

Video Article

Polygraphic Recording Procedure for Measuring Sleep in Mice

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Abstract

Recording of the epidural electroencephalogram (EEG) and electromyogram (EMG) in small animals, like mice and rats, has been pivotal to study the homeodynamics and circuitry of sleep-wake regulation. In many laboratories, a cable-based sleep recording system is used to monitor the EEG and EMG in freely behaving mice in combination with computer software for automatic scoring of the vigilance states on the basis of power spectrum analysis of EEG data. A description of this system is detailed herein. Steel screws are implanted over the frontal cortical area and the parietal area of 1 hemisphere for monitoring EEG signals. In addition, EMG activity is monitored by the bilateral placement of wires in both neck muscles. Non-rapid eye movement (Non-REM; NREM) sleep is characterized by large, slow brain waves with delta activity below 4 Hz in the EEG, whereas a shift from low-frequency delta activity to a rapid low-voltage EEG in the theta range between 6 and 10 Hz can be observed at the transition from NREM to REM sleep. By contrast, wakefulness is identified by low- to moderate-voltage brain waves in the EEG trace and significant EMG activity.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53678/>

Introduction

Technical advances have often precipitated quantum leaps in the understanding of neurobiological processes. For example, Hans Berger's discovery in 1929 that electrical potentials recorded from the human scalp took the form of sinusoidal waves, the frequency of which was directly related to the level of wakefulness of the subject, led to rapid advances in the understanding of sleep-wake regulation, in both animals and humans alike.¹ To this day the electroencephalogram (EEG), in conjunction with the electromyogram (EMG), *i.e.*, electrical activity produced by skeletal muscles, represents the data "backbone" of nearly every experimental and clinical assessment that seeks to correlate behavior and physiology with the activity of cortical neurons in behaving animals, including humans. In most basic sleep research laboratories these EEG recordings are performed by using a cable-based system (**Figure 1**) wherein acquired data is subjected off-line to pattern and spectrum analysis [*e.g.*, applying a fast Fourier transform (FFT) algorithm] to determine the vigilance state of the subject being recorded.^{2,3} Sleep consists of rapid-eye movement (REM) and non-REM (NREM) sleep. REM sleep is characterized by a rapid low-voltage EEG, random eye movement, and muscle atonia, a state in which the muscles are effectively paralyzed. REM sleep is also known as paradoxical sleep, because the brain activity resembles that of wakefulness, whereas the body is largely disconnected from the brain and appears to be in deep sleep. By contrast, motor neurons are stimulated during NREM sleep but there is no eye movement. Human NREM sleep can be divided into 4 stages, whereby stage 4 is called deep sleep or slow-wave sleep and is identified by large, slow brain waves with delta activity between 0.5 - 4 Hz in the EEG. On the other hand, a subdivision between phases of NREM sleep in smaller animals, like rats and mice, has not been established, mostly because they do not have long consolidated periods of sleep as seen in humans.

Over the years, and on the basis of EEG interpretation, several models of sleep-wake regulation, both circuit- and humoral-based, have been proposed. The neural and cellular basis of the need for sleep or, alternatively, "sleep drive," remains unresolved, but has been conceptualized as a homeostatic pressure that builds during the waking period and is dissipated by sleep. One theory is that endogenous somnogenic factors accumulate during wakefulness and that their gradual accumulation is the underpinning of sleep homeostatic pressure. While the first formal hypothesis that sleep is regulated by humoral factors has been credited to Rosenbaum's work published in 1892⁴, it was Ishimori^{5,6} and Pieron⁷ who independently, and over 100 years ago, demonstrated the existence of sleep-promoting chemicals. Both researchers proposed, and indeed proved, that hypnogenic substances or 'hypnotoxins' were present in the cerebral spinal fluid (CSF) of sleep-deprived dogs.⁸ Over the past century several additional putative hypnogenic substances implicated in the sleep homeostatic process have been identified (for review, see ref. 9), including prostaglandin (PG) D₂,¹⁰ cytokines,¹¹ adenosine,¹² anandamide,¹³ and the urotensin II peptide.¹⁴

