Sleep and EEG Phenotyping in Mice

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ABSTRACT

Although alternative, noninvasive methods are being developed, current research on rodent sleep still almost exclusively relies on recording the electroencephalogram (EEG). EEG provides information about the electrical activity of the brain and is, in combination with the electromyogram (EMG), primarily used to distinguish the different sleep and wake states. This unit describes the technique used to record EEG and EMG signals in freely moving mice, under standard laboratory conditions or specific experimental protocols such as sleep deprivation (SD). In addition to its use to determine behavioral state, the EEG contains a wealth of information concerning rhythmic brain activity that can be extracted using signal analysis tools. We will describe the protocol for implantation of EEG and EMG electrodes, provide examples of experimental designs, and discuss data acquisition and analysis illustrating the type of information that can be obtained from the EEG in mice. *Curr. Protoc. Mouse Biol.* 2:55-74 © 2012 by John Wiley & Sons, Inc.

Keywords: EEG • sleep • mice • signal analysis • spectral analysis

INTRODUCTION

This unit describes the techniques used to record electroencephalogram (EEG) and electromyogram (EMG) signals in freely moving animals, under standard and specific conditions (e.g., sleep deprivation) in the laboratory. The methods to determine behavioral state based on these two signals and to quantify the brain activity that contribute to the EEG will be explained.

Electroencephalography (EEG) is a powerful technique to capture and differentiate sleep and wakefulness states, and various stages within each state, by their defining EEG and EMG characteristics. In humans, EEG techniques are a well-established clinical tool used not only to study sleep and its disorders (see Campbell, 2009), but also to understand and help treat other disorders such as epilepsy (Tsiptsios et al., 2010) and cerebral dementia (Adamis et al., 2005). More recently, EEG has also been explored as a 'brain-machine' interface (Leeb et al., 2011).

Animal models are of great interest to study cerebral function and to mimic human pathologies (see Martín del Campo et al., 2009). EEG recording is a widely used approach to examine the electrical activity of the brain both in the physiological and disease state. The mouse model has the advantage that it can be genetically modified with relative ease, and is therefore an interesting species to use to gain insight into the genetic determinants of physiology and behavior, as well as to forward our understanding of human pathology. In sleep research, mouse models of pathologies are well established, with perhaps the best known example being narcolepsy (Chemelli et al., 1999; Willie et al., 2003), and have contributed in important ways to our current understanding of the complex regulation of sleep. The aim of this unit is to introduce the procedures for obtaining EEG data in mice and to explain and illustrate how this signal can be utilized to determine behavioral state and quantify rhythmic brain activity. The protocols presented are currently in use in our laboratory at the University of Lausanne, Switzerland. Since their original implementation in 1996 (Franken et al., 1998, 1999), several thousand mice

Current Protocols in Mouse Biology 2: 55-74, March 2012
Published online March 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/9780470942390.mo110126

have been recorded in our facilities, first at the University of Geneva, subsequently at the University of Stanford (California), and now in Lausanne. Several other laboratories have meanwhile adopted our protocols. Nevertheless, as no standard protocols have agreed upon for recording EEG signals in rodents, the protocols described here will differ from those developed in other laboratories. Moreover, providers for the materials listed are mostly local (Swiss), and equivalent tools and materials can usually and easily be obtained elsewhere.

The detailed protocols provide instruction on the implantation of EEG and EMG electrodes in mice (Basic Protocol 1), EEG/EMG calibration and recording (Basic Protocol 2), data acquisition (Basic Protocol 3), determination of behavioral states based on EEG/EMG signals (Basic Protocol 4), and spectral analysis of the EEG signals (Basic Protocol 5).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

BASIC PROTOCOL 1

IMPLANTATION OF EEG AND EMG ELECTRODES

Compared to human electroencephalography (EEG) using scalp electrodes (Campbell, 2009), in rodents the electrodes are put in direct contact with the cortex. The signal is therefore sometimes and more appropriately referred to as the electrocorticogram (ECoG) or epidural EEG. Similar to human EEG, EEG in rodents primarily detects the signal generated by the synchronized postsynaptic potentials occurring in the cortical pyramidal cell, and this signal varies between the different behavioral states (see Basic Protocol 4). In contrast to humans, in the mouse coherent signals from deeper brain structures, such as the hippocampus, can also importantly contribute to the EEG. The following protocol describes the implantation of EEG and EMG electrodes in the mouse, using a stereotaxic frame and standard coordinates.

Materials

Xylazine (Rompun, 2%, Provet AG; *http://www.provet.ch/*)
Ketamine (Ketasol 100, Graeub Veterinary Products, *http://www.graeub.com/*)
0.9% (w/v) NaCl
100% ethanol
Isoflurane (Isoflurane Attane, Provet AG; *http://www.provet.ch/*; optional)
Ophthalmic gel (e.g., Viscotears, Novartis Pharma AG; *http://www.novartis.com*)
Betadine
Adhesive Resin Cement (RelyX ARC Adhesive Resin Cement, 3 M ESPE Dental Products, *http://solutions.3msuisse.ch*)
Dental Cement (e.g., Paladur, Heraeus Kulzer; *http://heraeus-dental.de*)
Analgesic (e.g., Fynadine, 50 mg/ml)

Soldering iron (e.g., Weller WS 81, Distrelec; *https://www.distrelec.com/*)
Stainless steel miniature screws (e.g., Precision Screws and Parts, 303 SS, diameter 1.1 mm, Morris Co.; *http://www.morris01550.com/*; gold plated by a local gilding/galvanic workshop; the gold plating is not essential to the protocol and stainless-steel screws can be used as such, but it makes the soldering of the screws easier)
Copper electrical wire (Wire-Wrap AWG30 from Distrelec; *https://www.distrelec.com*)
Gold wire (e.g., Cendres + Métaux SA; *http://www.cmsa.ch/*)
Electric clippers

Stereotaxic frame (e.g., Small Animal Stereotaxic System, David Kopf Instruments)
Heating pad (e.g., DC Temperature Control System, FHC; *http://www.fh-co.com/*)
High-intensity light source (e.g., Fiber-Lite MI-150, High Intensity Illuminator, Dolan-Jenner Industries)
Sterilized surgical instrument kit including scissors, forceps, scalpels and clamps
Micro drill (e.g., Quetin MOD.CC 1000, Micro Mega; *http://www.micro-mega.com/*)
Sterile cotton swabs
Micro screwdriver ~1 mm (e.g., Minicraft TS-6N, Distrelec; *https://www.distrelec.com/*)
Connector (BPHF2-O6S-E-3.2, OHS RoHS; ENA AG; *http://www.ena.ch/*)
Suture (e.g., Ethilon II 5/0, 45 cm, Ethicon Products; *http://www.ethicon.com*)

NOTE: Although we only very seldom observe signs of infection in mice after surgery, some precautions have to be taken before starting the operation. All instruments should be sterilized before starting and, if necessary, again during the operation (e.g., autoclave or glass beads); the experimenter should wear protective clothes (clean lab coat, sterile gloves, and covering mask) and use a dedicated sterile surgical area.

Anesthetic reagent preparation

1. Put 0.8 ml xylazine (Rompun, 2%) in a 15-ml tube. Add 1.0 ml ketamine (Ketazol-100, Graeub) and 9.2 ml of 0.9% NaCl.

   The mixture can be aliquotted and stored at 4°C for a few weeks until use; protect from light. If not freshly prepared, the mixture should be allowed to reach room temperature before injection.

   A 1 ml/kg body weight injection of this mixture (see step 4) will deliver 75 mg of ketamine and 10 mg xylazine per kg body weight.

Preparation of electrodes

2. Prepare two EEG electrodes by soldering stainless steel miniature screws to a piece (~8 mm) of plain copper electrical wire (0.5-mm diameter). Clean thoroughly in ethanol. Also prepare two gold wires to be inserted into the neck muscles as EMG electrodes. Cut two pieces of gold wire to the desired length and bend them in shape so that they follow the skull curvature from the top of the skull to its base where the neck muscle attaches.

Animal selection and pre-surgical preparation

Many inbred strains of mice have been used for EEG recordings, and important genotype differences in the distribution and amount of sleep and in the EEG have been reported (Franken et al., 1998, 1999, 2001). For routine sleep phenotyping, we use adult subjects around 12 weeks of age with a body weight of 20 to 30 g. Usually, we do not implant mice younger than 9 weeks, because the size of the skull might cause some problems for the electrode implantation. Male mice are more commonly used in EEG studies because of the absence of hormonal fluctuations that could influence sleep. When recording both genders at the same time, one should be aware that there could be an influence of sexual excitation on the sleep pattern.

