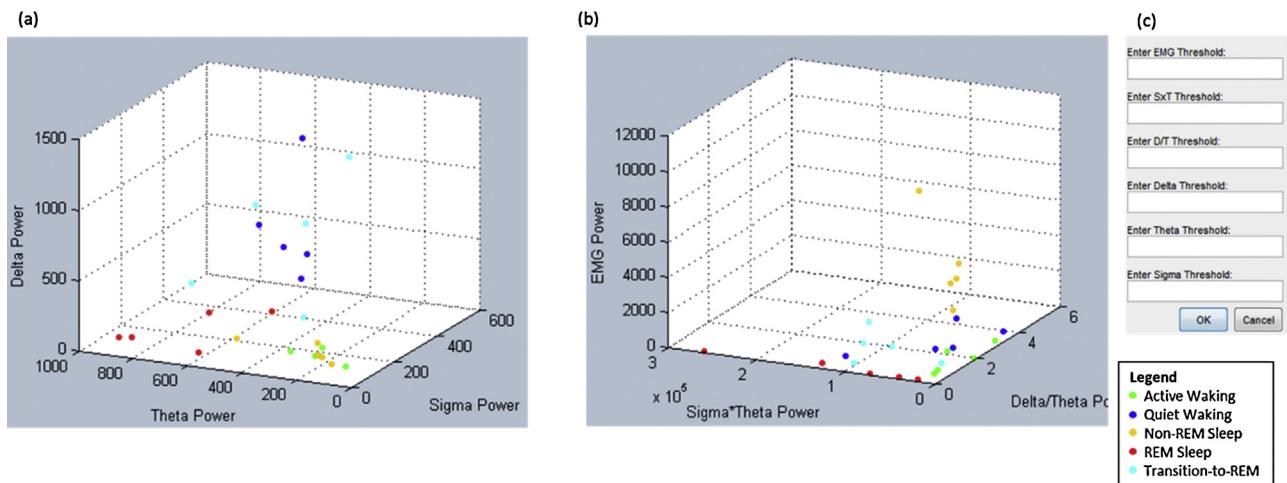


Fig. 2. (a and b) Illustration of GUI displaying power densities for EMG and EEG (delta, theta, sigma, delta/theta, and sigma \times theta). Thresholds settings for autoscoring are determined according to Gross et al. (2009) and (c) entered to begin autoscoring in real-time.

board; Neuralynx Inc., Bozeman, MT) connected to a computer running Neuralynx Cheetah software (digital acquisition [DA]-CPU). However, any data acquisition system that amplifies then digitizes continuous signals at a minimum of 500 samples per second per channel can be used. A second computer ran the automated air puff sleep deprivation software in MATLAB (air puff [AP]-CPU). The two computers were connected to a router on an internal network in order to maintain stable communication speeds. The digital I/O card of the DA-CPU was connected to an arbitrary waveform generator (33220A; Agilent Technologies, Santa Clara, CA), which was in-turn connected to a custom trigger switch (described in Section 2.2.1). A DC power supply (HY3006D; Mastech, Taipei, Taiwan) and an electronic air valve (Red-Hat II 8262G90, ASCO, Florham Park, NJ) were also connected to the trigger switch. A pressure-regulated air supply was connected to the electronic valves gas input, while the output was connected to tubing which ended at ports in the animal cage.

For every epoch in which sleep was detected, the AP-CPU sent a command to the DA-CPU to trigger the arbitrary waveform generator. The arbitrary waveform generator then sent out a single cycle burst square wave (250 ms width) to the custom switch which allowed electricity provided by the power supply to open the electronic air valve. Air from the air supply (built-in, but it could instead be from an air tank) with the pressure regulator manually set to

16 psi passed through the valve and into a silicone tube that split the air into four smaller tubes of equal length and diameter. The tubes were mounted near each of the four corners of a 33 cm long by 20 cm wide by 43 cm high plexi-glass cage ~2.5 cm above the level of the corn cobb bedding toward the bottom of the cage. Air puffs had a pressure of approximately 4 psi at each of the four ports in the cage. On rare occasions in which rats attempted to block the ports with their bedding, the experimenter intervened by moving bedding to unblock the port. This problem was likely mitigated by using a minimal amount of bedding and the height of the ports not being too proximal to the level of the bedding.

2.2.1. Custom trigger switch

Neither the electrical current supplied by a standard computer TTL pulse nor a typical arbitrary waveform generator were sufficient to open the electronic valve. It was necessary to design a custom trigger switch that would close the circuit between a power supply and the electronic valve, allowing the valve to open when a TTL pulse triggered the switch to close. While there are commercial solutions to meet the power requirements, such as arbitrary waveform generators or power supplies that contain their own TTL input, we include our design as it is an affordable solution (Fig. 4).