Experimental work by Economo^{15,16}, Moruzzi and Magoun¹⁷, and others in the early and mid-20th century produced findings that inspired circuit-based theories of sleep and wakefulness and, to a certain degree, overshadowed the then prevailing humoral theory of sleep. To date, several "circuit models" have been proposed, each informed by data of varying quality and quantity (for review, see ref. 18). One model, for example, proposes that slow-wave sleep is generated through adenosine-mediated inhibition of acetylcholine release from cholinergic neurons in the basal forebrain, an area mainly consisting of the nucleus of the horizontal limb of the diagonal band of Broca and the substantia innominata.¹⁹ Another

popular model of sleep/wake regulation describes a flip-flop switch mechanism on the basis of mutually inhibitory interactions between sleep-inducing neurons in the ventrolateral preoptic area and wake-inducing neurons in the hypothalamus and brain stem.^{18, 20, 21} Moreover, for the switching in and out of REM sleep, a similar reciprocally inhibitory interaction has been proposed for areas in the brain stem, that is the ventral periaqueductal gray, lateral pontine tegmentum, and sublaterodorsal nucleus.²² Collectively, these models have proven valuable heuristics and afforded important interpretative frameworks for studies in sleep research; however, a yet fuller understanding of the molecular mechanisms and circuits regulating the sleep-wake cycle will require a more complete knowledge of its components. The system for polygraphic recording detailed below should aid in this goal.

Protocol

Ethics Statement: Procedures involving animal subjects have been approved by the Institutional Animal Experiment Committee at the University of Tsukuba.

1. Preparation of Electrodes and Cables for EEG/EMG Recordings

1. Prepare EEG/EMG recording electrode according to the following procedure.
Note: The electrode is disposable and can be used only for 1 animal. Plan carefully the wiring configuration for all connectors. Place marks on the connectors for the correct orientation.
 1. Solder each pin of a 4-pin header to a 2-cm stainless steel wire. In brief, hold one end of the wire to the pin, place a hot soldering iron onto the wire-pin joint, and melt some solder to ensure that just enough solder runs smoothly into the joint. Be careful not to apply too much heat to the pin; otherwise, the plastic around the pins will melt.
 2. Solder the free end of each of 2 wires attached to the pin header to the head of a 1.0-mm-diameter stainless steel screw. In brief, hold the free end of the wire to the thread below the screw head, place a hot soldering iron onto the wire-screw joint, and melt some solder to ensure that just enough solder runs smoothly into the joint. The 2 wires with screws serve as EEG recording electrodes, whereas the 2 wires without screws serve as EMG recording electrodes.
 3. Use scissors to strip off 1 mm of the insulation at the end of the EMG electrodes to increase the quality of the EMG signal.
 4. Completely cover all soldered pins with epoxy adhesive by using a thin wooden stick or tooth pick to reduce the electrical noise during EEG/EMG recordings.
2. Prepare a cable for connecting the electrode with the slip ring as described below. This cable can be reused.
 1. Solder each pin of a 4-pin FFC/FPC connector with a wire of a 30-cm flat cable. In brief, hold the stripped end of the wire to the pin, place a hot soldering iron onto the wire-pin joint, and melt some solder to ensure that just enough solder runs smoothly into the joint.
Note: Choose the length of the flat cable which is appropriate for the height of the experimental animal cage used for EEG/EMG recordings.
 2. Solder crimp sockets to a tip of the wires on the other end of the flat cable. In brief, hold a crimp socket to the stripped free end of the wire, place a hot soldering iron onto the wire-socket joint, and melt some solder to ensure that just enough solder runs smoothly into the joint.
 3. Insert each crimp socket into a 4-position crimp housing.
 4. Completely cover the crimp sockets with epoxy adhesive by using a thin wooden stick or tooth pick.