3. Keep animals under standard housing conditions, in appropriate housing cages, either singly (minimal surface: 330 cm^2) or by group. Provide appropriate rodent litter and food and water ad libitum. Keep animals under a 12:12 hr light-dark cycle in an experimental room with controlled temperature at 25°C, controlled humidity levels (50% to 60%), and light intensity at 70 to 90 lux.

Figure 1 Mouse placed in the stereotaxic frame for EEG and EMG implantation. In this picture, the head is kept in place by earbars and a nose clamp. Note that the earbars are not inserted into the ear canal but put backwards just to restrain head movements in the horizontal plane. The nose clamp is sufficient to keep the head fixed in the vertical plan. The eyes are protected with eye protective gel (encircled). The nose is fixed by placing the teeth of the mouse into the tooth holder part of the custom-made nose clamp (arrow).

4. Anesthetize the mouse with the ketamine-xylazine mixture (step 1) delivered i.p. at a dose of 1 ml/kg, which should provide 30 min of anesthesia.

   The mouse should become unconscious within approximately 5 min after injection.

   IMPORTANT NOTE: *During the operation, regularly test the reflex of the animal to check anesthetic depth by pinching the hind leg or the tail. Also closely monitor the respiratory rhythm and, if anesthesia depth is no longer sufficient, complement with a gaseous anesthesia (isoflurane, carrier gas 100% oxygen, flow: 1.5%).*

   Other, gaseous anesthetics can be used (e.g., isoflurane), but the depth of anesthesia has to be carefully monitored throughout surgery to avoid respiratory depression that could lead to death. Moreover, once the animal awakens, the ketamine mixture provides a residual analgesic activity, which is not the case with gaseous anesthetics.

5. When the mouse has been anesthetized, first shave the scalp with electric clippers, so that hair will not enter the wound or become stuck to the cement afterwards. Apply protective gel to the mouse's eyes to prevent them from drying out.

6. To facilitate the surgical implantation, place the anesthetized mouse into a stereotaxic frame to fix its head in a well positioned manner (Fig. 1). Put the teeth of the mouse into the tooth holder and tighten the fixation screw. Place the tongue out of the mouth to avoid suffocation. Place a tube capable of providing gas into the hole that is in front of the tube holder, so that the mouse can be given anesthetic gas during the experiment if needed. Use the ear bars to fix the head inserting them into the auditory canal.

   The cone of each ear bar fits into the meatus hole, but it serves only for accurate positioning of the head, without damaging the skull. The shape of the bar provides a large surface contact to the bone of the skull around the outer ear hole. However, be very careful when using the earbars, because when inserted too deeply they can severely damage the ear and lead to death.

   It is also possible to put the earbars into the ears in reverse, so that the pointed end does not enter into the ear canal. In this way the bars mainly serve to avoid movements of the

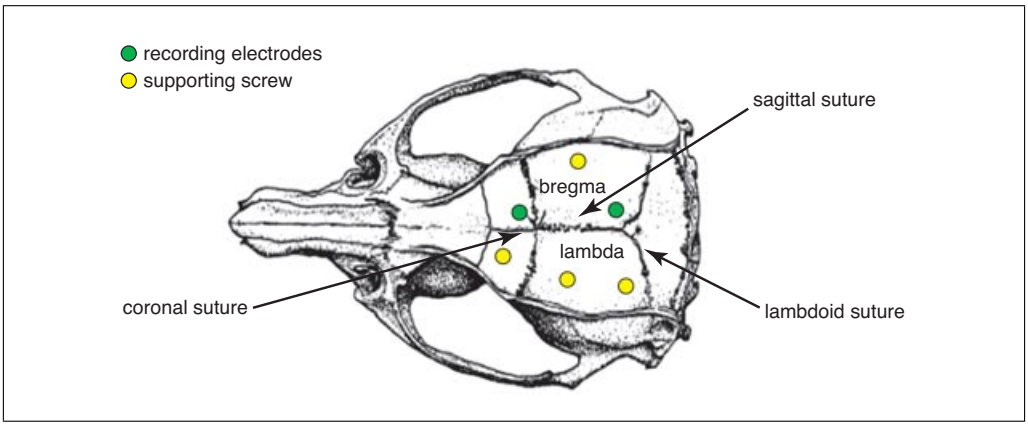

Figure 2 Schematic view of the mouse skull, indicating the position of the two recording electrodes and the four anchor screws over the cerebral hemispheres. In this configuration, the EEG signal is acquired as a bipolar derivation.

mouse head in the horizontal plane. Together with the nose clamp, this provides sufficient stability to hold the head in place (Fig. 1).

To protect the mouse from hypothermia during anesthesia, place a heating pad below the mouse and maintain a constant temperature at 36° to 37°C. Take care not to overheat the mouse. Ideally, use a heating pad connected to a rectal temperature probe to monitor and adjust body temperature automatically.

7. Illuminate the surgical area with a high-intensity light source. After having cleaned the scalp with ethanol, make a longitudinal incision using a scalpel or a scissor, along the midline, starting between the eyes and measuring ~2 cm backwards (Fig. 1). Produce a broad window by placing some clamps on the edge of the cut skin, on both sides of the head. Completely remove the skull periosteum, clean thoroughly with betadine and then with ethanol, and allow the bone to dry.

Drilling holes in preparation for implantation of EEG electrodes and screws

8. Cautiously drill six holes into the cranium with an adapted drill. Apply enough counter-pressure to avoid perforating the dura mater when the drill bit passes through the skull.

Holes are positioned bilaterally over the frontal and parietal cortex (Fig. 2). The holes for the two EEG electrodes are placed over the right cerebral hemisphere (frontal: 1.7 mm lateral to midline, 1.5 mm anterior to bregma; parietal: 1.7 mm lateral to midline, 1.0 mm anterior to lambda); the remaining four screws serve as anchor screws (see Fig. 2 for the approximate positions of these screws). The electrode positions are chosen to ensure an optimal distinction between the different behavioral states. The caudal electrode is placed over the hippocampal structures to capture the theta oscillation characteristic of REM sleep (REMS) and exploratory behavior (see Basic Protocol 4), and the rostral electrode placed over the frontal cerebral cortex captures EEG slow waves characteristic of NREM sleep (NREMS) (see Basic Protocol 4). In this manner, the EEG is acquired as a bipolar derivation, in which the potential difference between a pair of electrodes is measured. It is also possible to use a monopolar derivation, in which the potential of the recording electrode is compared to a neutral reference electrode (Osselton, 1965). In rodents, such a reference electrode can, e.g., be placed in the cerebellum. Moreover, it is possible to add electrodes to record from several brain areas simultaneously.

CAUTION: The holes should go through the skull bone, but not perforate the underlying meninges. If the dura mater is punctured, cerebrospinal fluid (CSF) and/or blood will emerge from the hole. In this case, carefully dry the skull and wait until no more CSF is emerging; the operation can be continued, but the risk of infection becomes higher.

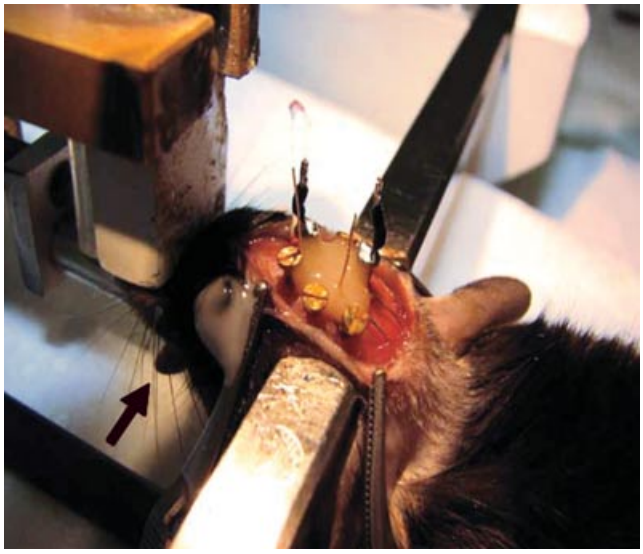

Figure 3 EEG electrodes are screwed into the skull, EMG electrodes are placed into the neck muscles and the entire assembly is fixed to the screws and skull with dental cement. All electrode leads are oriented in such a fashion that they can be easily soldered to the connector. Note that screws are not yet completely covered with cement. Also note tongue sticking out (arrow).