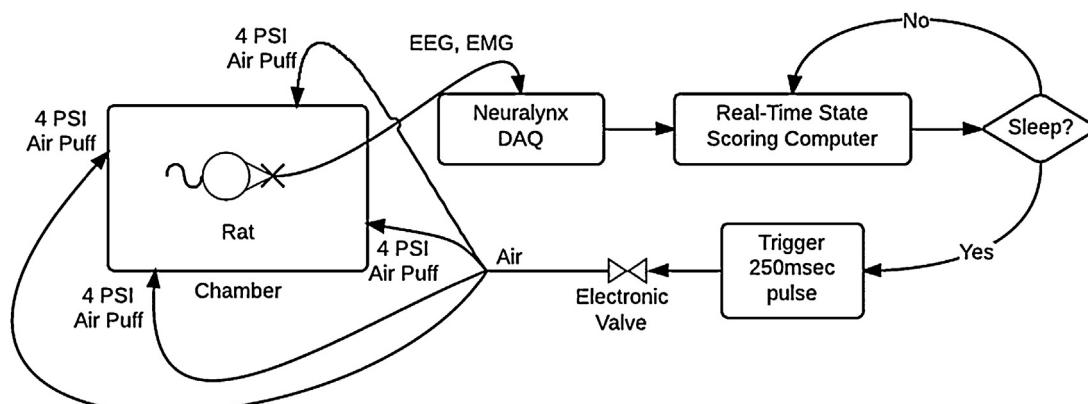


Fig. 3. An automated system uses nuchal EMG and cortical EEG signals to score sleep states in 2-s epochs in real-time, delivering air puffs for 250 ms (at a maximum rate of 1 air puff/5 s) when sleep is detected in the prior epoch. Briefly, a Neuralynx system connected to a real-time state scoring computer automatically calculates sleep states based upon data from Auto-Scorer. The on-line system is capable of automatically scoring every 1.76 s (a 0.24 s overlap between adjacent epochs). When a sleep state is detected, a trigger connected to an electronic air valve releases air (16 psi) into four chamber ports (4 psi/port), which awakens the animal.

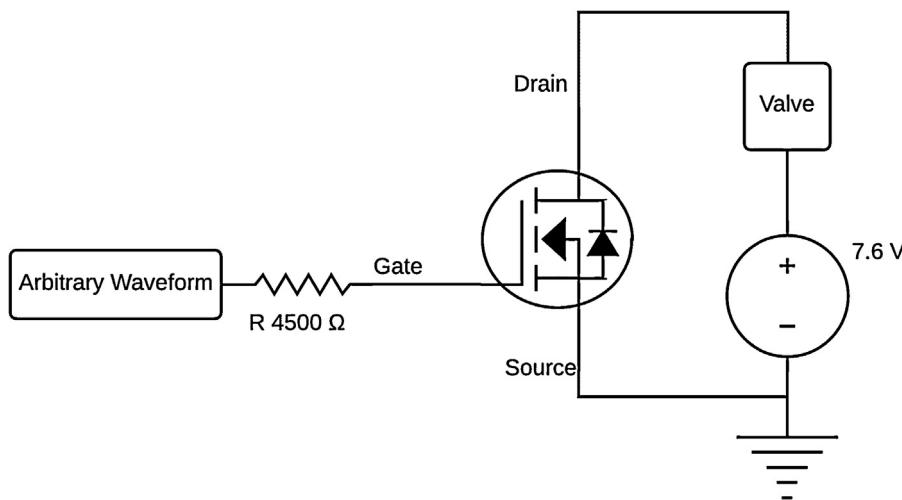


Fig. 4. A circuit diagram of the custom trigger used in the control of the electronic air valve. The transistor is an N-channel power MOSFET (max 30 A and 50 V; drain-to-source resistance = 0.040 Ω).

The switch consists of an N-Channel power MOSFET (BUZ11, Fairchild Semiconductor Corp., San Jose, CA) with a maximum current of 30 A and maximum voltage of 50 V and a drain to source resistance equal to 0.040 Ω when on, a resistor used to reduce the voltage from the trigger, and three female BNC connectors for the trigger and power input and output ports.

2.3. Animal experiments

In order to validate whether the air puff automated system actually sleep deprives animals, and to determine the stressfulness of the system, we carried out two sets of experiments. The first was performed to determine the speed of the algorithm in scoring 2-s epochs from signals being acquired from a rat in real-time. Upon validating the system for use in freely-behaving rats, we carried out a second set of experiments to determine the stress response of rats to sleep deprivation by puffs of air. Animal procedures were in compliance with both the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of Michigan Committee on the Use and Care of Laboratory Animals.

2.3.1. In vivo real-time performance validation

The ability of the automated system to score in real-time was initially evaluated by acquiring data in 2-s epochs from a freely behaving 6-month-old male Sprague-Dawley rat implanted with nuchal EMG and both frontal and parietal EEG electrodes.

2.3.1.1. Surgical methods used in validation experiment. The protocol for the electrode implantation is similar to one described in detail in (Watts et al., 2012). Briefly, the rat was anesthetized with a mixture of 90 mg/kg ketamine and 10 mg/kg xylazine (i.m.) and placed in a stereotaxic frame. An incision was made to expose the skull, and bregma was identified. Holes were pre-drilled using a Dremel rotary tool at locations for placement of screw electrodes. Two electrodes were screwed into the skull over the frontal cortex 1 mm in front of bregma and 1 mm to right and left of the midline. Parietal electrodes were screwed in 2 mm behind bregma and 1 mm to the left and right of the midline. Flexible wire electrodes were inserted through the incision from the rostral base of the skull into the dorsal neck muscles. A gold pin was crimped to each electrode at the backend of the electrode wire. All 6 gold pins were inserted into a 6-pin plastic pedestal head stage (Plastics One, Inc., Roanoke, VA). The assembled head stage was secured to the skull of the rat

using dental acrylic. The rat was returned to its home chamber once it had regained mobility, given food and water *ad libitum*, provided antibiotics for 3 days, and allowed to recover for at least one week prior to recording.

2.3.1.2. Real-time validation method. Once the rat was hooked up to the recording system, the time to analyze 2 s of streaming data, score the behavioral state as asleep or awake, and trigger an air puff (if scored as sleep—either non-REM or REM as detected by the algorithm) was measured. In addition, manual scoring of the data was performed offline to confirm that the system was capable of depriving the animal of sleep.

2.3.1.3. Acute stress response experiments. After validating the functionality of the automated air puff sleep deprivation system *in vivo*, we carried out more extensive experiments to determine whether or not our technique could serve as a feasible alternative to existing sleep deprivation methods. Both sleep architecture and stress responses were analyzed under normal sleep conditions in six male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN), during 8 h of sleep deprivation using the automated air puff system, and during 8 h of sleep deprivation via gentle handling. The 8 h length of deprivation was chosen in order to extend beyond the 4 to 5 h critical window in which the post-task sleep-dependent memory consolidation has been shown to occur.

