Development of electrophysiological tools to record sleep across life stages

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1. Abstract

It was frequently suggested by previous research that sleep plays a crucial role in the cognitive development of young animals, including humans. The exact mechanisms by which sleep interacts during healthy brain maturation are yet to be discovered. During this study, our lab had been developing and refining an experimental design, which would provide tools to explore that question extensively. We have successfully refined a device, which allows the recording of both ECoG (electrocorticogram), EMG (electromyogram) and LFP signals (with intracerebral tetrodes) of rodents (rats) of varying ages. Furthermore, the design includes the refinement of data acquisition as well as the development of an analytical tool, which will provide the automatized scoring and oscillation analysis of sleep recordings. I have successfully obtained preliminary data from these experiments, which would be of reference for further experiments. In my opinion, this tool will allow scientists to analyse and compare great amounts of data and explore sleep more extensively.

2. Introduction

Sleep has been proposed to affect several physiological functions, such as the thermoregulation, hormonal regulation, immunity, and metabolism of the body. It has been also implicated in cognitive processes (Cirelli & Tononi, 2008; Walker, 2017). Sleep need – and therefore functions – differs widely between animals (Siegel, 2008) and even across the individuals within a specie (Walker, 2017). In humans, and possibly in mammals in general, sleep quality and quantity varies with age (Roffwarg et al., 1966), possibly indicating the distinct cognitive demands of the different life stages. The alteration of the sleep architecture is the most pronounced during development, with an extensive amount of sleep, and during aging, with the decline of both sleep duration and intensity (Mander et al., 2017; Roffwarg et al., 1966).

Sleep is crucial for healthy cognitive development in mammals – yet we mostly do not know why. Specifically, we know very little about the underlying mechanisms occurring during sleep that are essential for brain maturation. This gap in our knowledge is notably due to a lack of tools to investigate these mechanisms. My master thesis aims to bridge that gap by refining experimental designs that would allow us to properly measure (and eventually manipulate) sleep in rodents across life stages. To that end, I have helped with (1) the design of new electronic, surgical, and computational tools suitable to record and automatically score sleep in animals across their development; and with (2) testing and optimizing these new tools while mapping sleep architecture in animals at different life stages from adolescence (P26) to young adult (~2 months) to adult (~6 months) and aging (1 year).

The experimental design includes several stages, that need optimization in order to provide valid results on the changes of sleep. (1) A crucial part of the experiment is to develop the appropriate **electronics:** a microdrive recording device, able to transmit both intracerebral (tetrodes), cortical (ECoG: electrocorticogram) and muscle (EMG: electromyogram) signals. Most importantly, the device must be developed so to be adapted to animals across life stages – therefore size and weight of components must be considered. Intracerebral, ECoG and EMG wires need to be chosen with consideration of e.g., the impedances for the applicable signal/noise ratio or the probable contact with air and the animal. (2) **The experimental procedures** include several variables such as the animal handling, surgical implantation, and postoperative care, as well as the design of the recording environment, and the sleeping environment. In order to obtain comparative results, it is important to reduce the stress caused by the surgery and to have the animals habituated to the different environments beforehand. As for the surgical implantation, the procedures must be adapted to the specific parameters appropriate for the different ages: the size of the animal, the thickness of the skull, physiological characteristics that determine the response to anaesthetics and the length of the

recovery period, the speed of growth or the post-surgical activity of the animal. The process and circumstances of data acquisition also need to be optimized. This includes finding the right environment for the sleep and the wake phases, determining the beginning of sessions, along with finding methods to motivate the animals to cooperate. Lastly, (3) **the analytical methods** as data pre-processing and automatized sleep scoring also need to be developed and fine-tuned. Depending on the quality of the data, certain measures need to be performed to prepare that for the analytical evaluation (e.g., applying filters, smoothing of the tracking signal, …). More importantly, the analysis methods need to be refined and improved based on experience to provide the valid results.

Precising these features provides us with adaptive tools to measure the differences in sleep across the developmental stages such as the emergence of sleep wake cyclicity, variation in the length of sleep boots, percentage of REM, NREM and wake states as well as the changes in the oscillatory activity. My secondary aim during my master thesis was to gather preliminary results on differences in sleep characteristics across the lifespan. Once these tools established, we will be able to address questions like why and how is sleep necessary for the healthy development? How to disturb and manipulate sleep? What exactly in sleep is that the healthy brain requires? What can be done to improve sleep - in special consideration to its decline with aging observed in humans?

