Cellular and Molecular Life Sciences

Technologies of sleep research

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Abstract. Sleep is investigated in many different ways, many different species and under many different circumstances. Modern sleep research is a multi-disciplinary venture. Therefore, this review cannot give a complete overview of all techniques used in sleep research and sleep medicine. What it will try to do is to give an overview of widely applied techniques and exciting new developments. Electroencephalography has been the backbone of sleep research and sleep medicine since its first application in the 1930s. The electroencephalogram is still used but now combined with many different techniques monitoring body and brain temperature, changes in brain and blood chemistry, or changes in brain functioning. Animal research has been very important for progress in sleep research and sleep medicine. It provides opportunities to investigate the sleeping brain in ways not possible in healthy volunteers. Progress in genomics has brought new insights in sleep regulation, the best example being the discovery of hypocretin/ orexin deficiency as the cause of narcolepsy. Gene manipulation holds great promise for the future since it is possible not only to investigate the functions of different genes under normal conditions, but also to mimic human pathology in much greater detail.

Keywords. Electroencephalogram, hypocretin, microdialysis, orexin, pathology, rest-activity recording, sleep regulation, temperature.

Introduction

This review attempts to give an overview of the most important methods and technologies used in sleep research. In addition, it tries to give an idea of recent developments in the use of animal models to investigate sleep regulation and sleep pathology. As will become clear from this review, sleep research has become a multi-technology and multi-disciplinary endeavor, and it is therefore impossible to list all the methods and animal models used.

The first part focuses on different techniques used in sleep research, starting with non-invasive methods and building up to invasive methods, which are mainly used in animal research. The second part focuses on animal models in sleep research, and where appropriate, will draw connections with human sleep pathology.

Rest-activity recordings

Rest-activity recordings are applied in many different species and have become an important supporting tool in sleep research because they are non-invasive and relatively easy to use. Actigraphy is the standard procedure to observe rhythms in human subjects who are entering an experiment, or to do a preliminary screen for sleep or circadian rhythm pathology in patients. In animal research rest-activity recordings have become an important tool as a preliminary screen for changes in sleep and circadian rhythms in genetically manipulated mice.

Actigraphs are miniaturized, wrist-worn activity monitors based on an omnidirectional acceleration sensor that records physical activity [1]. In most sleep laboratories subjects wear these 1 or 2 weeks prior to an experiment. Activity counts are accumulated over a predetermined epoch and stored in internal

memory. Inbetween recordings the data can be downloaded and analyzed.

Actigraphy is also used on children and infants [2]. In infants the actigraph is usually attached to the left ankle. With this method it is possible to obtain clear, long-term records. A disadvantage is that infants are also moved passively, and it is not always possible to exclude these artifacts reliably. Another application of the actigraph is the recording of activity in larger mammals in natural conditions, for instance reindeer under polar light conditions [3].

Rodents cannot carry an actigraph, and their rest activity is usually obtained by recording running wheel rotations. These recordings are influenced by several environmental cues. It is therefore important to perform wheel-running experiments under defined environmental conditions. The availability of a running wheel [4, 5], and even the size and type of running wheel [6, 7] have been found to influence the recorded activity pattern.

Another way to record rest-activity patterns in rodents is with passive infrared (PIR) detectors. The PIR detector is placed above the cage. It detects changes in heat radiation and can therefore only 'see' movements of euthermic animals. The threshold of detection can be changed to increase or decrease sensitivity. Also, here the closing of an electrical circuit is the signal to the recording computer.

The general pattern of PIR recorded activity is comparable with running wheel activity. However, a running wheel increases the activity of an animal, and therefore without a wheel more activity may be recorded during the rest phase and less during the active phase. Another difference may occur under constant conditions because availability of a running wheel influences the free running period of the animals [4, 5].