Given the variability of gentle handling between labs, our protocol will be described briefly. EMG, EEG, and live video of the rat in his home cage were monitored in real-time using the Neuralynx setup. Whenever the rat showed signs of prolonged quiescence via the video feed as well as low EMG coupled with large irregular EEG activity, the experimenter would intervene to prevent the rat from sleeping. The procedure began with opening the chamber door and proceeded in an escalating manner until the rat was active with lightly tapping on the home cage, using a gloved hand to gently nudge the rat and introducing Nylabones or knotted Kimwipes into the home cage as novel objects (hours 7 and 8 only).

2.3.1.4. Catheter surgery. In addition to being implanted with cortical screw EEG (2 frontal; 2 parietal) and 2 nuchal EMG electrodes connected to a 6-pin head cap as previously discussed, each rat was implanted with an indwelling intravenous catheter into the right external jugular vein with an access port implanted between the shoulder blades. This catheter construct remained potent for the length of the study (several weeks); therefore, it allowed us to use

the animals as their own controls, thereby reducing the minimum number of animals required for the study from 72 had we obtained core blood samples to 6.

Rats were prepared with indwelling intravenous catheters as reported by others (Saunders et al., 2013; Yager and Robinson, 2013). Pre-surgical administration of carprofen (2 mg/kg; s.c.) induced analgesia, while ketamine (90 mg/kg; i.m.) and xylazine (10 mg/kg; i.m.) were used for anesthesia. An incision was made 4 cm behind the shoulder blades, followed by an incision above the right external jugular vein. The catheter construct was inserted between the shoulder blades, and the tubing implanted around the neck and into the right external jugular vein. A silicone bead provided resistance to finalize placement and catheter patency was tested by flushing tubing with sterile saline via a cannula.

After testing patency, the front and back incisions were closed with absorbable sutures (Catgut Chrom, USP 3/0; B. Braun Melsungen AG; Melsungen, Germany), and triple antibiotic ointment was applied. This was immediately followed by the 6-pin head stage surgery described above. Further experimentation did not occur until rats recovered pre-surgical body weight (approximately 5–7 days). During this postoperative recovery period, catheters were flushed daily with 0.1 mL of a gentamycin solution (10 mg/mL in sterile saline) to prevent occlusions and potential infections. Upon recovery, a tether was connected to the rat's 6-pin head cap. The other end of the tether was connected to 6-channel commutator (Airflyte Electronics Co.; Bayonne, NJ) to prevent torsional stress from building up along the tether cable.

2.3.1.5. Protocol to measure stress response to acute sleep deprivation. All manipulations took place during the rats' light cycle and in their home cages (08:30–20:30). EEG and EMG signals were recorded under four conditions. The first was a baseline day in which the animal was not disturbed (sleep control, SC). This was immediately followed by a control day in which blood samples were taken and the animal was allowed to sleep (blood control, BC). After at least two full days had passed, the animal was exposed to 8 h of sleep deprivation using the automated air puff system with blood samples taken at the appropriate time points (air puff, AP). We then waited at least 1 week before subjecting the animal to 8 h of sleep deprivation via gentle handling along with taking blood samples (gentle handling, GH). Sleep deprivation began 30 min into the light cycle for both techniques.

2.3.1.5.1. Serum samples. On days in which blood samples were collected, a volume of 0.15 mL of blood was extracted from the catheter at Zeitgeber time (ZT) 0, 4, 8, and 12 h, where ZT0 is lights on and ZT12 is lights off. While sleep deprivation ended at ZT8, the ZT12 serum sample was intended to measure recovery from sleep deprivation should the measures show any significant differences by ZT4 or ZT8. Samples were allowed to coagulate at room temperature for 30 to 40 min and were centrifuged at 2000 rcf for 10 min (Centrifuge 5424; Eppendorf, Hamburg, Germany). Serum was then extracted and stored at $\leq -20^{\circ}\text{C}$. Once all of the samples had been collected, samples were thawed and prepared according to the protocol in the Milliplex Map Rat Stress Hormone Magnetic Bead Panel Kit (Cat. #RSHMAG-69K, EMD Millipore, Billerica, MA). The kit was used to measure concentrations of corticosterone (CORT) and adrenocorticotrophic hormone (ACTH) in each sample.

2.3.2. Data acquisition & analyses

2.3.2.1. Electrophysiological recordings. EMG and EEG signals were recorded simultaneously at 1000 samples per second with hardware filters set to pass signals in the frequency range of 0.1 to 325 Hz. Sleep and waking states were manually scored in 10-s epochs based on the cortical EEG and nuchal EMG. Scoring performance of the automated system was evaluated by the commonly used measure of percent agreement, in which the number of a given

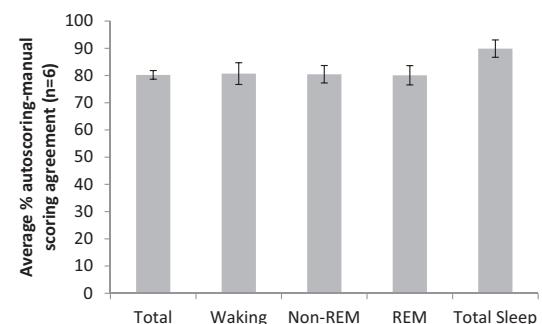


Fig. 5. Average percent automated scoring to manual scoring agreement of states in 2-s epochs ($n = 6$; error bars = S.E.M.). Performance is on par with Auto-Scorer (Gross et al., 2009).

state scored by the automated system matching that scored manually is divided by the total number of the state scored manually (Benington et al., 1994; Diba and Buzsáki, 2007; Havekes et al., 2014; Kamphuis et al., 2015; Kempler and Richmond, 2012; Meerlo et al., 2008; O'Callaghan et al., 2007; Paul et al., 2014; Prince et al., 2014; Prince and Abel, 2013; Ruigt et al., 1989; Sanford et al., 2014; Smith and MacNeill, 1994; Witting et al., 1996). In order to analyze differences in sleep architecture between manipulations, the amount of time in each state was analyzed, and percentages were compared across experimental conditions.