In the first part of my thesis (Chapter 0**Error! Reference source not found.**), I will start with introducing the main concepts and background related to sleep research across life stages. Specifically, I will focus on defining sleep, including the important aspects of sleep scoring (Chapter 2.1); the role of sleep and consequences of sleep loss (Chapter 2.2); the emergence of sleep patterns during development (Chapter 2.3); and the changes in sleep in result of aging (Chapter 2.4). In Chapter 3, I will both describe our methodology and the steps we took to refine that. In Chapter 4, I will present our results both regarding the methodologic refinements and the preliminary results of the experiments. Finally, I will discuss these results and consider the limitations of the project as well as introducing some ideas for further improvement (see Chapter 5).

2.1. The definition of sleep

"Sleep is a reversible state of reduced responsiveness usually associated with immobility" occurring naturally and periodically (Boccara, 2021; Cirelli & Tononi, 2008). The activity of sleep can be viewed either as a behavioural or a physiological state determined by behavioural or physiological criteria, respectively. Whether to describe sleep based on brain activity or the behavioural criteria is a long-debated question among scientists. However, before the development of the electrophysiology (1929, measuring the electrical activity of the brain), sleep had to be characterized and recognized by the former – consequently, well-pronounced behavioural criteria has emerged, described earliest by

Piéron in 1913, and later revisited by other scientists. However, one must note than EEG are the main tool to define sleep in clinical context.

Behavioural indicators of sleep by consensus are the following: 1) animals sleep in a species-specific stereotypical position (e.g. humans lie horizontally); 2) they maintain behavioural quiescence with lowered muscle tone and the absence of voluntary movements; 3) sleep is reversible (with appropriate stimulation); 4) sleep is coupled with an increase of arousal (sensory) threshold, meaning that the response to the stimulus is delayed and only occurs above a certain intensity (intensive sounds or touch). (M. S. Blumberg, Karlsson, et al., 2005; Campbell & Tobler, 1984; Walker, 2017). Blumberg later defined four other requirements to precise how to identify sleep, some of which already include electrophysiological observations and are as follows: 5) Sleep happens spontaneously and naturally, in a circadian rhythm, 6) and it is regulated by homeostatic mechanisms. Homeostasis means, that the body tries to preserve a definite amount of sleep that it needs, performing compensatory actions when the need is not met. Sleep loss builds up sleep pressure – an increased drive to fall asleep – , with a subsequent sleep rebound, that is an increase in both sleep amount in quality (Siegel, 2008). 7) changes in neural functions (certain neurons become active, while others are silent) 8) and it has to be identifiable as a characteristic in each species. (M. S. Blumberg, Karlsson, et al., 2005).

The characterization of REM sleep in 1953 (Aserinsky & Kleitman, 1953) put the definition of sleep in another light as it allowed for scientists to identify sleep states by specific brain wave criteria. This is considered a more reliable and method and is widely used among scientists (Campbell & Tobler, 1984; Purves et al., 2018). In addition to that, the use of polysomnography also gained ground in research – it is a multiparametric test combining the electroencephalogram (EEG), electromyogram (EMG), electro-oculogram (EOG) and electrocardiogram (ECG). These methods made possible of the emergence of a complex scoring system for the proper characterization and identification of sleep and sleep stages in humans. (Adamantidis et al., 2019), which I will describe in more details in the following chapter (see Chapter 2.1.1 and 2.1.2).

2.1.1. Sleep characteristics and scoring

Rechtschaffen and Kales were the first ones to develop a complicated and precise scoring system based on human polysomnographic data, where they divided NREM sleep to four stages (from S1 to S4, representing increasing sleep depths) based on changes in the oscillatory activity of the EEG (Rechtschaffen A. & Kales A., 1969). This system was later simplified by the American Academy of Sleep Medicine in 2007, establishing a universal sleep scoring system still widely used today, including only

3 NREM stages coupled with respiratory, cardiac and movement actions (Academy of Sleep Medicine, 2007).

There are two fundamentally distinguished forms of sleep: **rapid-eye-movement (REM) sleep**, named after burst of quick eye activities and usually tied with the activity of dreaming, and **non-rapid eye movement (NREM) sleep**.