9. Clean the skull with betadine and then with ethanol by using some sterile cotton swabs, but take care to avoid the penetration of alcohol into the holes. Make sure that the skull is completely dry before proceeding to the implantation of the screws.

Implantation of screws

10. Place gold-plated screws (diameter 1.1 mm) into the holes using a screwdriver, so that the end of each screw is in contact with the surface of the cortex (with our screws this corresponds usually to two to three turns of screwdriver). Next, cement the screw with Adhesive Resin Cement without covering the top of the screws (Fig. 3). Let the cement flow to cover most of the exposed skull area, but it should be viscous enough to keep the EMG electrodes (next step) temporarily in place. Proceed rapidly to the next step before the cement dries.

    It is extremely important that the entire skull surface be completely clean and dry, and that there be no residual bleeding, before applying the cement.

Implantation of EMG electrodes and attachment of connector

11. Carefully insert the two gold-wire EMG electrodes into the neck muscle. Bend both electrodes into shape before insertion, so that one end can be easily inserted down into the neck muscle near the base of the skull while the other end makes a 90° angle on the top of the skull in such a way that the wires can be easily soldered to the connector (see next step).

12. Place the electrodes between the screws onto the cement and take great care that the EMG electrode wires do not touch each other, the EEG electrodes, or the skull. Add some more Adhesive Resin Cement to thoroughly cover all electrodes (Fig. 3).

    The Adhesive Resin Cement can rapidly cure when exposed to UV. The light source mentioned in the material list above is sufficient. Position the end of fiber-optic arm close to the cement, turn the light intensity to maximal, and expose briefly (up to 30 sec) to the light. Protect your eyes and those of the mouse from the light.

13. When the cement is dry, take off the clamps and make sure the entire area is clean and that no bleeding occurs before soldering the connector plug to the EEG and EMG electrode ends, using soft solder wire and a soldering iron. Take care that the solder does not short circuit any of the four electrodes.

    If all contacts are solder-coated prior to surgery, the time needed to connect can be kept to a minimum thus avoiding heating the electrode tips.

Suturing of surgical site and post-surgical considerations

14. Take care that the entire area is clean before stitching the skin in front and in the back of the cement such that the skin fits snugly but not too tightly around the cemented electrode assembly. Use a sterile suture thread.

15. Cover all contacts with dental cement and make sure nothing flows down onto or under the sutured skin.

    Cut a small piece of adhesive tape and stick it on top of the connector to keep the contacts clean and free of dust.

16. Weigh the animal. Put it back in its cage and monitor constantly until anesthesia wears off and the animal starts moving.

    After the operation, the animal should be given analgesics to reduce post-surgical pain. For example, Fynadine can be administered subcutaneously at 50 mg/kg. Administration of analgesics can be repeated later during recovery, although it should be kept in mind that the analgesic drugs can alter the EEG and thus should not be given close to the start time of the experiment.

17. After the operation, allow the mouse to recover at least 10 days prior to an experiment under standard housing conditions (see Basic Protocol 2), and check health status and recovery of the animal. Weigh the mouse before and after operation. At a time point 4 days after surgery, weigh the mouse again before connecting it to the recording leads (see Basic Protocol 2), which allows the mouse at least 6 days to habituate to the cable.

    If the animal loses more than 25% of its body weight measured immediately after the surgery over this 4 days period, it has to be euthanized, because high body-weight loss constitutes a sign of sickness.

    IMPORTANT NOTE: *When the mouse has been implanted, it has to be single-housed to avoid damaging of the connector and recording cable by other animals.*

RECORDING EEG/EMG DURING EXPERIMENT

BASIC PROTOCOL 2

This section describes the experimental procedure to record EEG/EMG signals in freely moving mice under standard conditions and during a sleep deprivation (SD) experiment. SD is the protocol of choice to increase sleep need and analyze the consequences of sleep loss on subsequent recovery mechanisms. One can selectively deprive mice of one state of sleep (e.g., REMS) or one aspect of a sleep state (e.g., high amplitude slow waves during NREMS), or restrict the daily amount of sleep obtained. Here we describe a method of total SD that has been referred to in the literature as 'gentle handling' (see below) in contrast with other methods of sleep deprivation in rodents such as forced locomotion or automated EEG-driven methods that, e.g., move the cage floor upon the first EEG signs of the sleep state of which the mouse is to be deprived. An episode of several hours of SD is usually followed by an increase in sleep intensity as assessed by sleep and EEG parameters (see below), and by an increase in sleep duration, both seen as evidence of recuperation of the sleep time lost. In the mouse, depending on the strain, this recuperation can occur immediately after SD, can be delayed by several hours, or can be lacking.

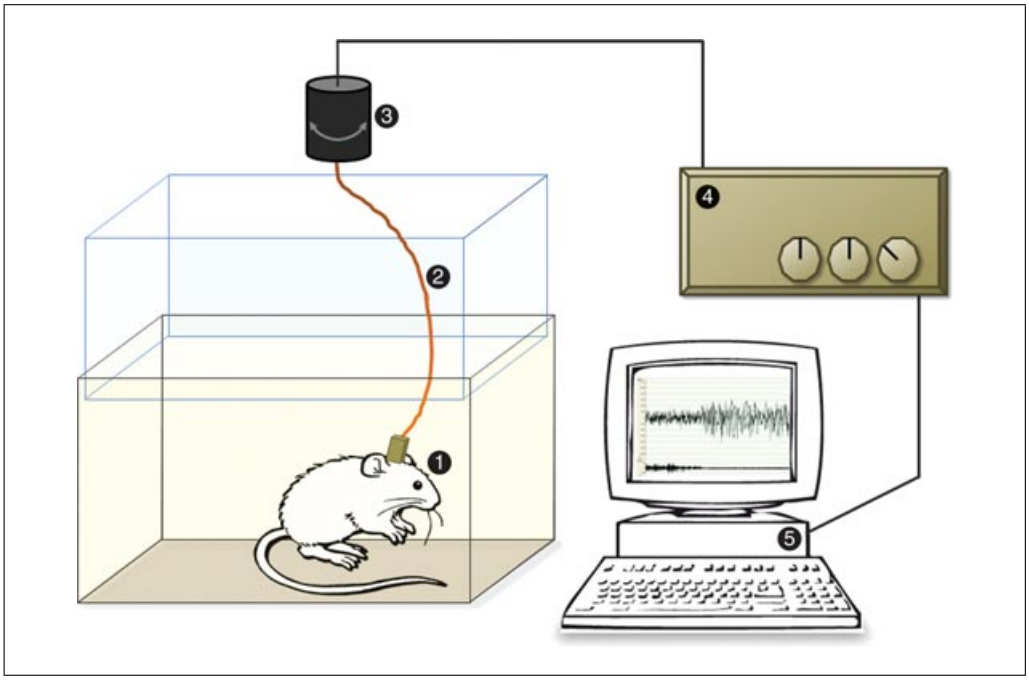

Figure 4 Schematic description of the experimental recording setup. The mouse has been implanted with EEG/EMG electrodes and connected to a head-mounted connector (1). The connector is attached to a recording cable (2) that is linked to a commutator (3) which transfers the signal to the acquisition unit (4). The processed signal is then transferred to a computer installed with a specific EEG/EMG acquisition and analysis software (5).

Materials

Mouse with electrodes implanted as described in Basic Protocol 1
Housing cages (e.g., Charles River Type F1, 30.5 × 18 × 18.4 cm) with food and water ad libitum
Electric flat-ribbon cables (e.g., 510640, Distrelec, *https://www.distrelec.com/*)
Connector 1 and 2 (OHS RoHS ENA AG; *http://www.ena.ch/)*
Commutators with counter balance mechanism (3 M, *http://solutions.3msuisse.ch*)

Connector construction

Prepare a recording cable with a connector plug to connect the mouse to the recording device (Fig. 4). The cable couples the connector mounted on the head of the mouse to a commutator or swivel contact allowing the tethered animal to turn. The cable is a standard flat-ribbon cable consisting of four insulated leads. The cable has a certain stiffness so that it can turn the commutator easily without first twisting itself. The weight of the cable should be counterbalanced by either using a counterbalancing arm or, in the case of the commutators we use, a direct current (DC) motor slightly lifting the recording cable via an attached nylon wire with constant adjustable force.