Ancillary to activity patterns, drinking rhythms can be monitored by recording drink nipple contacts. One method is to prepare the cage with a metal grid on the bottom. The bottom, the animal, and the nipple are part of an electrical circuit which closes upon drinking [8]. A more animal-friendly alternative is to place the drinking nipple between an infrared light and an infrared sensor. Drinking activity patterns will differ from activity records obtained with running wheels because most rodents need to drink during their rest phase, a time when they will hardly run in the wheel.

Electroencephalography and brain imaging

Modern sleep research began with the development of technology that allowed electrophysiological monitoring of sleep. The first person to record electro-physiological phenomena from the brain was Richard Caton (1842–1926). He presented his findings to the British Medical Association in 1875 [9], and a more detailed report appeared 2 years later [10]. Hans Berger (1873–1941) is generally credited as the 'discoverer' of the EEG. He developed the first instrument that recorded electrical activity generated by the human brain. His recordings were published in 1929 [11], and he already showed that the EEG differs between sleep and waking. Twenty-four years later Aserinsky and Kleitman [12] showed that sleep could be differentiated into two distinct states [13]. Nowadays the recording of the EEG, muscle activity (electromyogram) and eye movements (electro-oculogram) are combined in polysomnography (PSG) to differentiate sleep states. In modern sleep research many different variables are recorded with many different techniques, but the EEG remains the backbone of sleep research. PSG is regularly applied in adolescents and adults, but it is possible to obtain repeated recordings in the first year of life as well [14], showing that PSG can be recorded in virtually all age groups.

Using multiple EEG derivatives, interhemispheric asymmetry in the EEG in human [15], rat [16], and mouse [17] was detected after unilateral sensory stimulation. Even running wheel availability was shown to change the EEG regionally during sleep [18]. In humans the number of EEG channels can extend up to 256, enabling detailed localization of regional changes across the brain [19]. Given the distribution of scalp electric potential differences, it is in theory possible to localize current sources in the brain. One technique for source localization is low-resolution electromagnetic tomography (LORETA) [20,21], which was developed at the beginning of the 1990s. LORETA is based on multichannel EEG recordings. It directly computes a current distribution throughout the full brain. Based on evidence from single-cell recordings in the brian, the method assumes that neighbouring neurons are simultaneously activated. The computational task is to select the smoothest of all possible three-dimensional (3-D) current distributions. The result is a 3-D topography with the characteristic that localization is preserved with a certain amount of dispersion, i.e. it has relatively low spatial resolution. In the course of the years the method was refined, excluding white matter from the calculation of possible sources.

However, because of fundamental physical limits, the exact configuration of neuronal activity in the brain cannot be derived solely on the basis of the EEG. Two new techniques, positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), provide an opportunity to fill this gap. Both ap-

proaches rely on metabolic alterations at the neuronal level expressed as variations in glucose and oxygen consumption and hemodynamic changes, and both display brain structures and aspects of brain function with millimeter resolution. PET is silent and relatively non-invasive. Thus, PET scans can be acquired during natural sleep in healthy humans. After pioneering work of, among others, Maquet et al. [22], Braun et al. [23] and Nofzinger et al. [24] PET has been applied successfully in many sleep studies in humans, but is not a real-time measurement. fMRI provides real-time imaging so neuronal activity can be observed as it happens. The application of fMRI in sleep research has suffered technical problems due to high noise levels in the scanner, but much progress has been made in this area as well [25].