REM sleep is often called *paradoxical sleep (PS)* or *active sleep (AS)* because the EEG brain activity is very similar to the awake activity (Bear et al., 2020). It was first isolated in 1953 by Aserinsky and Kleitman (Aserinsky & Kleitman, 2003). It is characterized by fast, irregular, low voltage oscillations. REM sleep is known for vivid dreams with visual details and the rolling movements of the eye (hence the name rapid-eye-movement sleep) (Siegel, 2005a). A unique feature of REM sleep is muscle atonia: almost all muscles lose their tone and are paralysed, so the active brain cannot give commands to the body which to act on (Bear et al., 2020). This feature can be compromised in people with parasomnias, dissociated sleep states e.g., REM sleep behaviour disorder (RBD) or sleep paralysis (Hu, 2020; K. Pavlova & Latreille, 2019). Breaking this atonia, twitches occur naturally during REM sleep. These twitches are brief, irregular, spasmodic movements of the limbs, (whiskers in rodents) or other parts of the body (M. Blumberg, 2010). They are more frequent during development (M. Blumberg, 2010). The energetic demands (and oxygen consumption) of the brain are very high during REM sleep: that can be even higher than in wake states (Bear et al., 2020). REM is coupled with the sympathetic activity of the nervous system (activity responsible for the "fight and flight" responses): body temperature decreases, the heart and respiratory rates increase and are quite irregular (Bear et al., 2020). Brainstem neurons (that are operating vital functions) are highly active – similar to or even exceeding the wakeful rates –, and cortical neurons show activity similar wakefulness (Purves et al., 2018; Siegel, 2008). Although most neurons behave similarly during REM and waking, noradrenergic, serotonergic, and histaminergic neurons (that are usually tonically active throughout waking states) are silent in REM (Siegel, 2008). The activity reduction of noradrenergic neurons can explain the loss of muscle tone, while the quiescence of histaminergic neurons might be related to the loss of environmental awareness (John et al., 2004). "It is an active, hallucinating brain in a paralyzed body" described briefly and concisely by William Dement, sleep researcher at Stanford University. (Purves et al., 2018)

NREM sleep is also referred to as *slow-wave sleep*, due to the domination of slow, regular, large amplitude (high voltage) EEG oscillations, called slow oscillations (<1 Hz) and delta waves (0,5-4 Hz). This is due to the spread synchronized activity of cortical neurons. The more synchronized, the higher the amplitude and the deeper the sleep is. During deep sleep, neurons are highly synced, causing high arousal threshold and therefore, the sensory inputs are generally unable to reach the cortex. This

results in that the sleeper does not wake up unless higher external stimulus (Bear et al., 2020). NREM is characterized by a reduced activity in brainstem systems and the forebrain, meanwhile high-voltage slow waves and spindles are present in the cortex (Siegel, 2008). The overall brain activity and the muscle tension is reduced and there are only minimal involuntary movements. It is important to emphasize that in this state the body is capable of movement, however normally the brain does not command it to do so(Bear et al., 2020). NREM is coupled with the activation of the parasympathetic nervous system: the heart rate and the respiration slow down but the digestive processes speed up. The body temperature and the oxygen consumption of the brain falls as neurons are at their lowest activity (Purves et al., 2018). William Dement described it “the idling brain in a movable body”. (Bear et al., 2020).

Behaviour	**Wakefulness**	**REM sleep**	**NREM sleep**
EEG and state specific continuous oscillations **(Frequency are in reference to values obtained with human EEG)**	Fast, low voltage oscillations: • Theta oscillation (4-8 Hz) • Alpha oscillation (8-11 Hz) • Beta oscillation (15-30 Hz) • Gamma oscillation (30-90 mV)	Fast, low voltage oscillations: • Theta oscillation (4-8 Hz) • Beta oscillation (15-30 Hz) • Gamma oscillation (30-90 mV)	Slow, high amplitude oscillations: • Delta oscillation (0.5-4 Hz) • Slow oscillation (> 1 Hz)
Transient/intermittent oscillations	Spindles (11-14 Hz)	Sharp-wave ripples (100-300 Hz)	Spindles (11-14 Hz), K-complexes and sharp-wave ripples (100-300 Hz)
Movements	Voluntary, continuous	Atonia, only twitches and ocular movements	Scarce, involuntary
Autonomous nervous system activated	Sympathetic and parasympathetic	Sympathetic	Parasympathetic
Breathing and heart rate	Fast	Fast and irregular	Slow
Metabolism	Fast	Fast	Slow

Neuromodulation	Most neurons are active (activity dependent)	Noradrenergic, serotonergic, and histaminergic neurons silent	Mostly silent; synchronized activity

Table 1 Sleep stages and their main characteristics. *Three vigilance states present in all mammalian species are wakefulness, REM sleep and NREM sleep. These states are characterized by the differences observed in brain oscillations (continuous and transient), movements, the activity of the autonomous nervous system, breathing and heart rate, metabolism and neuromodulation. Source: (Adamantidis et al., 2019; Bear et al., 2020; Purves et al., 2018).*