1. Take a flat-ribbon cable and split of a section with four leads.
2. Solder each of the four leads of the cable to the correct contact of connector 1.

   This part will be connected to the head-mounted mouse connector.
3. Do the same on the other side of the cable with the connector 2.

   This connector will be plugged into the commutator.

Animal setup and baseline recording

4. Place the animal into a separate housing cage with free access to food and water. Connect it to the recording cable (Fig. 5).

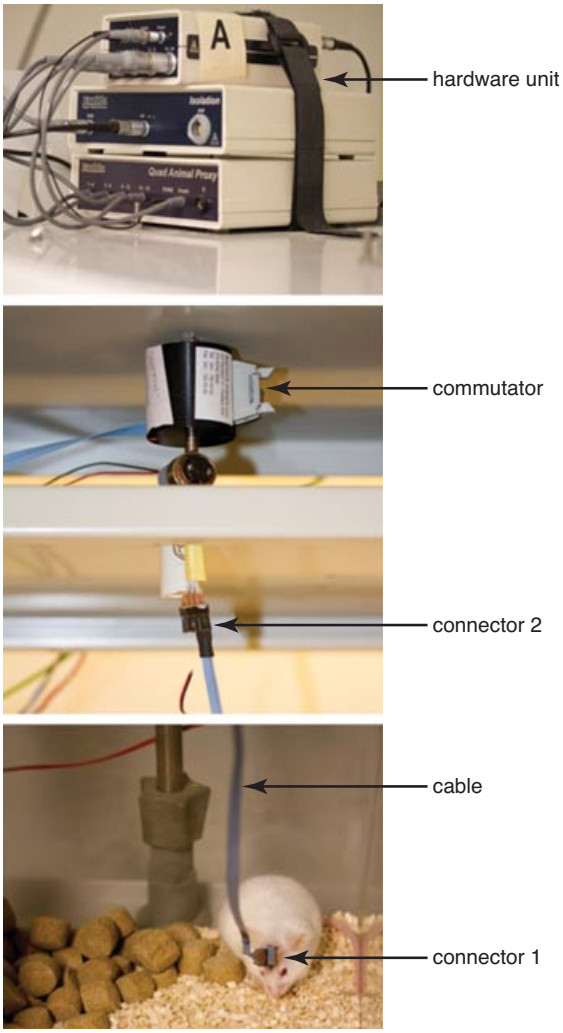

Figure 5 The mouse is placed into a cage, and connected via connector 1 to a recording cable that allows free movement of the animal within the cage but also allows the commutator to turn easily without first twisting the cable. The cable is linked to the connector 2 which joins the commutator. The latter brings up the signal to the hardware (see Basic Protocol 3).

Be very careful when connecting the animal to the cable; be sure to hold the connector on the animal's head when inserting the cable plugged into the head connector and avoid applying any pressure on the mouse's head.

Animals are singly housed in cages that have a bigger size than usual (e.g., Charles River Type F1, 30.5 × 18 × 18.4 cm). The cage should not have a wire-mesh top, because of the presence of the recording cable. To avoid the escape of the mouse from the open cage, we use a custom-made Plexiglas top inserted into the cage to raise the height to about 40 cm from the cage floor.

IMPORTANT NOTE*: Because of the presence of a recording cable, the cage should not contain objects that the mouse or cable can become stuck in or on (e.g., tunnels, mouse house, running-wheels. . .). Moreover, the length of the cable should permit the animal to move freely in the cage but with minimal risk of twisting of the cable.*

5. Allow the mouse to habituate to the cable and the experimental room for at least 6 days.

6. After sufficient recovery and habituation (>10 days), record the mice for a continuous 48 hr period, i.e., 2 consecutive days, without any disturbance.

   These 2 days serve as a baseline to access the effects of experimental interventions such as SD (see below). Recording 2 consecutive baseline days also allows determining day–to-day stability of the sleep and EEG variables being assessed.

   To avoid contamination of the signal from environmental electromagnetic field, the recording boxes can be put into a Faraday cage.

Experimental intervention: sleep deprivation

7. For the SD experiment immediately following a baseline recording, sleep-deprive the animals and subsequently follow by one or more days of recovery.

   We typically sleep deprive mice by "gentle handling," usually for a period of 6 hr, and recovery is measured for 24 hr. Gentle handling means that the animal is kept in its home cage attached to the recording cable and left undisturbed as long as it does not display behavioral or EEG signs of sleep.

   If needed, sleep is prevented by introducing paper tissue into the cage, changing the litter, bringing a pipet in proximity to the animal, or gently tapping the cage. The number of interventions needed to keep animals awake gradually increases over the course of SD, and keeping several animals awake simultaneously becomes challenging toward the end of a 6-hr SD. Some interventions, such as changing part of the litter, should not be scheduled toward the end of the procedure, since novelty and stress might interfere with recovery sleep. SD can be initiated at any time of the day or night, and duration of SD can be varied to construct 'dose'-response curves. However, usually, SD is performed at light onset; i.e., the onset of the main rest phase in the mouse, under the assumption that the effect of an SD will be largest then.

   The duration of SD can vary; however, if it is not long enough, little effect on the sleep will be observed, and if it is too long, sleep pressure becomes so great that the occurrence of short periods of sleep becomes unavoidable and the SD is no longer complete. Under these conditions, even short periods of sleep can impact the recovery variables assessed. Finally, extremely long SDs (multiple days) should be avoided, as these are deleterious and lethal and will not give much insight into the physiological regulation of sleep.

BASIC PROTOCOL 3

DATA ACQUISITION

The recorded EEG and EMG signals have to be filtered and amplified before analog-to-digital (AD) conversion. Various commercial amplifier systems are available, a few of which are tailored specifically for use in rodents. Most systems contain a "notch filter" to suppress the main environmental electrical noise (occurring at 50 or 60 Hz depending on country). Theoretically, the sampling rate of the AD conversion should be at least twice that of the frequency of the EEG activity of interest, although oversampling is recommended. Inappropriate filtering and sampling rates can lead to artifacts in subsequent signal analysis. This protocol does not give an introduction to the drawbacks and pitfalls of signal analysis, but instead lists the settings adopted in our laboratory to acquire and process the data from the EEG recording. As a signal-analysis algorithm, the example of the Fast-Fourier Transformation (FFT) is given. For this type of analysis, a window function has to be chosen; in our case we use a 4-sec window, which matches the resolution at which we determine behavioral state.

Materials

Computer

Acquisition hardware which includes an isolation unit, amplifier, AD converter, and filter components (e.g., EMBLA A10, Medcare Flaga; *http://www.embla.com*)

Acquisition software (e.g., Somnologica 3, Medcare Flaga; *http://www.embla.com*); this acquisition and analysis software automatically performs most of the steps listed in the following protocol

1. Perform analog/digital (AD) conversion of the analog EEG and EMG signals at a digitization rate of at 2000 Hz (i.e., a sampling interval of 0.0005 sec).
2. Down-sample the signals at 200 Hz.
3. Set a high-pass filter at 0.0625 Hz to reject the DC signal.
4. Set a notch filter to discard the activity in the 50 Hz band (or 60 Hz in some countries, e.g., U.S.A.) that is often caused by interference from surrounding electrical equipment, which can introduce line artifacts in the EEG.
5. Transform the EEG signal with an FFT to yield power spectra between 0.25 and 90 Hz with a 0.25 Hz frequency and a 4-sec time resolution (see Basic Protocol 5).
6. For EMG, rectify and integrate the signal over 4-sec windows.
7. Store digital signals on a hard disk and make sure to back up the recordings.

DETERMINING BEHAVIORAL STATE BASED ON THE EEG AND EMG SIGNALS (i.e., "SCORING")

BASIC PROTOCOL 4

The EEG provides a measure of brain electrical activity which differs according to behavioral state. In rodents, like in humans, one can distinguish three main behavioral states that can be differentiated by their distinct oscillatory EEG activities and their differences in muscle tone. Wakefulness is a state of alertness that includes a broad variety of behaviors (eating, grooming, exploring, resting...), and is characterized by EEG activity of mixed frequency and low amplitude. Muscle tone is present, and can be highly variable. Rapid Eye Movement sleep (REMS), also called paradoxical sleep, is a state in which rapid movement of the eyes can be observed. It is characterized by regular theta waves (6 to 9 Hz) in the EEG, and muscle atonia with occasional twitches. Exploratory behavior in the mouse is also accompanied by high-amplitude synchronized theta activity. Therefore, the EMG is helpful to unequivocally distinguish between REMS and other waking activities characterized by coherent theta activity (Fig. 6A). Non-rapid eye movement sleep (NREMS) is characterized by highly amplitude slow waves in the delta frequency range (1 to 4 Hz) and a low and stable muscle tone (Fig. 6B).