2.3.2.2. Statistical analyses. All statistical analyses were carried out using GraphPad Prism 6 for Windows (Version 6.02; GraphPad Software, Inc., San Diego, CA). Two-way ANOVA was used to test for differences between baseline BC and AP in amounts of waking, non-REM sleep, and REM sleep in 1-h bins. Repeated measures two-way ANOVA was used to test whether or not any differences in concentration of each hormone existed across time in a given day or between BC and AP or AP and GH. A one-way ANOVA was performed on the rate of air puffs delivered per minute in 1-h bins over the course of the 8-h sleep deprivation period. With a significant ANOVA, post-hoc comparisons were performed using a Bonferroni correction.

3. Results

3.1. Auto-scoring algorithm accuracy

The scoring performance of the automated algorithm in 2-s epochs as compare to manually scored data in 10-s epochs was consistent with our previous results (Gross et al., 2009) (Fig. 5). Agreement by state was approximately 80% when separated by waking ($80.7 \pm 9.8\%$ S.D.), non-REM sleep ($80.5 \pm 7.8\%$ S.D.), and REM sleep ($80.1 \pm 8.7\%$ S.D.). Given that the automated air puff sleep deprivation system was being validated for detection and deprivation of any sleep state, non-REM and REM sleep scored epochs were combined. The agreement between the auto-scoring algorithm and manual scorers for this combined sleep state was $89.9 \pm 7.8\%$ S.D.

3.2. Real-time system performance

Real-time validation of the air puff sleep deprivation system *in vivo* showed that the system was capable of analyzing the most recent 2 s of the EEG and EMG signals and stage scoring to decide if the animal was asleep or awake in 1.76 s. Therefore, no gap in time existed between epochs being evaluated in real-time. In addition, manual scoring of the recorded signals in 10-s epochs post-experiment showed the rats ($n = 6$) had significantly more waking during AP than during BC (Fig. 6a; $F(1, 72) = 121.3$, $p < 0.0001$), and significantly less non-REM sleep (Fig. 6b;

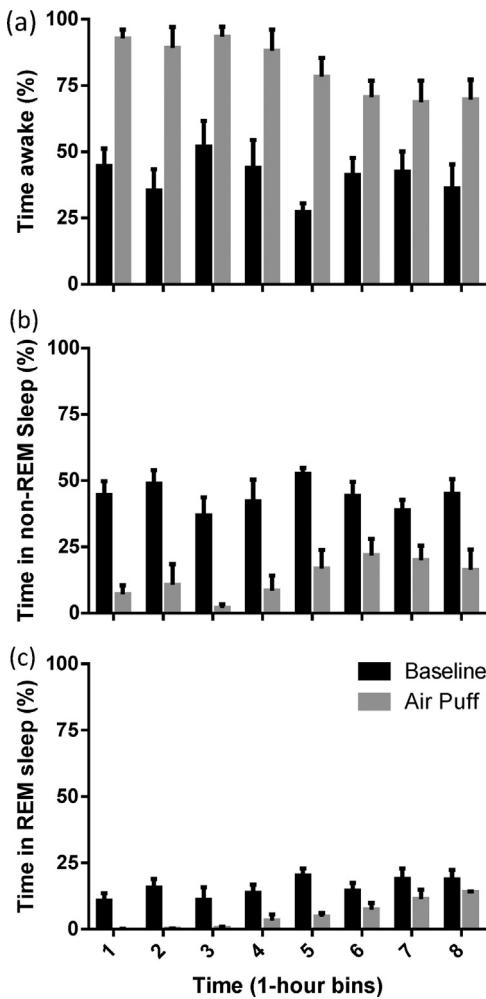


Fig. 6. Average percent time in (a) wake, (b) non-REM, and (c) REM sleep of rats ($n=6$) over 8 h (in 1 h bins) during baseline sleep or air puff sleep deprivation (AP). Data are mean \pm S.E.M.

$F(1, 72)=117.3$, $p < 0.0001$) and REM sleep (Fig. 6c; $F(1, 72)=55.97$, $p < 0.0001$). There was also a significant variance over time for REM sleep ($F(7, 72)=4.167$, $p = 0.0007$). Post-hoc analyses showed a lack of significance difference between AP and BC for the 6, 7, and 8-h bins ($p = 0.0975$, 0.0975 , and 0.1412 , respectively; Holm-Sidak's multiple comparisons test). Results from the one-way ANOVA of the rate of air puffs delivered per minute over the 8-h sleep deprivation period (in 1-h bins) showed a significant difference among the means (Fig. 7; $F(7, 34)=3.604$; $P=0.0052$). Post-hoc comparisons showed that air puffs significantly increased relative to the first hour in hours 6 ($p=0.0175$), 7 ($p=0.0126$), and 8 ($p=0.0210$).

3.2.1. EEG response to air puffs

An analysis of EEG power in the 2 s immediately preceding and following each air puff which fell within manually scored non-REM epochs was performed to investigate if there was an effect on the EEG in the absence of a behavioral change. Delta power was significantly greater just prior to compared to after air puffs (Fig. 8; paired t test; 1-tailed; $p=0.0431$).