Sleep scoring depends mostly on three factors to distinguish between wake and the two sleep states: muscle tone (measured by EMG), eye movements (measured by EOG) and cortical activity (measured in human by electroencephalogram – EEG). Wakefulness is recognized based on muscle activity and desynchronized cortical activity resulting in fast and low-amplitude oscillations. REM sleep is identified based on the occurrence of rapid eye movements (measured by EOG) the absence of muscle activity (atonia) and cortical oscillations similar to wakefulness. NREM sleep is primarily scored based on the synchronized slow wave activity, in addition small movements can be detected with the EMG (M. S. Blumberg et al., 2020). In human, NREM sleep has three distinct stages that follow each other as sleep gets deeper and deeper, and cortical neurons get more and more synchronized, resulting in increasing slow oscillation amplitude (Purves et al., 2018). I will define these different stages in Chapter 2.1.2.

2.1.2. Sleep architecture and sleep oscillations in humans

As humans fall asleep, they enter a cycle of REM and NREM sleep stages following each other in a relatively precise and regular order during the night. Each cycle lasts approximately 90 minutes and is repeated throughout the sleep period – this is called an ultradian rhythm. An average adult human spend about 75% of their total sleep time in non-REM and 25% in REM sleep (Bear et al., 2020; Purves et al., 2018).

Most often, the first stage of a sleep period is stage 1 of non-REM sleep (NREM-1) (Bear et al., 2020). This is the lightest stage of sleep coupled with irregular EEG of decreasing frequency (**alpha (8-11 Hz)** and then **theta rhythm: 4-8 Hz**) (Adamantidis et al., 2019). NREM-1 typically lasts for only a couple minutes, before transitioning to stage 2 non-REM sleep (NREM-2)(Bear et al., 2020). NREM-2 is somewhat deeper and longer lasting (for about 15 minutes) (Bear et al., 2020). The frequency of the EEG oscillation is further decreasing and is interrupted by occasional **sleep spindles**: 12-15 Hz oscillation bursts (50-100 μV) of activity that last about 1-2 seconds. Spindles result from interactions between the corticothalamic networks and the reticular nucleus of the thalamus (De Gennaro & Ferrara, 2003). High amplitude (~100 μV) sharp wave **K-complexes** (with 0.5 second duration) are also typical transient oscillations of NREM-2, supposedly resulting from a synchronized cortical network,

spreading through the cortex and transferred to the thalamus (De Gennaro & Ferrara, 2003) and can be induced by sensory stimulation (Adamantidis et al., 2019). During Stage 3 (NREM-3) **delta waves (0.5-4 Hz)** dominate the EEG: the frequency continues to decrease, and the amplitude increases. Stage 4 (NREM-4) is the deepest stage of sleep and is called slow-wave sleep, as it is described by the large amplitude, 1Hz or less EEG delta rhythms (Adamantidis et al., 2019). The first NREM-4 of the night is typically the longest, it can last for 20-40 minutes (Bear et al., 2020). According to the new scoring system by the American Academy of Sleep Medicine, these two states are combined together and called NREM-3 (Academy of Sleep Medicine, 2007). After the last stage of NREM, the sleep lightens and from a brief (10-15 minutes) transition through stage 3 and 2 the sleeper enters REM sleep with **fast beta (15-30 Hz) and gamma (30-90 Hz) rhythms** and frequent eye-movements(Bear et al., 2020). During the night within each cycle NREM sleep length reduces and the time spent in REM sleep increases – half of REM sleep occurs during the last third of sleep and the longest REM can last 30 to 50 minutes. On average, about four REM sleeps occur during one night's sleep. (Bear et al., 2020; Purves et al., 2018)