States are usually determined by visual inspection of the EEG and EMG traces (Figs. 6A-C) for discrete time intervals (or windows), although automatic or semi-automatic scoring algorithms are employed as well. Another more precise approach when determining behavioral state would be to determine the transitions between the states, instead of using a fixed window. However, we score the signals by windows so that the behavioral scores can be aligned with the results of the Fast Fourier Transformation (FFT), an analysis for which a fixed window size is a requirement (see Basic Protocol 5). We have chosen a 4-sec window to accommodate both sufficient frequency resolution (0.25 Hz) and time resolution, as states in the mouse change more rapidly as compared to humans (with shorter window size, frequency resolution of the FFT decreases, while with longer windows, the fine sleep-wake architecture can no longer be reliably determined).

Here we will describe the procedure of "scoring" behavioral state visually using Somnologica. The specific commands will be different according to the analysis software used. Information on state provides information on sleep onset latency, time spent in the various states, their distribution over the day, and the fine architecture of sleep indexing the quality of sleep. Please note that the state-specific EEG amplitude and patterns differ greatly among inbred strains, rendering scoring of behavioral state sometimes difficult (Franken et al., 1998; Hasan et al., 2010). Moreover, in lines carrying targeted or spontaneous mutations, various types of EEG abnormalities can be observed (e.g., epileptiform

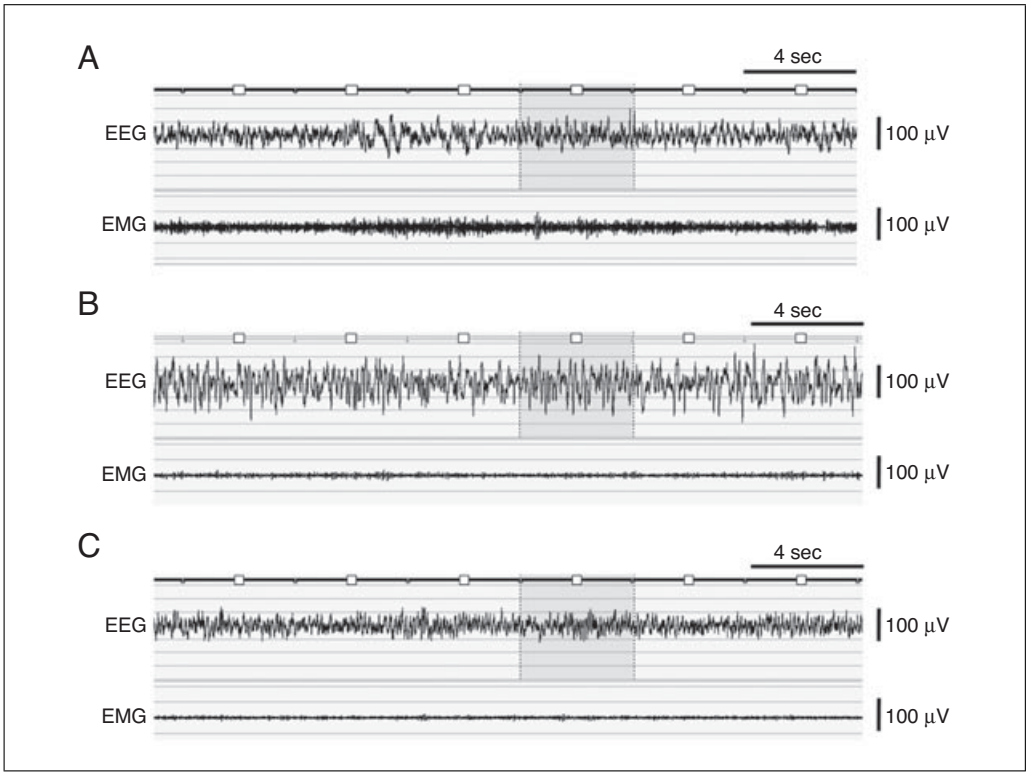

Figure 6 Example of EEG/EMG signals for wakefulness, NREMS, and REMS. (**A**) During wakefulness, the EEG trace is composed of mixed frequencies, and muscle tone is present on the EMG. (**B**) NREMS is characterized by high amplitude and low frequency EEG oscillations, ranging from 1 to 4 Hz; muscle tone is low. (**C**) During REMS, EEG shows regular theta waves (6 to 9 Hz) and EMG is flat except for rhythmic heart-rate artifact.

or other paroxysmal EEG activities), requiring specific state annotations that will not be discussed here.

Materials

Computer for EEG analysis
EEG analysis software (e.g., Somnologica 3, Medcare Flaga; *http://www.medcare.com*)

Scoring of behavioral vigilance state and masking EEG artifacts

Before starting to score the signals, the EEG and EMG traces can be filtered by the computer software for display purposes.

1. For the EEG, set a low-pass filter at 0.7 Hz, and for the EMG, set a low-pass filter at 10 Hz.

   This will not change the signals themselves, but only how they are presented on the screen.

2. Go to "event," "new scoring" in the Somnologica software and enter the precise date and time of the beginning of your recording.

   You have to take care to set the exact start time that was used for the FFT analysis, so that the behavioral scores exactly match the 4-sec windows of the spectral analysis.

3. Visually score each 4-sec window as wake (w), NREMS (n), or REMS (r). Mark EEG artifacts occurring in each state as follows: assign "1" for artifacts that occur in the wake state, "2" in NREMS, and "3" in REMS.

4. Save all the scoring data in a database that will later be linked to the EEG analysis.

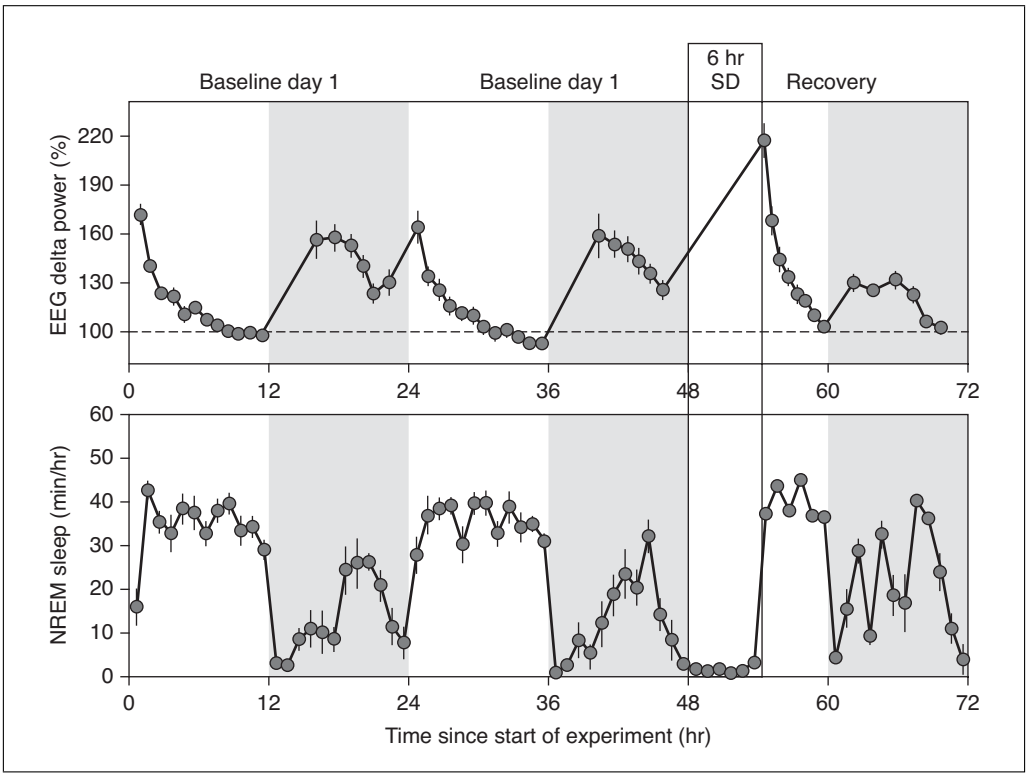

Figure 7 EEG delta power during NREMS (upper panel) and distribution of NREMS during 48-hr baseline, 6-hr sleep deprivation (SD), and 18-hr recovery (lower panel). Delta power increases during the dark period (gray parts) and decreases during the light period (white parts). After 6 hr of SD, delta power reaches its highest values, and decreases rapidly during the rest period. Changes in delta power are said to be driven by the distribution of NREMS; during the light period, when NREMS is prevalent (~40 min/hr), EEG delta power gradually decreases, while during the dark period, when less time is spent in NREMS especially immediately following dark onset, delta power is increased in subsequent NREMS. After a 6-hr SD, NREMS distribution is changed compared to the baseline periods.