3.3. Stress response comparisons

Repeated measures two-way ANOVAs between BC and AP showed no significant interactions between time and manipulation for CORT ($F(3, 15)=1.100$; $p=0.3797$) or ACTH ($F(3, 15)=2.639$; $p=0.0875$). In addition, there was no main effect of manipulation

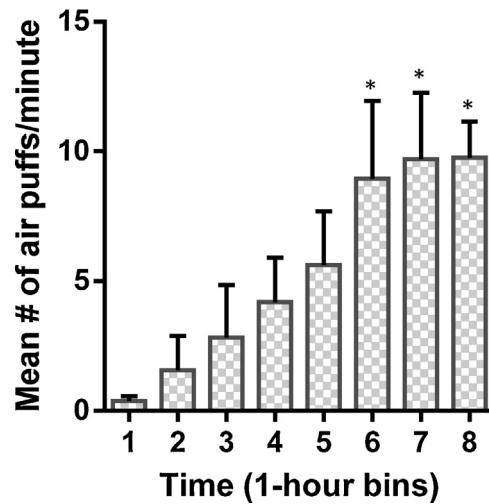


Fig. 7. Average rate of air puffs per minute in 1 h bins over the course of 8 h of sleep deprivation via air puffs ($n=6$; error bars = SEM). Asterisks indicate a significant difference from hour 1 using Bonferroni's multiple comparisons test (adjusted p value <0.05).

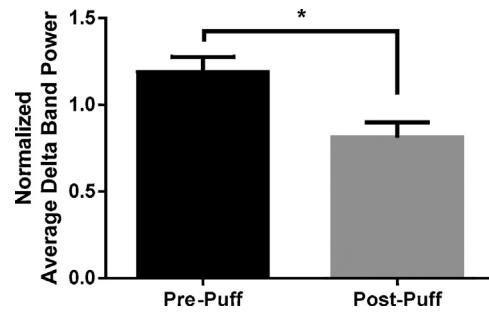


Fig. 8. Average delta power in the 2 s preceding and 2 s following air puffs within epochs scored offline as non-REM sleep ($n=6$ animals; error bars = SEM). Power was normalized to the overall delta band power average for each animal. * Delta power was significantly greater before the air puffs than after (paired t test; 1-tailed; $p=0.0431$).

for CORT ($F(1, 5)=0.3737$; $p = 0.5677$) or ACTH ($F(1, 5)=0.07635$; $p = 0.7934$). While there was no main effect of time for ACTH ($F(3, 15)=0.9498$; $p = 0.4415$), there was a main effect of time for CORT ($F(3, 15)=8.157$; $p = 0.0019$). Bonferroni's multiple comparisons tests showed that the significant differences occurred for both BC and AP at ZT0 versus ZT8 (adjusted $p=0.0328$ and 0.0012 , respectively) and at ZT0 versus ZT12 (adjusted $p=0.0316$ and 0.0166 , respectively).

No significant interactions between time and manipulation were found in the repeated measures two-way ANOVAs between AP and GH for CORT ($F(3, 12)=1.326$; $p=0.3116$) or ACTH ($F(3, 12)=1.881$; $p=0.1866$), nor were there significant variations between manipulations for CORT ($F(1, 4)=1.075$; $p=0.3584$) or ACTH ($F(1, 4)=2.073$; $p=0.2233$). There was no main effect of time for ACTH ($F(3, 12)=1.046$; $p=0.4075$), but there was a main effect of time for CORT ($F(3, 12)=6.188$; $p=0.0087$). The significant difference occurred for only AP at ZT0 versus ZT8 (adjusted $p=0.0497$) according to Bonferroni's multiple comparisons tests.

3.4. Post-deprivation sleep characterization

A comparison of the sleep architecture was performed between BC and AP during the remainder of the light cycle after sleep deprivation had ended. Scored states were separated into 30 min bins in

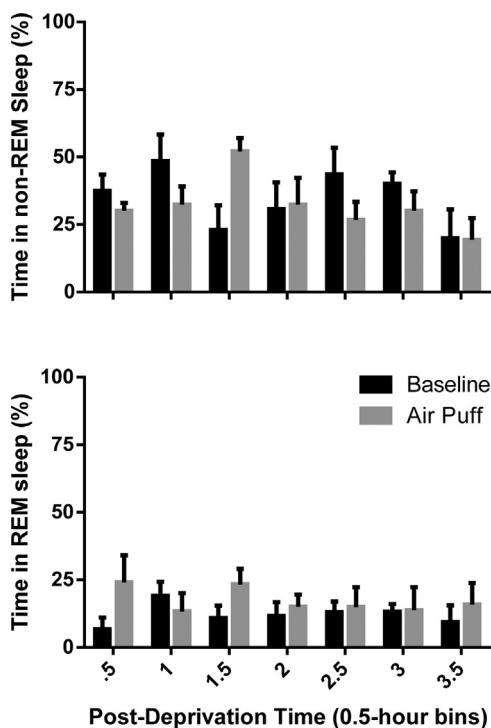


Fig. 9. Percent time in (a) non-REM and (b) REM sleep separated into 30 min bins beginning after the completion of sleep deprivation with air puffs and ending at lights off ($n = 6$ animals; error bars = SEM). Baseline data taken from the same animals during the same timeframe on the sleep with blood sampling day. No significant differences were found for either sleep state between baseline and air puff days (2-way ANOVA with repeated measures; all p -values $\gg 0.05$).