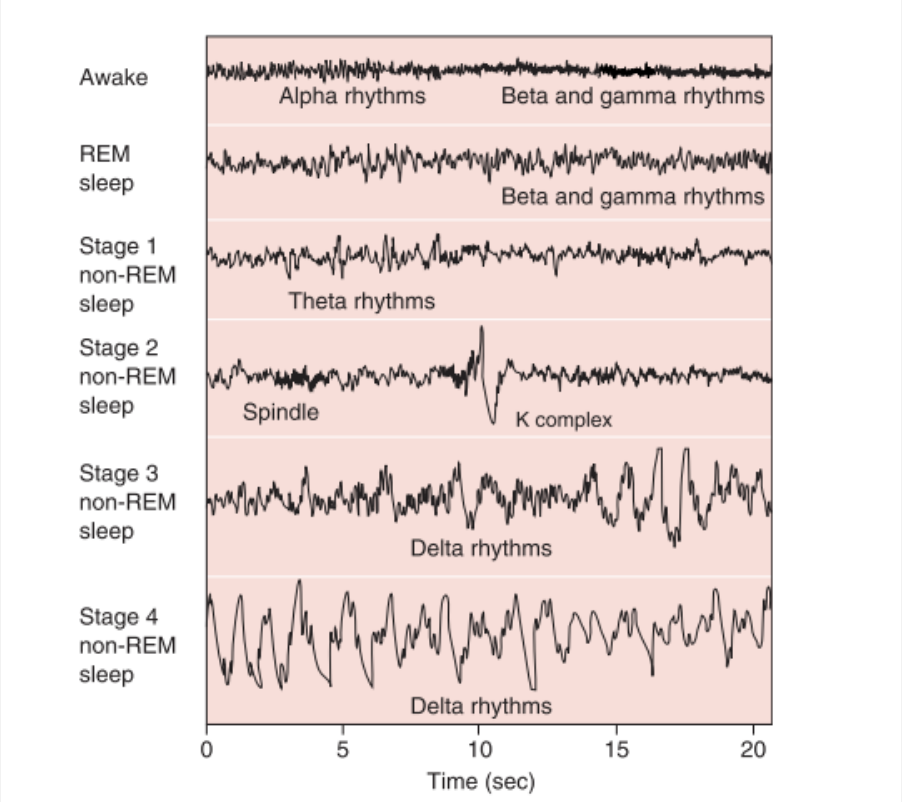

Figure 1 Characteristic oscillations of the different sleep stages in humans. *Six stages of vigilance are characterized in humans. Awake states are characterized by the low voltage, fast alpha, beta, and gamma rhythms. REM sleep has similar, but more irregular oscillations with dominating beta and gamma rhythms. NREM-1 is recognized by slightly slower theta oscillation and as sleep gets deeper, these oscillations get even slower, as during NREM-2. K-complexes and spindles are transient oscillations specific to this stage. NREM-3 are recognized by the domination of delta rhythms and NREM-4 by delta and even slower, slow-wave oscillations. Source: (Bear et al., 2020)*

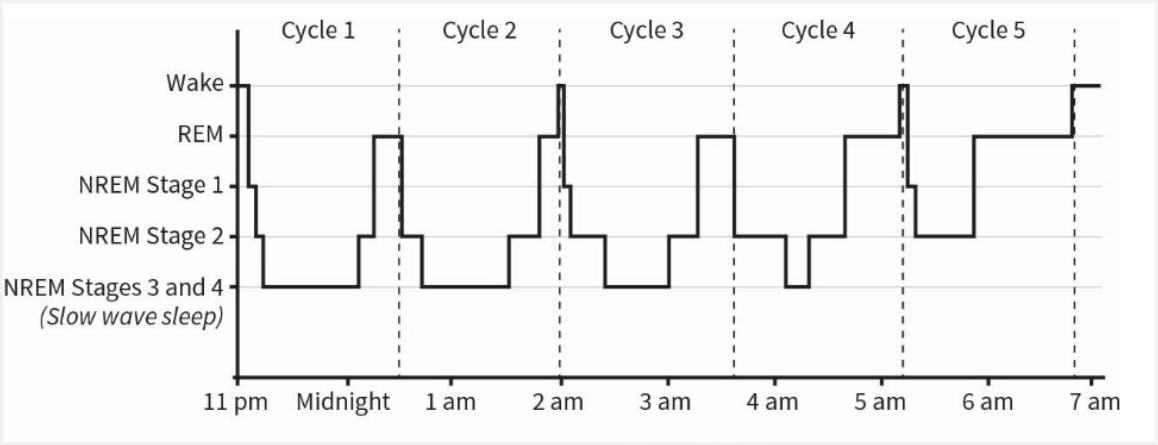

Figure 2 Schematic picture of the human sleep architecture. *The figure shows how sleep stages follow each other in an orderly fashion: wake is followed by the lightest NREM sleep, then proceed to the deeper NREM stages relatively fast. During some of the cycles certain NREM stages can be skipped or are very short. REM is usually the last stage of an ultradian cycle (e.g., Cycle 1) and is often followed by short wake bouts. As the sleep progresses the time spent in REM sleep becomes more and more pronounced – thus sleep gets lighter. Source: (Walker, 2017)*