Body and brain temperature

Another standard in sleep research PSG is the recording of body temperature, which was already performed before the 1900s [26]. Temperature at different locations on or in the body are in some cases recorded simultaneously to determine heat-loss changes in the course of sleep and wakefulness to obtain insight into the relation between sleep and thermoregulation [27].
Body temperature is regulated in the hypothalamus. Therefore, it is of great interest to measure brain temperature as well. In humans a whole 24-h recording of brain temperature has been accomplished only once [28]. In animals this type of recordings is performed on a regular basis, investigating the relationship between sleep states and brain temperature [29–32], but also to investigate the relationship between sleep and hypometabolic states like hibernation [33, 34] and daily torpor [35, 36], or to investigate the effects of changing brain temperature on the EEG [37, 38].
In many cases brain temperature is recorded with a temperature-sensitive resistor, which is placed between the skull and the *dura mater*. Simultaneous recordings from hypothalamus and cortex in the rat with this method have shown that temperature fluctuates in parallel in both brain areas [39]. With these probes core body temperature can also be recorded, but 'temperature-sensitive' transmitters can be used as well [35, 40]. This way, temperature cannot be sampled as frequently, but the recordings can be obtained without a limiting recording cable. A recent cheaper development is the use of a small data logger which logs the temperature on an internal memory. The data are downloaded after the experiment [41].

Stereotaxically placed instruments

The EEG is recorded from the cerebral cortex. However, vigilance state control does not originate in the cortex, but in deeper brain structures. To investigate these areas, measuring instruments need to be placed accurately deep in the brain by use of a stereotaxic instrument.
The stereotax fixes the head of the animal in a standard orientation with respect to a probe. The probe (i.e. electrodes, cannulas or microdialysis probes) or the animal (and its brain) can be moved in the medial-lateral and anterior-posterior direction. In addition, the probe can be lowered in stereotaxic space in the vertical direction in the brain through a hole in the skull. For accurate placement on a target deep within the brain a 3-D atlas of the brain is needed. This provides research opportunities in animals which are not available in healthy human subjects.
Electrophysiology in sleep research concerns recording the electrical activity of neurons in the intact, freely moving animal. Although it is possible to answer some questions with anesthetized animals or in brain-slice preparations, true sleep electrophysiology can only be done with the occurrence of changing vigilance states [42]. The observed activity consists of extracellularly recorded action potentials fired by different neurons in the area underneath the electrode. Isolating the activity of a single cell requires more or less elaborate manipulations. When the electrode is in a fixed position it should be possible to isolate the activity of a single neuron with a characteristic action potential. How successful this is depends on the size of the neurons relative to the size of the electrode. Figure 1a shows an extracellular recording obtained from the suprachiasmatic nucleus (SCN) of the hypothalamus. SCN neurons are small and densely packed [43], which results in a recording with a relatively small signal-to-noise ratio. In general, though, neurons close to the electrode tend to be less abundant, and because of the short distance, their signal will be large (i.e. higher signal-to-noise ratio). In the example a few of those larger spikes are visible. In some cases it is sufficient to select the highest-amplitude spike to isolate the activity of a single neuron. This is easier in areas with larger neurons like the hippocampus where, with the additional help of tetrode techniques, it is possible to investigate the activity of 'place cells' in relation to sleep and wakefulness [44, 45]. Sometimes the shape of the spike reveals something about the type of neuron it originates from [46]. In areas with small and densely packed neurons it is more complicated, although some single neurons have been recorded from the SCN
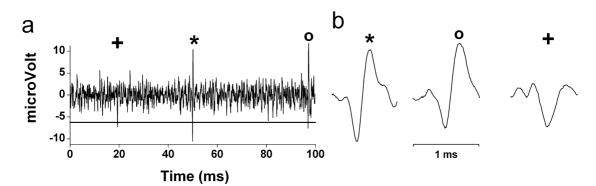
Figure. 1. (**a**) Example of multiunit activity oscilloscope traces recorded from the suprachiasmatic nucleus, with three examples of above-threshold (horizontal line in **a**) spikes enlarged in (**b**). Textbook theory says that the shape of an action potential within a single neuron is always the same. Therefore, the shape of the spikes is sampled, and discrimination between the three occurs on the basis of amplitude of the up- and downward swing of the signal or, if necessary, on the entire shape of the recorded spikes. The spike marked with '+' is sufficiently different from the other two, and it can be concluded that the three spikes are produced by at least two different neurons. The shape of a single spike can be followed over time, and the activity of a single neuron can be documented in different vigilance states.

successfully over several days [47]. With mathematical software, e.g. a multiple component analysis, the shape of the recorded spikes can be compared, increasing the chance of isolating the activity of individual neurons.