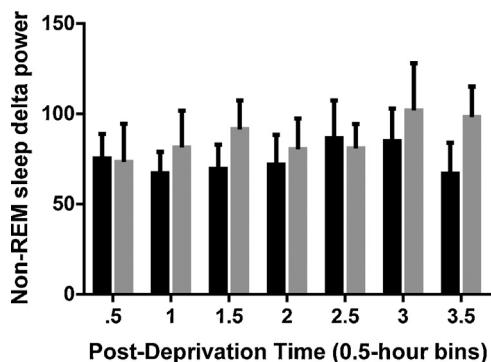


Fig. 10. Average delta power for non-REM sleep separated into 30 min bins beginning after the completion of sleep deprivation with air puffs and ending at lights off ($n = 6$ animals; error bars = SEM). Baseline data taken from the same animals during the same timeframe on the sleep with blood sampling day. No significant differences were found between baseline and air puff days (2-way ANOVA; interaction, method, and time as well as Holm-Sidak's multiple comparisons test at each time point resulted in p -values $\gg 0.05$).

order to increase the likelihood of discovering differences between groups. No significant differences were found in the repeated measures two-way ANOVAs between BC and AP in the amount of non-REM (Fig. 9a; time: $F(6, 30) = 1.584, p = 0.1862$; method: $F(1, 5) = 0.8114, p = 0.4090$; interaction: $F(6, 30) = 1.712, p = 0.1526$) and REM sleep (Fig. 9b; time: $F(6, 70) = 0.7773, p = 0.5905$; method: $F(6, 70) = 0.1417, p = 0.9901$; interaction: $F(1, 70) = 2.417, p = 0.1245$). In addition, delta power during non-REM sleep was compared over this time period (Fig. 10). A two-way ANOVA showed no significant differences in non-REM sleep delta power between BC and AP days during the recovery period (time: $F(6, 62) = 0.3004, p = 0.9344$; method: $F(1, 62) = 1.659, p = 0.2025$; interaction: $F(6, 62) = 0.2609, p = 0.9529$).

3.5. Sound level measurements

For our method, ambient noise within the home cage placed in the temperature and light-regulated chamber with the door closed was ~ 47 A-weighted decibels (dBA). The average noise level for our air puff pulses was 68 ± 3 dBA (S.E.M.). As a comparison, the ambient noise of the animal housing room was 63 ± 1 dBA (S.E.M.) with the chamber door open.

4. Discussion

Our goal in developing the automated air puff sleep deprivation system was to create an automated alternative to manual sleep deprivation techniques (e.g., gentle handling) that deprives an animal of all stages of sleep during a critical 4 to 5 h window in which sleep must occur after the completion of a learning task for hippocampal memory consolidation to take place (Bjorness et al., 2005; Hagedowd et al., 2010; Prince et al., 2014). The results from both the off-line auto-scoring validation and the on-line animal experiments suggest that the real-time version of Auto-Scorer (Gross et al., 2009) can be used confidently to automatically score 2 s epochs of sleep and waking states from EEG and EMG recordings in rats. This on-line version is capable of running in real-time and has time for additional calculations to be included in the logic algorithm without dropping data between epochs. Yet, it is recognized that the processing time is dependent on the capabilities of the computer that is running MATLAB, so the run-time may vary under different hardware configurations.

The system is effective for the first 5 h (see Section 3.2) (the critical memory consolidation window identified in many studies) using the defined air puff parameters. The results demonstrated that the system was not significantly effective in hours 6, 7 and 8—beyond the critical window. This may have been due to several factors. For example, some rats in our study found a zone of tolerance in the middle of the cage where the air puffs did not behaviorally awaken them and learned to stay there. When this occurred, we placed an inverted water dish in the middle of the cage so that rats could not rest in this zone, which put the rat in closer proximity to some of the air ports. However, the decrease in delta power after air puffs indicate that there is still an EEG response to the stimulus in the absence of a behavioral arousal scored by 10 s epoch (see Fig. 8). The fact that the air puff technique remained effective at suppressing delta, even in the latter hours, implies that there was no habituation. Due to our programmed “blackout” period allowing stimulation only every 5 s, the rats were able to obtain about 3 s of delta before the next possible air puff. The deprivation technique, with this “blackout” period, became, in effect, a sleep disruption technique. Because they did not habituate, it is likely that total sleep deprivation could be obtained in later hours simply by eliminating the blackout period. The sleep obtained in hours 6 through 8 may also explain why we saw no differences in the sleep architecture between baseline controls and the air puff groups; neither did we see an increase in delta power during the post deprivation period that is typically seen in deprived animals.

Although not the goal of our study, researchers interested in developing the system for longer periods of sleep deprivation could vary the air puff pressure, duration, and pulse pattern; especially during REM when the arousal threshold is at its highest. However, stress responses would need to be characterized to make sure that any changes to the stimulus do not induce significant stress.

We did not design the study to target the dark period. In Borberly and Neuhaus (1979), rats were deprived using a slowly rotating cylinder, starting during the dark phase or during the light phase, and found no difference in the deprivation efficiency, but did find a difference in what occurred during rebound sleep. In a paper

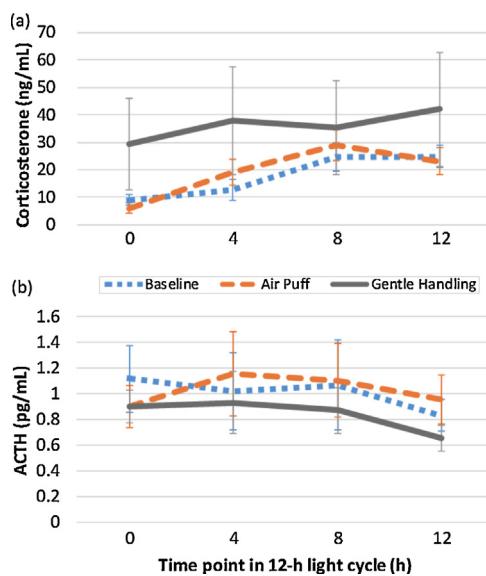


Fig. 11. Hormone concentrations of (a) corticosterone (CORT) and (b) adrenocorticotropic hormone (ACTH) were measured from serum samples to determine the effect of air puff (AP) to baseline and gentle handling (GH) sleep-deprivation. Samples were collected at four time points (0, 2, 4, and 6 h) throughout the light-cycle during baseline or manipulation.

recently published depriving for 12 h (using a gradually increasing rotation variability) in either the light or dark phase, researchers showed that sleep deprivation was equally effective in the light and dark phases, but because there is less overall sleep to deprive from the dark phase, rebound was not significant in the subsequent light phase (Leenaars et al., 2011). Circadian and homeostatic sleep drive is highest during the light period in rats. According to the above two studies, the technique that works in the light phase, works as well in the dark phase. Our study confines deprivation to the light phase when most studies look at sleep dependent memory consolidation.