Time spent in sleep and wake states

5. Sum all the windows that have been scored as wake, NREMS, or REMS in three separate categories. If artifacts have been marked according to behavioral state (e.g., 1, 2, or 3; see above), they should be included in the sum of each state.

6. Calculate the total time spent in each behavioral state over the light period, the dark period, and/or the whole experiment.

   By calculating the time spent in each behavioral state, you can obtain a first profile of the sleep and wake pattern of your animal. Indeed, most mice have a sustained period of wakefulness following dark onset and are predominantly asleep during most of the light period, whereas some others consistently show a 'nap' in the middle of the active period. One can calculate time-spent-asleep and -awake over various time intervals, such as over 24 hr (for the daily amount), over 12 hr (for the amounts in the light and dark period), and at hourly values (to construct a profile of the distribution of sleep over the day; Fig 7). Moreover, to examine the ability of the mice to maintain behavioral state, you can analyze the distribution of behavioral states as a function of episode length.

Effects of sleep deprivation on time spent in each state

7. Quantify the effect of SD by contrasting the amount of sleep obtained during recovery to that obtained during the same recording time intervals during baseline for each individual (Fig. 7).

   SD is thought to lead to an increase of sleep need as evidenced by the increased time spent asleep during subsequent recovery. The effects of SD are modulated by genotype, sex, and time of day.

Sleep latency

8. Calculate the latency to fall asleep as the time from, e.g., the beginning of the light period in baseline, drug administration, or the end of SD.

9. Express the latency to REMS either with respect to the same time references as sleep onset or relative to the first occurrence of NREMS.

   Usually, the first period of NREMS after an extended period of wakefulness is characterized by high delta waves and lasts for several minutes without any interruption by awaking, and it mostly ends with a REMS period.

BASIC PROTOCOL 5

SPECTRAL ANALYSIS

Spectral analysis is a tool to quantify the contribution of various frequencies that constitute the EEG signal. The goal of spectral analysis is thus to explore and quantify the rhythmic activity patterns present in the EEG, by decomposing a complex time series with cyclical components into the underlying sinusoidal functions of particular wavelength. A commonly used and efficient method is the Fast Fourier Transformation (FFT). The contribution of the various frequency components of the EEG signal can be determined for each behavioral state. These analyses can be used to construct an average spectral signature of each behavioral state during baseline, which can serve as a starting point for genetic studies underlying the relative importance of the various EEG components or to quantify the effect of a drug on global brain activity. One can also quantify the dynamics over time of EEG activity within distinct frequency bands. A good example of this are the sleep-wake dependent changes in EEG power in the delta frequency range (1 to 4 Hz), referred to slow-wave activity (SWA) or, more accurately, EEG delta power. Besides yielding power in specific frequency bands, the FFT can also give information about the prevalent frequency of EEG oscillations of large amplitude. In this section, we will describe how to do spectral analysis on EEG data, and how to analyze the delta power of the signal. Again, for a detailed discussion on the assumptions, limitations, and pitfalls of spectral analyses in general and the FFT specifically, we refer to the literature (Semmlow, 2004).

Materials

Software to manage the data (e.g., algorithms written in R or Matlab or other programming languages)
A statistical analysis tool (e.g., SAS Institute software, version 9.1)
Software for graphics (e.g., SigmaPlot 10.0, Systat Software)

NOTE: Most of the EEG recording and analysis software applications such as Somnologica offer the possibility to generate EEG spectra for discrete windows using FFT. Be sure that the chosen 4-sec windows for scoring state match the windowing of the FFT spectra.

Spectral profiles

The power spectrum graph displays the distribution of the power over the frequency range of the EEG signal for each state (Figs. 8 and 9). The frequency range depends on the sampling rate (with a sampling rate of 200 Hz the full frequency range will be between 0 and 100 Hz) and the frequency resolution depends on the window size (with a 4-sec window the frequency resolution will be 1/window size or 0.25 Hz). With this analysis, you can compare power spectra of a state for different genotypes or different conditions.

1. Calculate the mean EEG spectra for each state by adding for all the artifact-free 4-sec windows scored as that state, the power density in each frequency bin, and then dividing by the total number of windows obtained for each state and animal.

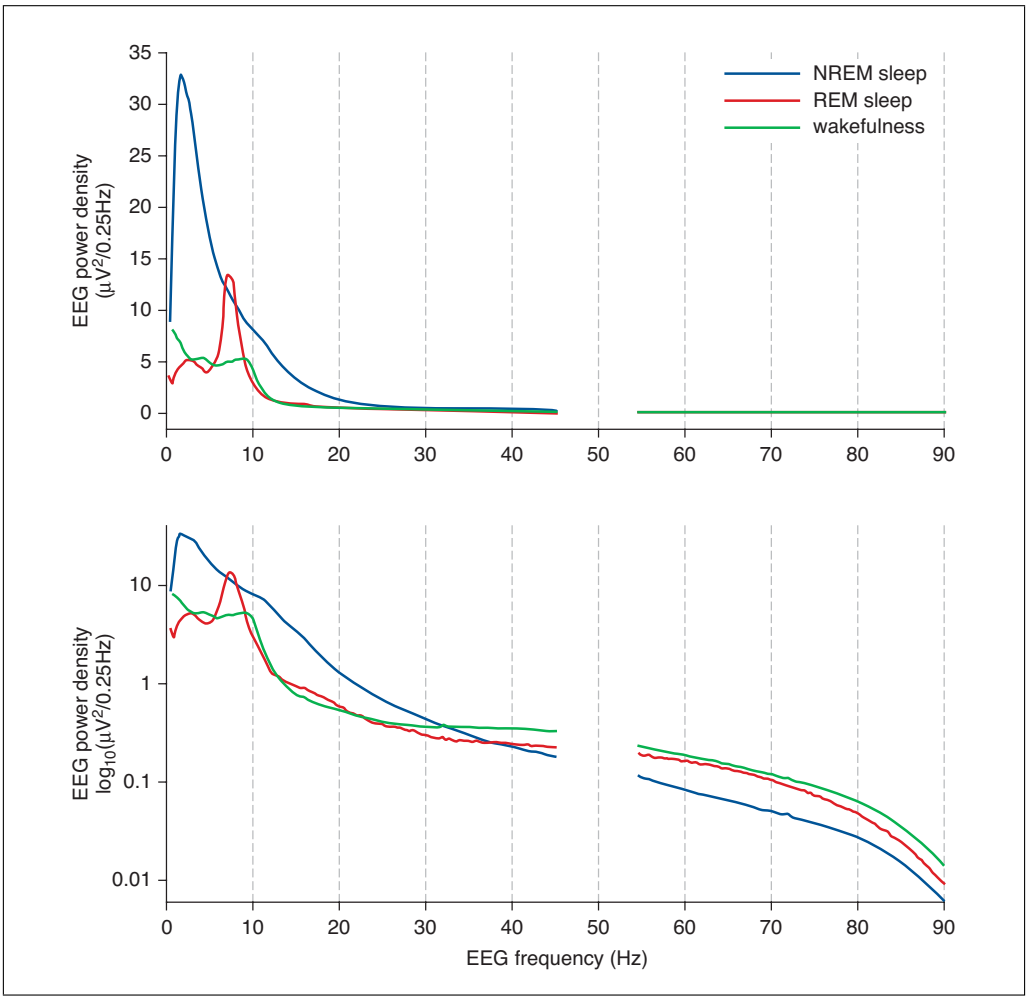

Figure 8 Average EEG power spectrum density for wakefulness (green line), NREMS (blue line), and REMS (red line) calculated over a 24-hr baseline recording period (n=7 C57Bl/6J mice). The upper graph represents the power density of the signal (in μV^2/0.25 Hz) for each frequency band. To illustrate EEG activity at higher frequencies in the lower graphs, the same spectra are plotted on a $\log_{10}$ scale. The interruption of the power density signal at around 50 Hz is due to the notch filter that has been used to remove line artifacts. Wakefulness is characterized by a low power in the low frequencies, whereas the EEG during REMS shows a prominent peak of power density at around 7 Hz, i.e., in the theta frequency range, and NREMS has high power in the delta band (1 to 4 Hz), consistent with the presence of slow waves in the EEG. Please note that wakefulness is not further differentiated into specific behaviors, each with a specific spectral profile; the spectral profile during, e.g., exploratory behavior will resemble that of REMS with its prominent theta peak.