With microdialysis the chemistry of extracellular space in living tissue can be monitored, and a preview of chemical changes in the tissue is obtained before these events are reflected in blood. To this end a physiological salt solution is slowly pumped through the stereotaxically placed microdialysis probe, which equilibrates through a membrane with the surrounding extracellular tissue fluid. It will then contain a representative proportion of the tissue fluid's molecules. The microdialysate is then extracted and analyzed. Note that the exchange of molecules can occur in both directions. The difference in concentration governs the direction of the gradient. Therefore, endogenous compound can be collected at the same time as exogenous compounds (drugs) are introduced into the tissue.

One of the highlights of the use of microdialysis in sleep research was the investigation of adenosine levels in the basal forebrain in a control situation and during and after sleep deprivation (SD) [48]. More recently microdialysis was used in combination with EEG and brain-temperature measurements to analyze hyocretin levels in the lateral hypothalamus [49]. Also in humans it has recently been possible to investigate extracellular adenosine with microdialysis in epileptic patients [50].

Cannulas enable the delivery of relative large quantities of liquid and soluble substances and have been used for a wide range of brain areas and substances in the last 30 years. Another possibility with cannulas is the retrieval of cerebrospinal fluid (CSF) [8, 51, 52]. This technique is less common, but may be an alternative to microdialysis when larger amounts of fluid are needed or when a comparable situation to human CSF analysis is preferred. For this purpose a cannula is placed in the *cisterna magna* (Fig. 2a). An alternative is puncturing the neck area of the animal [53].

We have recently collected CSF with cannulas to measure circadian fluctuations at 2-h intervals in hypocretin levels in the rat [8]. For the analysis approximately 50 µl CSF per time point was needed. Collecting this amount of CSF within a 24-h period is not possible in a rat. Therefore, a schedule was adopted, collecting over a 3-day period with intervals of 6–8 h (Fig. 2b). By recording drinking behavior simultaneously, the circadian time point of every sample could be determined, and the circadian waveform of hypocretin release over a 24-h period was reconstructed (Fig. 2c).

Animal models in sleep research and sleep pathology

Before the middle of the 1990s all animal models of sleep pathology were either achieved through lesions or spontaneous mutations. The first publication of sleep in a gene knockout mouse model [54] heralded the genetic era for sleep research. Although the use of genetically modified animals holds great promise, it is clear that also with this tool research mainly involves eliminating possibilities and seldom provides straightforward answers.

Sleep is regulated by homeostatic and circadian processes [13, 55]. In mammals sleep homeostasis is reflected by EEG slow-wave activity (SWA, EEG power density between ~1 and 4 Hz) in NREM sleep. In all mammalian species investigated, SWA increases as a function of prior waking duration, and in several
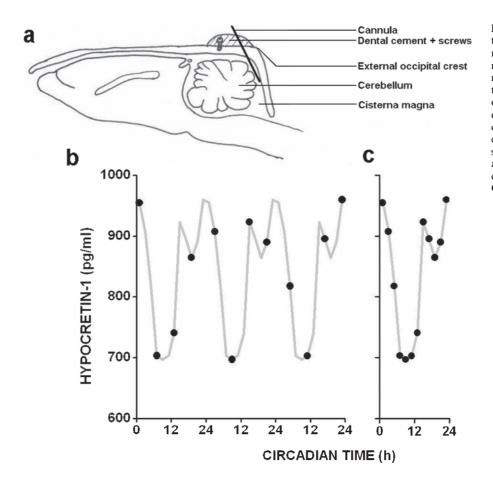
Figure 2. (**a**) Schematic representation of the position of the cannula when extracting cerebrospinal fluid (CSF) from the cisterna magna to analyse hypocretin content. (**b**) The schedule shows the original order of sampling (black dots) over 3 days. (**c**) Simultaneous recording of drinking activity enabled precise localization of sample time in each individual animal, which results in the circadian pattern of hypocretin in the CSF [8].