CORT and ACTH are hormones indicative of hypothalamic-pituitary-adrenal (HPA) activity (Borberly and Neuhaus, 1979). Chronic sleep restriction techniques have been shown to activate the HPA axis, a primary neuroendocrine response to stressors (Meerlo et al., 2002; Rechtschaffen and Bergmann, 2002); however, acute sleep deprivation using the same methods (e.g., gentle handling or rotating disc) do not result in significantly different HPA axis activity compared to controls (Rechtschaffen et al., 1999). Results from the immunoassay of isolated serum samples suggest that AP is no more stressful than GH as an acute sleep deprivation technique. Furthermore, the data also suggest that hormone concentrations during AP are not significantly different than during sleep (see Section 3.3 and Fig. 11).

The lack of a significant stress response to the air puffs used in acute sleep deprivation may at first seem to conflict with previous studies in which air puffs were used for the purpose of causing stress; however, the parameters of the delivered air puffs in our method are drastically different from these studies (Furlong et al., 2014; Porter and Hayward, 2011; Spencer et al., 2005). The pulse length of air puffs used for these stress-inducing studies was 8 to 4800 times longer than that used in our method. In addition, the pressures of the puffs used in these studies were 5 to 11 times greater than in our method. Furthermore, it is unclear as what effect on stress sound of the air puffs may play in the higher pressure studies as noise levels were not reported in two of the studies, while the study using a continuous air stream at 20 psi stated that the noise level was ~75–80 decibels without further details on the measurement (Porter and Hayward, 2011). Given that the noise level for air puffs in our study was lower and that the control animals exposed

to air noise alone did not have significant stress-induced changes, it is unlikely that the noise level of our air puffs would induce stress under acute exposure. Therefore, our results do not conflict with previous findings, but instead emphasize the importance of the parameters used to accomplish the intended goals of the method and support the use of air puffs as a means to disrupt sleep in acute studies on cognition.

Based on the real-time capabilities of the system, the accuracy of the auto-scoring algorithm, and the ability of the system to automatically deprive rats of sleep without a significant stress response, we have shown that the automated air puff sleep deprivation system is a viable alternative to existing sleep deprivation techniques (i.e., gentle handling, multiple platforms over water, noise, and forced locomotion). Further, use of this system avoids questions of exercise as a confounding variable that has been raised in techniques using forced locomotion to disrupt sleep (Franken et al., 1991; Rechtschaffen et al., 1999). Future studies will focus on the cognitive effects of AP using both sleep-dependent and independent cognitive tasks in order to further characterize this novel technique.

While our goal was to create an automated system to deprive sleep in the critical window in order to study the mechanisms of sleep-dependent memory consolidation in the hippocampus, the system that we have developed could easily be used to disrupt or fragment, rather than totally deprive, sleep by modifying the logic algorithm in the open source code, for example, to trigger air puffs for 1 out of every n detected 2 s sleep epochs in an ordered or random fashion. In addition, the algorithm could be used to target individual stages of sleep (e.g., REM-only) with the caveat that additional optimization of the state detection algorithm may be needed to achieve the intended goals of the study. Furthermore, our system could be modified by simply replacing the electronic air valve connected to the trigger switch to automatically deliver other types of real-time feedback such as sounds or brain stimulation (e.g., electrical or optogenetic).

The software component of this system will be made available via an open-source license. As mentioned in our previous publication on Auto-Scorer (Gross et al., 2009), our auto-scoring algorithm has been developed for our specific experiments using rats with cortical screw EEG and neck EMG electrodes. Given the ease of access to our logic-based algorithm in MATLAB, even sleep researchers without any prior programming experience should be able to modify the algorithm to meet their experimental goals.

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References

- Benington JH, Kodali SK, Heller HC. Scoring transitions to REM sleep in rats based on the EEG phenomena of pre-REM sleep: an improved analysis of sleep structure. *Sleep* 1994;17:28–36.
- Bjorness TE, Riley BT, Tysor MK, Poe GR. REM restriction persistently alters strategy used to solve a spatial task. *Learn Mem* 2005;12:352–9.
- Borberly AA, Neuhaus HU. Sleep-deprivation: effects on sleep and EEG in the rat. *J Comp Physiol* 1979;133:71–87.
- Diba K, Buzsáki G. Forward and reverse hippocampal place-cell sequences during ripples. *Nat Neurosci* 2007;10:1241–2.