2.1.3. Sleep characteristics in rodents

The rodent life span can be divided as followed: After birth rodents are called neonatal until the seventh postnatal day (P0-P7), then become infants (P7-P21) until the end of the weaning (~P21). Adolescence is from P21 to sexual maturity (~P50-60) and can be divided to early adolescence (P21-P36), puberty during peri-adolescence (P37-48) and late adolescence. Animals can be called young adults from (P49-60) (Nelson et al., 2013; Sengupta, 2013). Rodents are considered old after 18 months (or older, depending on the phenotype) (Flurkey et al., 2007). The mature adult of 3-6 months are considered to be equivalent of humans of 20-30 years old, the middle aged rodent category of 10-14 months are equivalent to the 38-47 years old in human age, and old 18-254 months old rodents are compatible with 59-69 years old humans (see **Figure 3**) (Flurkey et al., 2007).

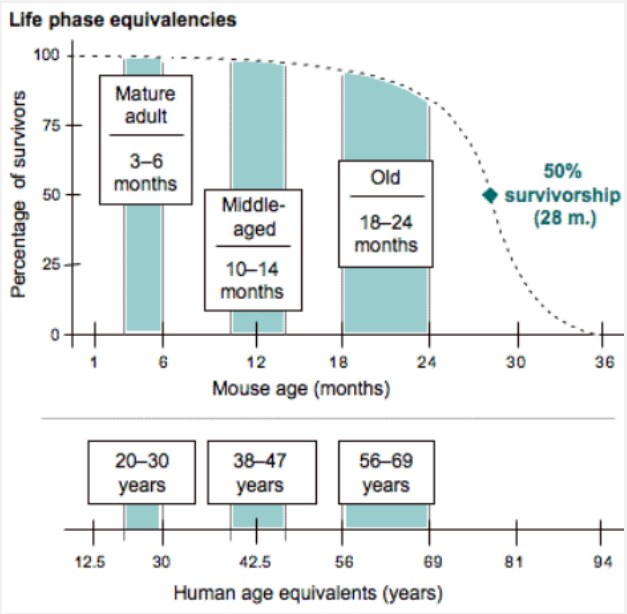

Figure 3 Comparison between the human and the rodent life cycle. *Scientists have made attempts to align the human and the rodent lifespan based on the most important developmental events (e.g. weanling, sexual maturation). Rodents become mature adults when 3-6 months old in contrast with humans who are considered as such between 20-30 years old. Middle aged rodents are 10-14 months old, that corresponds to 38-47 human years. 18-24 months are old age for a rodent, which is compatible with 59-69 of human years. Source: (Flurkey et al., 2007)*

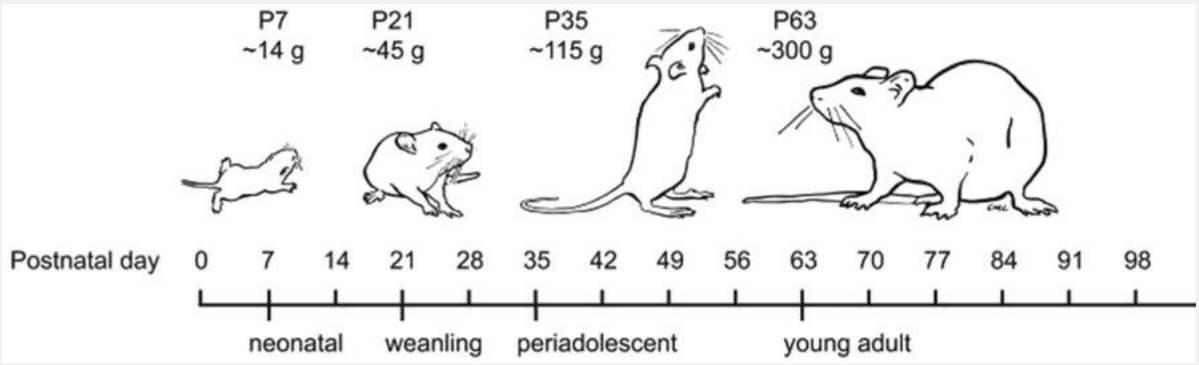

Figure 4 The life cycle (and the corresponding weight) of the rat*. Neonatal(P7) rats are around 14 grams, weanling rats (until P21) are around 45 grams, preadolescent animals (P35) are approximately 115 grams, and a fully mature adult rat (P63) weighs around 300g. Source: (Sengupta, 2013)*

Laboratory rats are widely used in sleep research for they have many similarities to human. Rodents have the same circuits involved in the regulation and control of sleep, similar oscillations (slow wave, delta, and theta) dominate their sleep stages, moreover two distinct stages, REM and NREM sleep alternate similarly to humans (Adamantidis et al., 2019). There is only one NREM stage (SWS) defined well in rodents, although some attempts have been made to divide it to stages resembling the ones found in human (Adamantidis et al., 2019; Lacroix et al., 2018). The dynamics of the sleep cycle

is also similar in rodents to what was described in humans. According to this novel classification, sleep starts with NREM-1, followed by N2 and N3 respectively, and REM sleep terminates the cycle. N3 is more prominent in the beginning of the sleep period and the amount of REM increases towards the end (Laventure et al., 2018).