2. Implantation of Electrodes in the Mouse Head (Duration: Approx. 20 min)

1. Sterilize all surgical tools in a hot bead sterilizer before the surgery. Anesthetize a male mouse (10 - 20 weeks old, 20 - 30 g) with an intraperitoneal injection of pentobarbital (50 mg/kg). After checking that the mouse is deeply anesthetized by pinching a toe, shave the hair on the head and neck with clippers.
2. Move the mouse to the stereotaxic frame, and fix the head between the 2 ear bars. Apply petroleum jelly on the eyes to prevent dryness. Cleanse the shaved-skin with alcohol and cut it along the midline with a scalpel to expose the skull. Clip the skin to keep the surgical area open.
3. By using a carbide cutter (drill size: 0.8-mm diameter), drill 2 holes into the skull, one over the frontal cortical area (1 mm anterior to bregma, 1.5 mm lateral to the midline) and the other over the parietal area (1 mm anterior to lambda, 1.5 mm lateral to midline) of the right hemisphere, according to stereotaxic coordinates of Paxinos and Franklin.²³
4. By using a jeweler's screw driver, place stainless steel EEG recording screws in the holes and make 2 - 2.5 turns for each screw for epidural positioning over the cortex.
Note: Do not insert screws too deep to prevent damage to the brain tissue. Check that the screws are tightly fixed on the skull. This is important to have stable EEG signals during a long period of multiple recordings (typically, more than 1 month). Wiggly screws produce EEG artifacts and may come off before the end of the experimental schedule.
5. Fix the electrode assembly (cf. Section 1.1, pins turned upward) with instant glue to the skull and cover with dental cement. Make bilaterally small holes with forceps in the trapezius (neck) muscles and insert the stainless steel wires that serve as EMG electrodes into the holes. Suture the skin with a silk thread (0.1 mm diameter) to avoid exposure of the muscle.
6. Remove the mouse from the stereotaxic frame. Administer ampicillin (100 mg/kg) and meloxicam (1 mg/kg) intraperitoneally to the mouse to avoid bacterial infection and to reduce post-surgical pain, respectively. Keep the mouse on a heat pad and monitor it until it has regained sufficient consciousness to maintain sternal recumbency. House the mouse individually during recovery to avoid removal of electrodes by other animals, and administer meloxicam (1 mg/kg) intraperitoneally on the first day after the surgery.

3. Recording and Acquiring EEG/EMG Data

1. After a 1-week recovery period, house each mouse individually in an experimental cage placed in a soundproof recording chamber. Maintain an ambient temperature of 23 ± 1 °C and automatically control cycles of 12-hr light/12-hr dark (lights on at 08:00, illumination intensity ~ 100 lux).
2. Connect the EEG/EMG electrode assembly on the mouse head to a recording cable. Ensure that the recording cable is connected to a slip ring (which is designed so that movements of the mouse are not restricted by twisting of the cable) and an EEG/EMG signal amplifier. Filter EEG/EMG signals (EEG, 0.5-64 Hz; EMG, 16-64 Hz), digitize at a sampling rate of 128 Hz by an analog-to-digital converter (A/D) and finally record on a computer running EEG/EMG recording software (Table 'Materials', 4th entry from bottom).
3. Habituate the mouse for 2 - 3 days in the recording chamber. If the EEG/EMG recording includes intraperitoneal drug administrations, gently handle the mouse on each habituation day at the time of the drug administration.
4. Subsequently, start EEG/EMG recording software (Table 'Materials', 4th entry from bottom).
 1. Select the 'Data file information' tab and click the box next to the file name. Enter a file name and click 'Save'.
 2. Select the 'Recording condition' tab and select all EEG/EMG channels which will be recorded.
 3. Select the sampling frequency (128 Hz) in the 'Recording condition' tab.
 4. Check if the selected channels are displayed properly in the 'Channel information' tab.
 5. Select the 'Timer setting' tab and click 'Monitor' to display EEG and EMG.
 6. Check if EEG/EMG signals are displayed correctly.
 7. Select the 'Timer setting' tab and set the clock time for the beginning and end of the recording in the 'Main Timer' area.
 8. Click 'Monitor' in the 'Timer setting' tab to start the recording.
5. Record EEG/EMG signals under baseline (*i.e.*, sleep/wake behavior of a freely behaving mouse) and different treatment conditions (*e.g.*, caffeine administration or sham treatment) over several days. Euthanize the mouse with an intraperitoneal injection of pentobarbital (200 mg/kg) after the last experimental day.