- Ferrara M, Iaria G, Tempesta D, Curcio G, Moroni F, Marzano C, et al. **Sleep to find your way: the role of sleep in the consolidation of memory for navigation in humans.** *Hippocampus* 2008;18:844–51.
- Franken P, Dijk D, Tobler I, Borbely AA. **Sleep deprivation in rats: effects on EEG power spectra, vigilance states, and cortical temperature.** *Am J Physiol* 1991;261:R198–208.
- Furlong TM, McDowall LM, Horiuchi J, Polson JW, Dampney RAL. **The effect of air puff stress on c-Fos expression in rat hypothalamus and brainstem: central circuitry mediating sympathoexcitation and baroreflex resetting.** *Eur J Neurosci* 2014;39:1429–38.
- Gross BA, Walsh CM, Turakhia AA, Booth V, Mashour GA, Poe GR. **Open-source logic-based automated sleep scoring software using electrophysiological recordings in rats.** *J Neurosci Methods* 2009;184:10–8.
- Hagewoud R, Havekes R, Novati A, Keijser JN, Van der Zee EA, Meerlo P. **Sleep deprivation impairs spatial working memory and reduces hippocampal AMPA receptor phosphorylation.** *J Sleep Res* 2010;19:280–8.
- Havekes R, Bruinenberg VM, Tudor JC, Ferri SL, Baumann A, Meerlo P, et al. **Transiently increasing cAMP levels selectively in hippocampal excitatory neurons during sleep deprivation prevents memory deficits caused by sleep loss.** *J Neurosci* 2014;34:15715–21.
- Kamphuis J, Lancel M, Koolhaas JM, Meerlo P. **Deep sleep after social stress: NREM sleep slow-wave activity is enhanced in both winners and losers of a conflict.** *Brain Behav Immun* 2015, in press.
- Kempfer L, Richmond JL. **Effect of sleep on gross motor memory.** *Memory* 2012;20:907–14.
- Leenaars CH, Dematteis M, Joosten RN, Eggels L, Sandberg H, Schirris M, et al. **A new automated method for rat sleep deprivation with minimal confounding effects on corticosterone and locomotor activity.** *J Neurosci Methods* 2011;196:107–17.
- Meerlo P, Koehl M, van der Borght K, Turek FW. **Sleep restriction alters the hypothalamic–pituitary–adrenal response to stress.** *J Neuroendocrinol* 2002;14:397–402.
- Meerlo P, Sgoifo A, Suchecki D. **Restricted and disrupted sleep: effects on autonomic function, neuroendocrine stress systems and stress responsivity.** *Sleep Med Rev* 2008;12:197–210.
- Mendelson WB, Guthrie RD, Frederick G, Wyatt RJ. **The flower pot technique of rapid eye movement (REM) sleep deprivation.** *Pharmacol Biochem Behav* 1974;2:553–6.
- O'Callaghan RM, Ohle R, Kelly ÁM. **The effects of forced exercise on hippocampal plasticity in the rat: a comparison of LTP, spatial- and non-spatial learning.** *Behav Brain Res* 2007;176:362–6.
- Paul S, Khanapur S, Elsinga PH, Ishiwata K, Meerlo P, Dierckx R, et al. **PET imaging of adenosine A1 receptor occupancy.** *J Nucl Med* 2014;55:1918.
- Poe GR, Walsh CM, Bjorness TE. **Both duration and timing of sleep are important to memory consolidation.** *Sleep* 2010;33:1277–8.
- Porter K, Hayward LF. **Stress-induced changes in c-Fos and corticotropin releasing hormone immunoreactivity in the amygdala of the spontaneously hypertensive rat.** *Behav Brain Res* 2011;216:543–51.
- Prince T-M, Wimmer M, Choi J, Havekes R, Aton S, Abel T. **Sleep deprivation during a specific 3-hour time window post-training impairs hippocampal synaptic plasticity and memory.** *Neurobiol Learn Mem* 2014;109:122–30.
- Prince TM, Abel T. **The impact of sleep loss on hippocampal function.** *Learn Mem* 2013;20:558–69.
- Rechtschaffen A, Bergmann BM. **Sleep deprivation in the rat: an update of the 1989 paper.** *Sleep* 2002;25:18–24.
- Rechtschaffen A, Bergmann BM, Gilliland MA, Bauer K. **Effects of method, duration, and sleep stage on rebounds from sleep deprivation in the rat.** *Sleep* 1999;22:11–31.
- Robert C, Guilpin C, Limoge A. **Automated sleep staging systems in rats.** *J Neurosci Methods* 1999;88:111–22.
- Ruitg GS, Van Proosdij JN, Van Wezenbeek LA. **A large scale, high resolution, automated system for rat sleep staging. II. Validation and application.** *Electroencephalogr Clin Neurophysiol* 1989;73:64–71.
- Sanford LD, Suchecki D, Meerlo P. **Stress, Arousal, and Sleep.** *Curr Top Behav Neurosci* 2015;25:379–410.
- Saunders BT, Yager LM, Robinson TE. **Cue-evoked cocaine craving: role of dopamine in the accumbens core.** *J Neurosci* 2013;33:13989–4000.
- Smith C. **Sleep states and memory processes.** *Behav Brain Res* 1995;69:137–45.
- Smith C, MacNeill C. **Impaired motor memory for a pursuit rotor task following Stage 2 sleep loss in college students.** *J Sleep Res* 1994;3:206–13.
- Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, et al. **Olfactory exposure to males, including men, causes stress and related analgesia in rodents.** *Nat Methods* 2014;11:629–32.
- Spencer SJ, Buller KM, Day TA. **Medial prefrontal cortex control of the paraventricular hypothalamic nucleus response to psychological stress: possible role of the bed nucleus of the stria terminalis.** *J Comp Neurol* 2005;481:363–76.
- Tobler I, Borbely AA. **The effect of 3-h and 6-h sleep deprivation on sleep and EEG spectra of the rat.** *Behav Brain Res* 1990;36:73–8.
- Watts A, Gritton HJ, Sweigart J, Poe GR. **Antidepressant suppression of non-REM sleep spindles and REM sleep impairs hippocampus-dependent learning while augmenting striatum-dependent learning.** *J Neurosci* 2012;32:13411–20.
- Witting W, Van der Werf D, Mirmiran M. **An on-line automated sleep-wake classification system for laboratory animals.** *J Neurosci Methods* 1996;66:109–12.
- Yager LM, Robinson TE. **A classically conditioned cocaine cue acquires greater control over motivated behavior in rats prone to attribute incentive salience to a food cue.** *Psychopharmacology* 2013;226:217–28.
- Youngblood BD, Zhou J, Smagin GN, Ryan DH, Harris RB. **Sleep deprivation by the flower pot technique and spatial reference memory.** *Physiol Behav* 1997;61:249–56.