2. Construct a behavioral state-specific spectral EEG profile for baseline.

Time course of EEG delta power

Delta power is the total power of the EEG signal that occurs within the delta band (1 to 4 Hz). Since coherent delta oscillations are usually observed only during NREMS, and since NREMS is not uniformly distributed over the day, a reliable time course can be constructed by defining percentiles of time to which an equal number of NREMS windows contribute, instead of using fixed time intervals during which very little or no NREMS can occur (Franken et al., 2001).

3. Divide the recording session into sections (percentiles) to which an equal number of 4-sec windows scored as NREMS contributed.

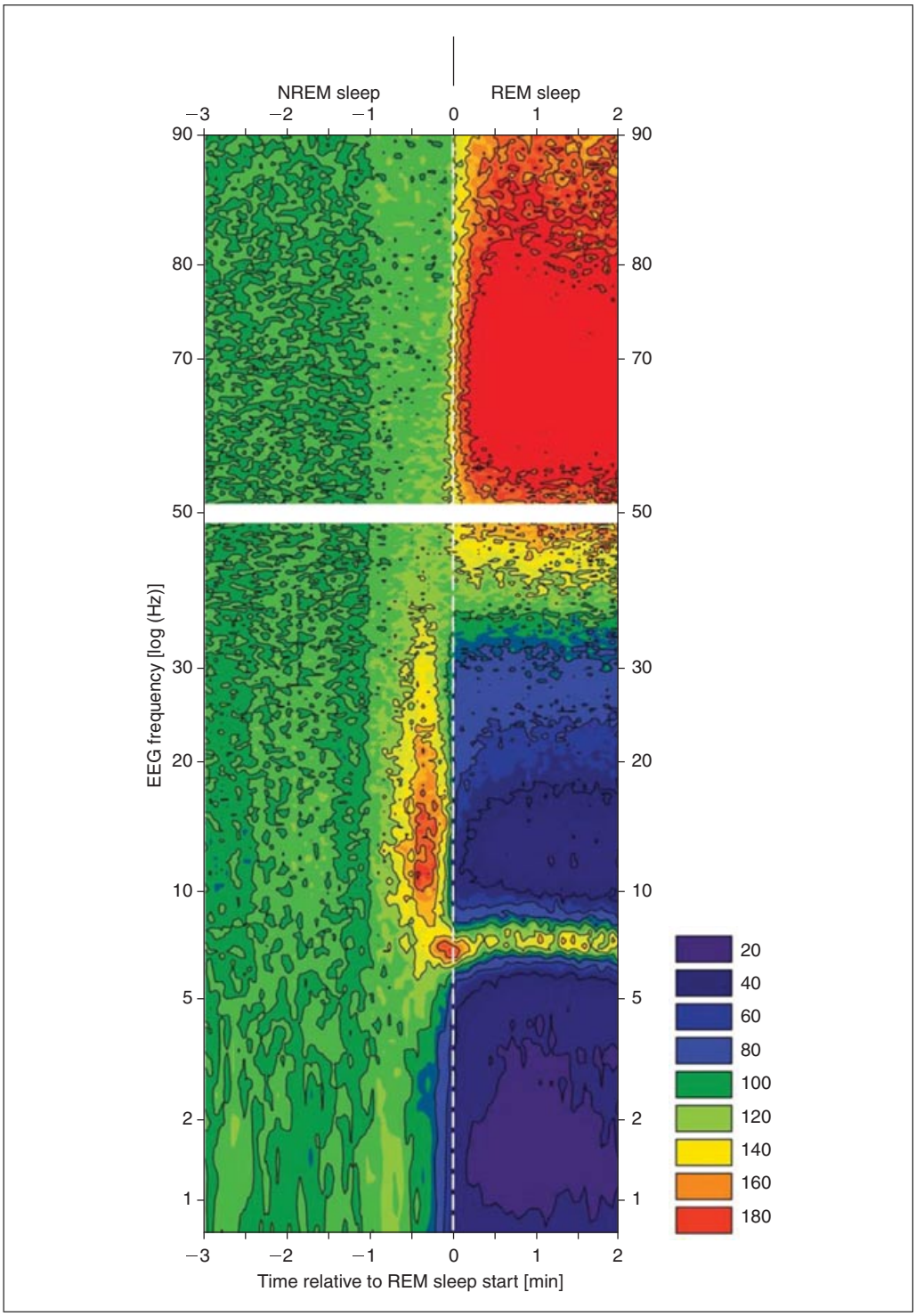

Figure 9 Plot of EEG spectral composition changes at the transitions from NREMS (−3 to 0 min) to REMS (0 to +2 min). Power density within 0.25 Hz bins is expressed as a percentage of the mean power density for that bin over 4-sec windows scored as NREMS in the first 2 min (i.e., −3 to −1 min) of the transitions to visualize relative spectral changes. Contour lines connect levels of similar relative power density in 8 color–coded 20% increments. Significant EEG changes start during the first minute prior to REMS onset and entail a marked increase in EEG activity in the spindle frequency range (10 to 15 Hz), shortly followed by an increase in theta activity (5 to 10 Hz). Maximum spindle activity is reached around −25 sec, while maximum theta activity is reached at the transition. After the transition, spectral values reach their typical REMS levels with, below 35 Hz, no other activity than theta, and above 35 Hz, high EEG activity including gamma (35 to 60 Hz).

a. For the baseline light periods, divide the first 6 hr of baseline into six such sections and the second 6 hr into four sections.
b. For the recovery light period, divide the second 6 hr into six sections.
c. For the dark period of both baseline and recovery, divide the first 6 hr into eight sections.

The choice of the number of sections per recording period depends on NREMS prevalence in each period.

4. Average EEG power density in the 1- to 4-Hz range for each 4-sec windows scored as NREMS.
5. Average EEG power density in the 1- to 4-Hz range for 4-sec windows scored as NREMS during baseline, and after SD for each section and animal.
6. Normalize the values by expressing them as a percentage of an individual mean delta power value reached in baseline such as, e.g., over the last 4 hr of the main rest period when delta power reaches stable minimal values not affected by eventual individual or genotype difference in the prior sleep-wake distribution (Franken et al., 2001).
7. Calculate group means (Fig. 7).

EEG dynamics at NREMS-to-REMS transitions

Changes in brain activity leading up to a state transition can be explored by spectral analyses of the EEG signal. As an example, we analyze here the time course of the EEG changes immediately before and after a transition from NREMS to REMS (Fig. 9), as previously published (Franken et al., 1998, 2006). This analysis constructs an average time course by aligning all transitions to sustained episodes of REMS (>3 4-sec windows) in a baseline recording. It reveals that in the 30 sec before REMS onset, EEG activity peaks in the sigma frequency range (11 to 15 Hz), followed by a peak in theta activity (6 to 9 Hz) at the transition. Moreover, during REMS, the EEG is dominated by theta and gamma (35 to 60 Hz) activity. This type of analysis might help shed light on the neurophysiological processes underlying the triggering of REMS during NREMS.

8. Based on the behavioral state scores, locate all REMS episodes of sufficient duration (e.g., a minimum of three 4-sec windows).
9. For each REMS episode found, determine for each frequency bin (0.25 to 90 Hz) the mean power density in all 4-sec windows scored as NREMS occurring in the 3 min prior to REMS onset. This average value serves as a normalization for each frequency bin (=100%). Include only 4-sec windows without EEG artifacts.
10. Express EEG power density within each frequency bin, and of each 4-sec window scored as NREMS in the 3 min before REMS onset or scored as REMS in the 2 min after REMS onset, as percentage of the mean power density for that bin over the 4-sec windows scored as NREMS in the first 2-min (i.e., –3 to –1 min) for each transition. Keep track of the number of 4-sec windows scored as NREMS and REMS for each transition, because not all 4-sec windows in the 3 min before the transitions will be scored as NREMS, and not all 4-sec windows in the 2 min after the transition will be REMS.
11. Repeat this for each REMS episode identified and construct an average 5-min time course of relative EEG power density for each frequency bin, first within individuals and then among individuals.