4. Scoring of Behavioral State Based on EEG/EMG Data

1. Start the software for EEG/EMG analysis (Table 'Materials', 4th entry from bottom). Open the EEG/EMG raw data (.kcd file) produced under the step 3. Click the 'Sleep' tab and select Epoch time. Select 10 sec.
2. Click the 'Sleep' tab and select 'Multi-screening' to automatically score all 10-sec epochs into 3 stages (*i.e.*, NREM and REM sleep, and wakefulness) on the basis of the amplitudes of EEG and EMG and power spectral analysis of the EEG.³
3. Click 'FFT condition for EEG'.
4. Set the parameters for the power spectral analysis [256 datum points (corresponding to 2 sec of the EEG), Hanning window function, and the average of 5 spectra per epoch].³ Click 'OK'.
5. Click 'Start Screening' to begin the automatic screening. Open the scored data (.raf file).
6. Check the results of the automatic screening and, if necessary, correct them manually based on the standard criteria (see the Representative Results, **Figure 1B** and **Table 1**).^{2,3} Briefly, click and hold left mouse button on an incorrectly scored epoch and drag the cursor across the string of incorrectly scored epochs. Release the left mouse button and select the correct behavioral state in the pop-up window.

Note: Occasionally, epochs at the transition between two vigilant states are difficult to score unambiguously to one state. In such cases, the epoch should be scored to the ostensible state and the same criteria should be applied to similar epochs throughout the experiment to ensure data reproducibility.

Representative Results

Figure 1B illustrates examples of the mouse EEG in the different vigilance states. As shown in **Table 1**, epochs are classified as NREM sleep if the EEG shows large, slow brain waves with a delta rhythm below 4 Hz and the EMG has only a weak or no signal. Epochs are classified as REM sleep if the EEG shows rapid low-voltage brain waves in the theta range between 6 and 10 Hz and the EMG shows low amplitude. Other epochs should be classified as wakefulness (*i.e.*, low-to-moderate voltage EEG and occurrence of EMG activity).

For instance, the EEG/EMG recording set-up described in this protocol can be used to determine the sleep amount and sleep/wake profile of C57BL/6 mice under baseline conditions or after treatment with caffeine (**Figures 2 and 3**).

Under baseline conditions, mice, which are nocturnal animals, exhibited a clear circadian sleep-wake rhythm, as seen in these figures, with larger amounts of sleep during the light period than during the dark one (**Figure 2A**). During the 12-hr light period, the mice showed 6.7 hr and 0.9 hr of NREM and REM sleep, respectively; whereas during the 12-hr dark period, wakefulness was predominant (**Figure 2B**). On the other hand, the quality of sleep can be evaluated on the basis of the vigilance state and EEG power spectrum analysis (**Figure 2C-F**). Typically, polygraphic recordings of EEG and EMG can be used to determine episode duration distribution, mean duration, and stage transition number for each vigilance state (**Figure 2C-E**). Moreover, the EEG power spectrum for NREM and REM sleep in mice during the light and dark period (**Figure 2F**) shows strong EEG power density in the frequency range of 0.5 - 4 Hz and 6 - 10 Hz, respectively. It should be noted that the EEG during REM sleep includes small amounts of delta waves (0.5 - 4 Hz), since the intermingled states of NREM and REM sleep are sometimes a contaminant.