Rodent NREM (or SWS) sleep is characterized by similar oscillations to human NREM-3: high-amplitude, low-frequency slow waves consisting of delta (0.5-4 Hz) and slow oscillations (<1 Hz) and sleep spindles (10-16 Hz). K-complexes are less defined in rodents (Adamantidis et al., 2019). Rodent REM sleep is dominated by theta (6-9 Hz) and gamma (30-150 Hz) oscillations (Adamantidis et al., 2019). The duration of a sleep cycle is remarkably shorter than in humans, it was proposed to lasts only about 10 minutes (Trachsel et al., 1991). Some studies have also used a transitional state, intermediate sleep (IS), that is defined by high theta/delta ratio in the hippocampus, while spindles and delta activity are still observed in the prefrontal regions of the brain. IS provides transition between NREM sleep to REM sleep (Gottesmann, 1996).

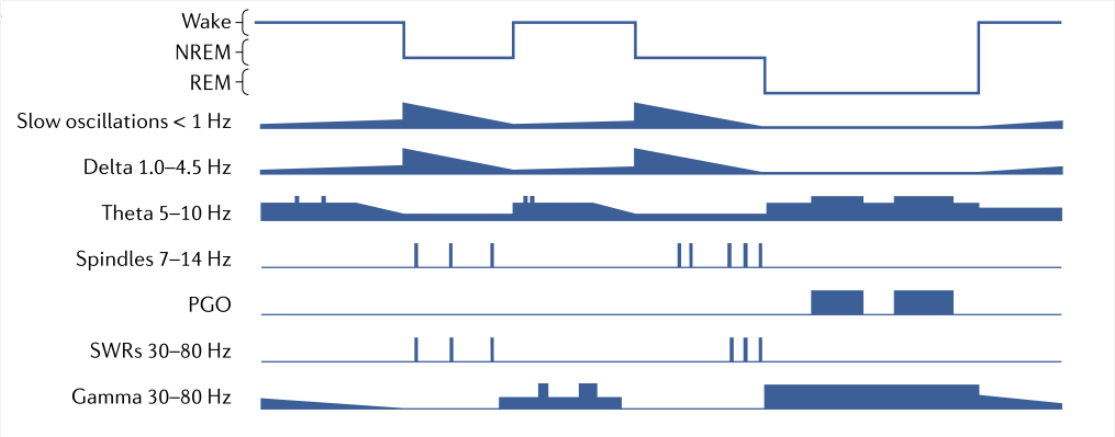

Figure 5 Rodent sleep stages and their characteristic oscillations. *Three vigilance states are described in rodents: wakefulness, NREM and REM sleep. These stages cycle similarly to human sleep stages and have similar characteristic oscillations. NREM sleep is dominated by delta and slow oscillations occasionally interrupted by spindles and sharp wave ripples. REM is prominent in theta oscillations and are also associated with PGO (ponto-geniculo-occipital) waves. Wakefulness is characterized with high gamma and theta oscillations. Source: (Adamantidis et al., 2019).*

It is clear, that a lot is unknown about the rodent sleep cycle and its compatibility with the stages described by human as sleep scoring methods are yet to be adapted and refined accordingly. My work offers an opportunity to establish tools to further investigate the sleep architecture in rodents across their lifespan.

2.1.4. Sleep regulation and homeostasis

The “sleep-switch” concept was developed by Saper and colleagues in 2001 to present their ideas about sleep-wake regulation (Saper et al., 2001). The “flip-flop” switch model represents two basic principles of sleep-wake regulation: the mechanisms creating sleep and wake states are reciprocally inhibitory; and transitions between the two states occur rapidly, without intermediate phases. According to their model, the first flip-flop decides between wake and sleep states, and a second flip-flop mediates transitions between REM sleep and NREM sleep. (Saper et al., 2001).