The resulting power density matrix (360 frequency bins × 75 4-sec windows) can be plotted as a heat map to visualize the relative spectral changes in the EEG triggered with respect to REMS onset (Fig. 9).

COMMENTARY

Background Information

It has become evident that sleep is essential for well being and health; a lack of sleep can contribute to weight gain, anxiety, depression, lethargy, and fatigue (Leproult and Van Cauter, 2010). Moreover, in western countries, there are a growing number of subjects suffering from chronic and pathologic sleep disorders, such as insomnia, sleep apnea, hypersomnia, parasomnia, or narcolepsy, and these diseases can be associated with immune deficiency or cancer. Mounting evidence indicates that sleep is beneficial for the correct function of immunity and metabolic processes, but it has also been shown that sleep is implicated in cognitive functions, such as learning, memory, and attention. To understand the precise role of sleep in these various functions and behaviors, sleep research mainly uses animal models, especially mouse model, which can be used to study the molecular and physiological pathways underlying sleep disorders and test potentially therapeutic treatments.

Alternative methodologies to record sleep

Other techniques than the one we have adopted in our laboratory can be used to study sleep. For example, biotelemetry can be used to measure a variety of behavioral and physiological parameters, including sleep (Tang and Sanford, 2002; Weiergräber et al., 2005). The advantage of telemetry is that the animal is not tethered to a recording cable, and can be kept together with other mice and in more enriched environments (e.g., nest chamber, running wheel, tunnels. . .). Telemetry is also more appropriate for behavioral studies that require transferring animals from their home cage to a test apparatus. For telemetry, a small transmitter is implanted either intraperitoneally or subcutaneously in addition to implanting the EEG/EMG electrodes. Usually, apart from EEG and EMG, several additional physiological parameters can be recorded in parallel (activity, body temperature). However, telemetry also has disadvantages compared to using a recording cable. First, the surgery is more invasive, and the surgical trauma is greater and minimal recovery time longer compared to simply implanting EEG/EMG electrodes. Second, although telemetry has been used successfully in the rat to record EEG and EMG for sleep studies, even the latest versions of radio transmitters are still rather big for mice; i.e., the smallest commercially available transmitter allowing the recording of both the EEG and EMG (DSI PhysioTel F20-EET; *http://www.datasci.com/*) weighs 3.9 g at a volume of 1.9 cm^3 (i.e., 13% to 20% of body weight). As technology further improves, telemetry will become an attractive alternative technique in the near future. Other developments making it possible to avoid using recording cables concern the filtering, AD conversion, and storage of EEG/EMG signals on the head of the mouse (e.g., Neurologger by NewBehavior TSE systems) for off-line analysis after the experiments.

Other, noninvasive methods to record sleep make use of video-recording and image-analysis software (Pack et al., 2007) or piezoelectric systems (Flores et al., 2007; Donohue et al., 2008; Signal Solutions LLC, *http://www.signalsolutionsllc.com/*). The latter systems use piezoelectric films placed on the bottom of the cage to detect the animal's movements with high sensitivity (Fig. 10). The resulting signal can be used to automatically distinguish sleep from wakefulness. During wakefulness, locomotor activity and even small movements result in a high-frequency, erratic, and irregular signal, while during NREMS, the principal movements are the rhythmic respiration-related chest-wall movements, producing a regular ∼2 Hz rhythmic signal. Although EEG activity and REMS cannot be measured yet, this system can be used for large-scale genetic sleep studies and rapid screening of sleep/wake-promoting drugs. The piezoelectric system outperforms systems depending solely on locomotor activity (beam breaks or passive infra-red sensors), because an animal that does not move is not necessarily asleep.

Critical Parameters

All animal experimentation requires approval of an Institutional Animal Care and Use Committee. The procedures and experiments described here should therefore be approved first, before being implemented. Special care has to be taken to ensure that pain and discomfort to the animal are kept to a minimum. This is of particular importance for the surgery. Post-operative care is essential to ensure the proper recovery of the animal. Moreover, it has to be kept in mind that EEG/EMG implantation requires some practice on the part of the experimenter, and that some mice should be dedicated for training before the beginning of the experiment.

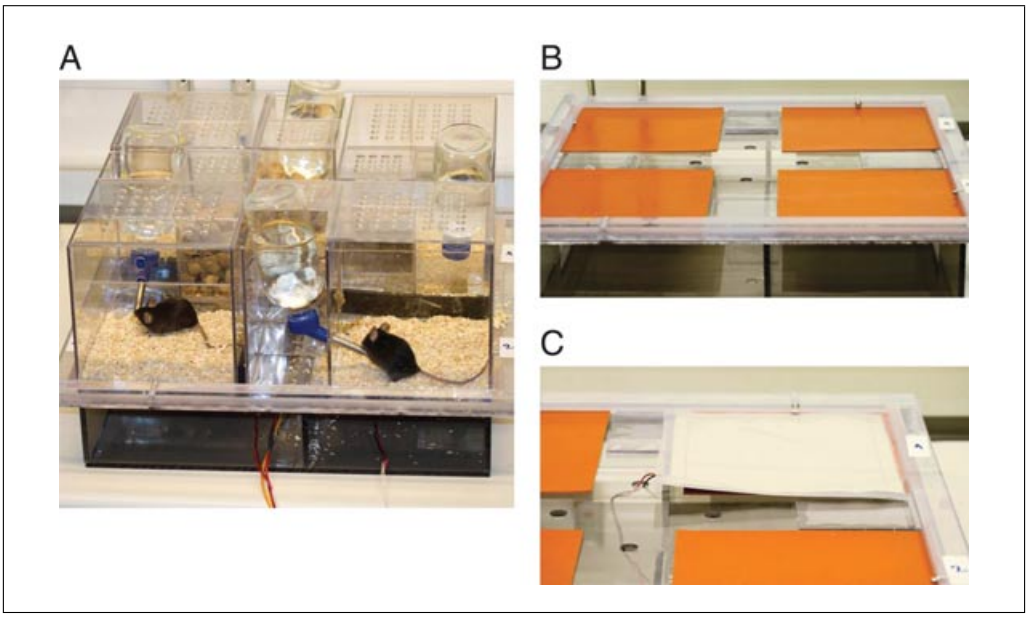

Figure 10 The piezoelectric system is a noninvasive alternative to EEG/EMG recordings. The mice are placed in separate housing cages containing litter and food and water ad libitum (**A**). In this setup, four mice can be recorded at the same time (**B**). The piezoelectric film (**C**) is placed on the bottom of each animal cage and used as an ultra-sensitive motion detector to identify NREMS based on rhythmic activity related to the chest-wall movements related to regular breathing patterns during this state.

Time Considerations

Total time for EEG/EMG implantation is about 1 hr, and with practice will decrease to ~45 min. When correctly implanted, high-quality EEG can be obtained for several months. To improve the quality and the time of EEG electrodes, implantation should not be done in mice younger than 9 to 10 weeks. Because scoring and artifact removal are done visually for consecutive 4-sec windows, the initial step of the analysis can take quite a long time (depending on the number of mice and duration of the recording). Also, genetic background plays a role, since discrimination among states can be more difficult in some strains and, also, the number of state transitions varies with genotype. 'Expert' scorers require on average 3 hr for a 24-hr recording of one mouse—i.e., annotating 21,600 4-sec windows. Many laboratories have developed 'in-house' algorithms to automatically score behavioral state based on the spectral composition of the EEG and EMG amplitude or variance. Scoring accuracy seldom exceeds 90%, however, and especially REMS and EEG artifacts often cannot reliably be detected without visual inspection. Such error might be acceptable for some types of more preliminary analyses (e.g., amount of sleep per day), but not for the fine architecture of sleep, the EEG spectral dynamics at state transitions, and determining if unanticipated pathological EEG phenomena occur. For these reasons, we do not rely on automatic scoring in our laboratory.

Acknowledgments

We would like to thank Yann Emmenegger and Francesco La Spada for their invaluable help with the illustrations. This work was supported by grants from the Swiss National Science Foundation (SNF 31003A-130825) and EUMODIC (Contract no.: 037188) supporting YE, the University of Lausanne, and the state of Vaud.

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