To assess drug effects on the sleep-wake behavior of mice,²⁴⁻³⁰ EEG and EMG are typically recorded for 2 consecutive days. To determine, for example, the arousal effect of caffeine on C57BL/6 mice,²⁴ the mice were treated with vehicle (10 ml/kg saline; intraperitoneally) on day 1 at 10:00 A.M. in the early phase of the light period. The animals were then treated with caffeine (15 mg/kg) 24 hr later, and the vigilance states were classified offline into waking, REM sleep, and NREM sleep. **Figure 3A** shows typical examples of EEG, EMG, and hypnograms after the administration of caffeine (lower polysomnographic panels) or vehicle (upper polysomnographic panels) in a C57BL/6 mouse. Caffeine increased the amount of wakefulness in C57BL/6 mice 2.8-fold for 3 hr after the injection (**Figure 3B**).

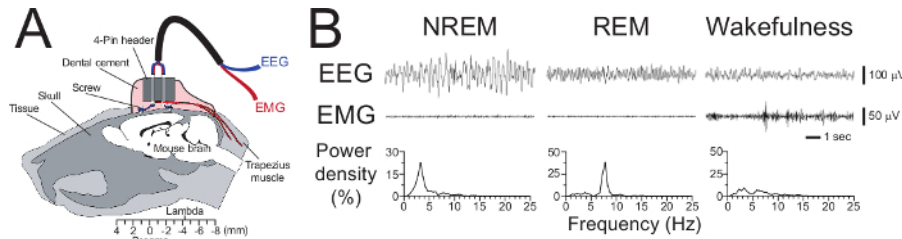


Figure 1. Sleep Bioassay System for Mice.

(A) To monitor EEG signals, stainless steel screws are implanted epidurally over the frontal cortical and parietal areas of 1 hemisphere. In addition, EMG activity is monitored by stainless steel wires placed bilaterally within the trapezius muscles. (B) Typical examples of EEG, EMG, and EEG power density for 10 sec during NREM or REM sleep or wakefulness in a mouse. In NREM sleep, EEG shows high-amplitude waves; and the delta band (0.5 - 4 Hz) is dominant (left). In REM sleep, EEG shows low amplitude waves, with the theta band (6 - 10 Hz) being dominant (middle). In wakefulness, the EEG shows low-amplitude waves, with no frequency being dominant (right). EMG signals are lower in both NREM and REM sleep than in wakefulness. [Please click here to view a larger version of this figure.](#)

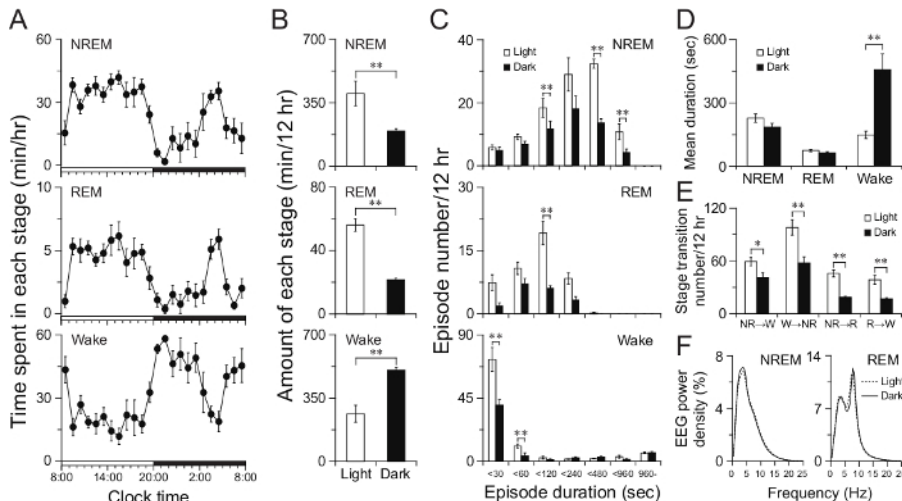


Figure 2. Sleep-wake Profiles under Baseline Conditions in C57BL/6 Mice Assessed by EEG/EMG Recordings.