species a dose-response relationship between waking duration and subsequent SWA was established [56–60]. Mathematical models, simulating the homeostatic response, have been applied successfully in human [61], rat [62] and mouse [59,63]. The circadian process is controlled by a pacemaker located in the SCN and provides the homeostatic process with a circadian framework [55].

Sleep regulation can be divided into three different components. The *circadian component* is reflected in the time at which the major sleep episode occurs. Sleep-wake *architecture* is reflected in the level of consolidation of sleep and wakefulness. The *depth* of sleep is reflected by EEG slow waves produced during NREM sleep and tells us something about its recuperative value. The three components are not completely independent from each other, but for all three animal models exist.

Circadian regulation

Separating circadian and homeostatic sleep components is complicated by the synchronous progression of circadian time and changes in sleep and wakefulness. In humans several protocols (ultra-short day, forced desynchrony) have been established to uncouple sleep homeostasis from the output of the circadian pacemaker [64, 65]. Only once has a forced desynchrony protocol performed in rats [66]. In animals, research is done either after SCN lesions [67–69] or in spontaneously arrhythmic animals [60,70,71]. Another possibility is to observe homeostatic and circadian responses as close to the source as possible by simultaneous recording of EEG and SCN neuronal activity [42].

Genomics has brought a new perspective. The search for clock genes has been highly successful, resulting in 5–7 putative clock genes (Period genes Per1, Per2, Per3, Clock, Bmal, Cryptochromes Cry1 and Cry2, reviewed by [72]). Knocking out one or more of these genes results in an animal with a changed or disabled circadian clock in which sleep regulation can be investigated. However, until now some of these models provided puzzling data.

Cry1 and Cry2 double knockouts showed increased NREM sleep time and consolidation, and increased EEG SWA [73], all signs of high non-REM sleep pressure. Also, the Clock mutation seems to alter sleep homeostasis since the mice sleep less than wild-type control animals [74]. This is remarkable because removing the SCN in rodents was never followed by

an overall change in sleep amount [67–69, 75]. The results seem to indicate that Cry and Clock are not only responsible for circadian rhythms but may also play a role in sleep homeostasis. However, it is unclear which part of the results is caused by loss of circadian regulation and which by loss and/or compensation of gene function in other regions of the brain. Until studies become available where the mutated mice and wild-type control animals of these particular mouse strains underwent SCN lesions, the meaning of these results may not be resolved.

The Per mutant mice may become an animal model for familial advanced or delayed sleep phase syndrome. Advanced sleep phase syndrome (ASPS) is a rare disorder. The first mutation responsible for a familial form was found in the PER2 gene [76]. Delayed sleep phase syndrome (DSPS) is less rare than ASPS, and familial forms seem to be associated with PER 3 gene polymorphism [77, 78]. Clock gene polymorphism was not found to be associated with sleep and circadian phenotypes in humans [79].

Sleep and sleep regulation have been investigated in mPer1, mPer2 and mPer3 mutant mice [80, 81]. The genotypes clearly influenced the distribution of sleep, reflecting especially the phase advance of motor activity onset in the mPer2 mutant, which corresponds to similar observations in humans with ASPS. However, the correspondence between mPer3 mutant mice and human point mutations in the Per3 gene associated with DSPS are less clear. The homeostatic response to SD is intact in all mutant mice, supporting the notion that the circadian and homeostatic components underlying sleep regulation function independently.