(A) Time-course changes in the hourly amount of each behavioral stage. White and black bars above the x-axes indicate the light and dark periods, respectively. (B) Total amount of each stage for 12 hr shows a larger amount of NREM and REM sleep during the light period compared with that in the dark period. (C) Distribution of episode duration of each stage. (D) Mean duration of each stage is longer for wakefulness in the dark period. (E) Stage-transition number of each stage shows more frequent transitions during the light period. (F) EEG power spectrum during NREM and REM sleep shows essentially no power-density differences between light and dark periods. Data are presented as the mean $\pm$ SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$, as assessed by paired two-tailed Student's t test. [Please click here to view a larger version of this figure.](#)

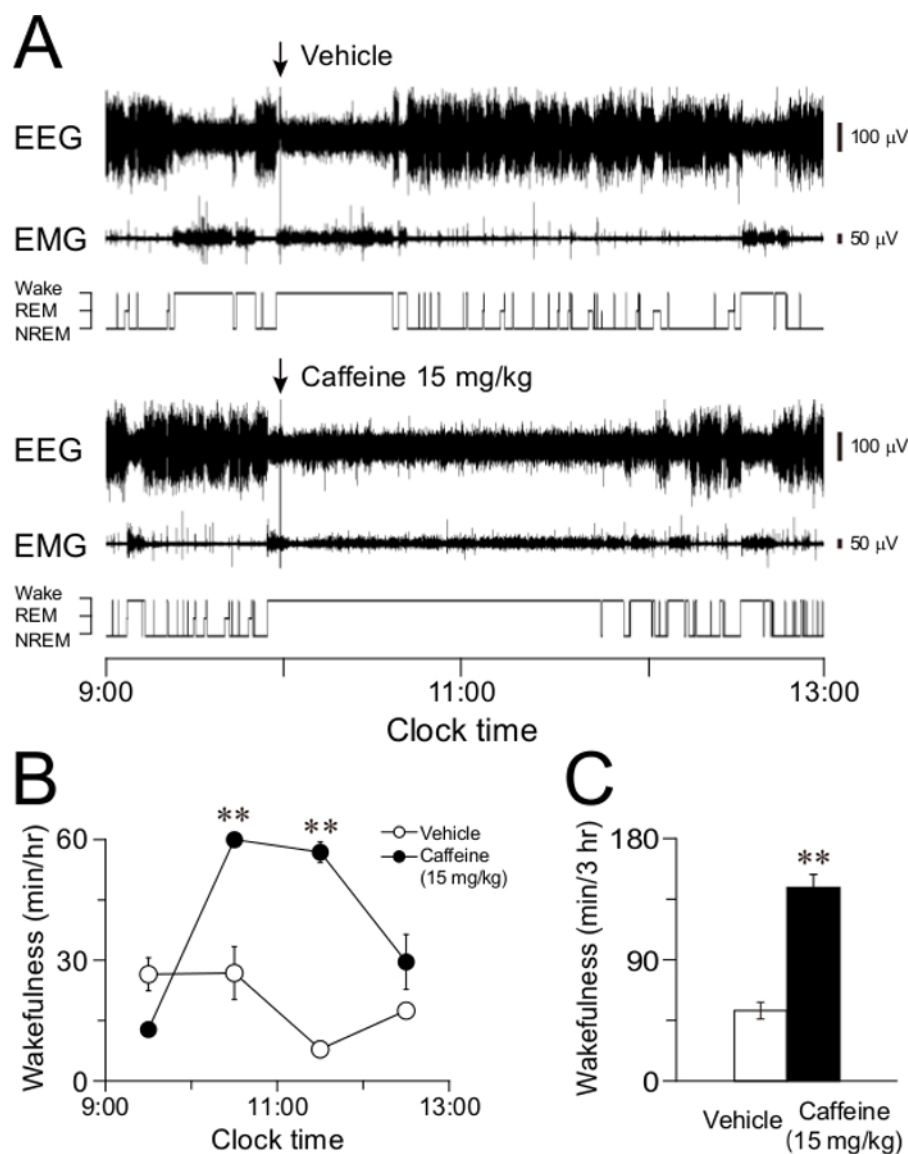


Figure 3. Arousal Effect of Caffeine Assessed by EEG/EMG Recordings.

(A) Typical examples of EEG, EMG, and hypnograms after administration of vehicle (upper panel) or caffeine at a dose of 15 mg/kg (lower panel). (B) Time courses of wakefulness in mice treated with caffeine. (C) Wakefulness over a 3-hr period after injection of caffeine. Data are presented as the mean $\pm$ SEM ($n = 5$). $**p < 0.01$ compared to vehicle injection, as assessed by paired two-tailed Student's t test. [Please click here to view a larger version of this figure.](#)

	NREM sleep	REM sleep	Wakefulness
EEG amplitude	High	Low	Low
Dominant EEG frequency	Delta band (0.5 - 4 Hz)	Theta band (6 - 10 Hz)	None
EMG amplitude	Low	Low	High

Table 1: General Criteria to Score Behavioral States by EEG/EMG Signals.

Discussion

This protocol describes a set-up for EEG/EMG recordings that allows the assessment of sleep and wakefulness under low-noise, cost-effective, and high-throughput conditions. Due to the small size of the EEG/EMG electrode head assembly, this system can be combined with other implants for intra-brain experiments, including optogenetics (optical fiber implantation) or, in conjunction with simultaneous cannula implantation, microinfusion of drugs into the mouse brain.³¹ Moreover, the design of the electrode head assembly with respect to multi-pin headers offers flexibility in the number of recording channels, if the measurement of additional electrical signals (e.g., contralateral EEG, electrooculogram or local field potential) is required.

However, individual housing is required for the cable-based design described in this protocol, which therefore limits the assessment of behavioral states, *i.e.*, sleep and wakefulness, in combination with social interaction or complex behavioral testing. In these cases, a wireless sleep monitoring system is likely more suitable, although telemetric devices are not without their own limiting features, especially battery cost and life.

The quality of EEG/EMG signals is important for the scoring of behavioral states according to the criteria shown in **Figure 1** and **Table 1**. Wiggly electrodes (*i.e.*, screws) are often the reason for electric noise resulting in artifacts in the EEG and FFT analysis. Moreover, it is important for the quality of the EEG signal to completely cover the electrode screws with dental cement to avoid air bubbles between the screws and cement, resulting in increased electric noise. The quality of EEG signals can be checked in an obviously sleeping mouse by visually confirming that they have a high amplitude and low frequency.

Cost and time to implant electrodes are critical factors for many sleep research laboratories and are considered a major drawback for large-scale screening of sleep-wake behavior in mice. The EEG/EMG recording system described here can be established and operated at low-to-moderate cost, including recurring cost for electrode materials and medical supplies (approximately 2 USD per mouse) and investments for EEG/EMG recording equipment (slip ring, amplifier, and A/D converter; approximately 2,000 USD per mouse).

With respect to time, a skilled researcher can conclude the electrode implantation for 1 mouse in less than 20 min; and thus, it is possible to operate on more than 20 mice per day. Another key factor for the overall efficiency of the assessment of sleep on the basis of EEG/EMG measurement is the use of software for acquisition and automatic scoring of EEG/EMG data. For these purposes, a variety of commercial and in-house developed software is available with a high variability in pricing or scoring accuracy.

Disclosures

The authors Yujiro Tauguchi and Sayaka Kohtoh are employees of Kissei Comtech Co., Ltd that develops SleepSign software for acquisition and automatic scoring of EEG/EMG data used in this article.

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