Sleep architecture

The largest advance in our thinking about sleep architecture has come from research into narcolepsy, a sleep pathology where sleep-wake architecture is profoundly disturbed. The association of episodes of muscle weakness triggered by excitement and sleepiness were first described in 1878 by Westphal [82] and Fisher [83]. In both cases hereditary factors were noted as well. In 1880 Celineau introduced the name narcolepsy [84]. In canine narcolepsy, which was first described in 1973 by Knecht et al. [85] and Mitler et al. [86], the trait is transmitted as a single autosomal recessive gene [87,88].

To identify the gene responsible for the disease, a library containing large DNA fragments from a dog that was heterozygous for a narcolepsy gene was built in the 1990s. The search narrowed down to a genomic region containing two candidate genes, one of which was expressed in the brain. It encoded the hypocretin 2 receptor [89]. Almost simultaneously it was shown that preprohypocretin knockout mice have sleep abnormalities similar to narcolepsy [90].

A year earlier hypocretin was identified by De Lecea et al. [91] and Sakurai et al. [92]. De Lecea identified a transcript that was only expressed in the lateral hypothalamus. It encoded a precursor molecule for two related peptides and showed a homology with the gut hormone secretin. Based on this the peptides were called hypocretin-1 and -2. Sakurai et al. [92] independently confirmed the existence of hypocretins. They reported increased feeding after central administration and increased messenger RNA (mRNA) expression after fasting, and decided to call the newfound protein orexin. Both names are still in use in the literature.

The role of hypocretins in sleep is still under investigation [93]. Undetectable levels of hypocretin in the cerebrospinal fluid of patients [94] suggest that human narcolepsy is caused by hypocretin production deficiency. Recently, a transgenic rat model was developed [95], which could provide a useful model of human narcolepsy. It loses its orexin neurons at approximately 17 weeks of age, mimicking the progression of the disease in humans [94]. The finding that human narcolepsy is associated with low or absent hypocretin levels opened up novel therapeutic and diagnostic perspectives, but it also pointed to new research avenues in basic sleep research. The hypocretin system in the lateral hypothalamus is uniquely positioned to modulate brain areas involved in sleep regulation. Narcolepsy being primarily characterized by disrupted state organization suggests that hypocretin may be important in stabilizing vigilance states. New conceptual models concerning vigilance state consolidation were generated [96], and new avenues of research were opened.

Depth of sleep

From the earliest days of sleep research it was clear that a correlation existed between depth of sleep and the amount of EEG slow waves [97]. This relation was confirmed over 50 years later in the rat [98]. As discussed previously there is large similarity between mammals in the regulation of slow waves in the NREM sleep EEG in response to SD.

Dramatic progress has been made in our knowledge of how these slow waves in the sleep EEG come about [99], but it is still unclear how the homeostatic response to SD is regulated. Research in this direction is mainly focused on the simulation of the time course of SWA [59,61–63] and less in anatomical localization of the process. Comparison between different species and strains suggests a species-dependent and genetic component for the build-up rate of sleep pressure [31, 59, 63].

An unexpected finding concerning slow wave EEG synchrony came from the comparison of the EEG of different mouse strains. Slow-waves are more prominent in some strains than in others [59, 100], and with a linkage analysis and subsequent targeted lesion analysis Maret et al. [101] showed that the gene encoding retinoic acid receptor beta has a large influence on the contribution of slow-wave oscillations to the sleep EEG. Retinoic acid signaling is known to be involved in the patterning of the brain and dopaminergic pathways during development [102, 103]. In the adult brain it seems to be involved in cortical synchrony during sleep. At the moment it is unclear what this eventually means for basic sleep research, but the link with the dopaminergic system suggests that age-related changes in sleep may depend to some extent on changes in the dopaminergic system. In addition the data may explain sleepiness and increased slow waves in the EEG induced by dopaminergic drugs [104].

Starting in 1878 with the first EEG recordings, sleep research has become a field that uses many different methods and technologies, including cellular signaling and genetics, as well as continuing to use EEG.

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