A sleep promoting network was identified in the anterior hypothalamus, specifically the ventrolateral preoptic (VLPO) area. It was proposed that the wakefulness side is promoted by networks in basal brainstem and caudal hypothalamus (Lu et al., 2000). Later, it turned out that the brainstem itself is enough to aid basic wake and sleep components, and candidate areas are the medulla and mesopontine region (M. S. Blumberg et al., 2014). Regulation of sleep is a complex mechanism with the interaction of many brain nuclei, which I am not going to describe in detail (for further information see (Scammell et al., 2017).

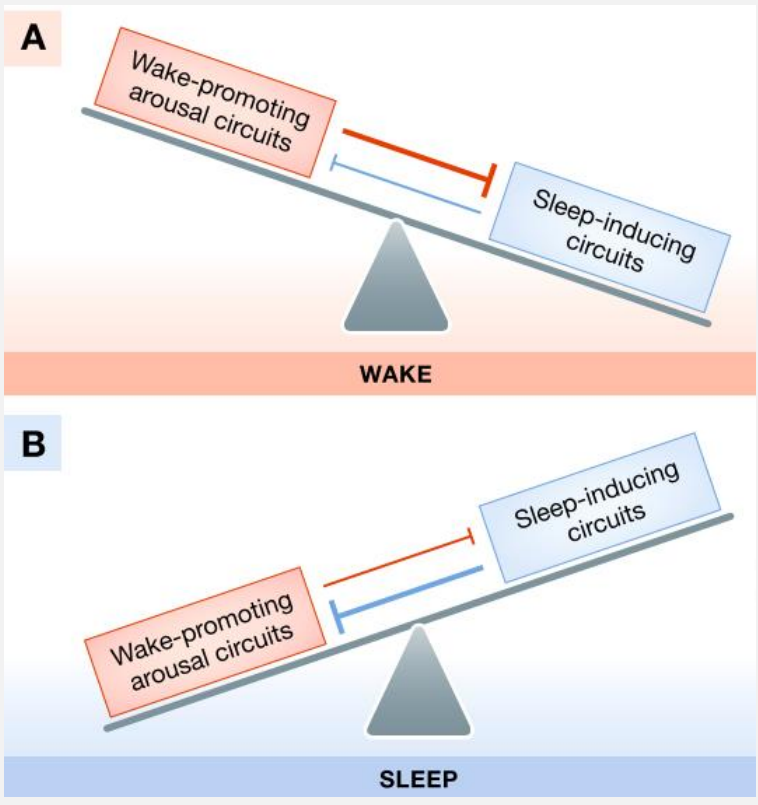

***Figure 6 Schematics of the sleep switch**. The figure indicates the principle of the sleep regulatory system: a reciprocal inhibition of sleep promoting networks and wake promoting networks, thereby working as a switch between the two states. (Bringmann, 2019)*

"Sleep homeostasis" means that after longer periods of sleep deprivation, animals display compensatory responses (Borbély & Achermann, 1999). Two dimensions of sleep homeostasis are sleep pressure and sleep rebound. Sleep pressure is the escalating drive to falling asleep and it accumulates during the deprivation (Boccara, 2021). Sleep rebound is the subsequent increase in sleep, both in terms of sleep amount and intensity (Boccara, 2021). Sleep amount is identified as an increase in the total sleep time, whereas sleep intensity is usually measured as the delta power during deep sleep (Borbély & Achermann, 1999; Cirelli & Tononi, 2008). **Slow wave activity (SWA)** is a universal marker of sleep intensity: it refers to the number and amplitude of slow waves during non-REM sleep (Achermann & Borbély, 2003). SWA is widely used to investigate sleep homeostasis and measure sleep rebound in adult animals.

2.2. The role of sleep

2.2.1. Sleep homeostasis and the consequences of sleep deprivation

We humans (and several other animals) spend a third of our life sleeping, nonetheless, we have little idea about what function it fulfils exactly. The most fundamental question would be to find out if sleep is essential, whether it achieves a vital and therefore universal function in animals. The duration and intensity of sleep varies widely across species, regardless in all species observed so far sleep seems to a role in maximizing energy savings by reducing the energy consumption of the body and the brain and initiating or simply providing opportunity for restorative functions (Siegel, 2005b).In most cases, being asleep is a quite disadvantageous behavioural state, as it is coupled with a reduction in alertness and lowered responsivity to surrounding events, exposing the animal to its environment and possible danger (Cirelli & Tononi, 2008). Considering this, sleep must achieve a vital purpose that cannot be accomplished during waking states – or else it would have been eliminated during evolution. Quoting Rechtschaffen: "if sleep does not serve an absolute vital function, then it is the biggest mistake the evolutionary process ever made"(Allen Rechtschaffen, 1971; Rial et al., 2007).

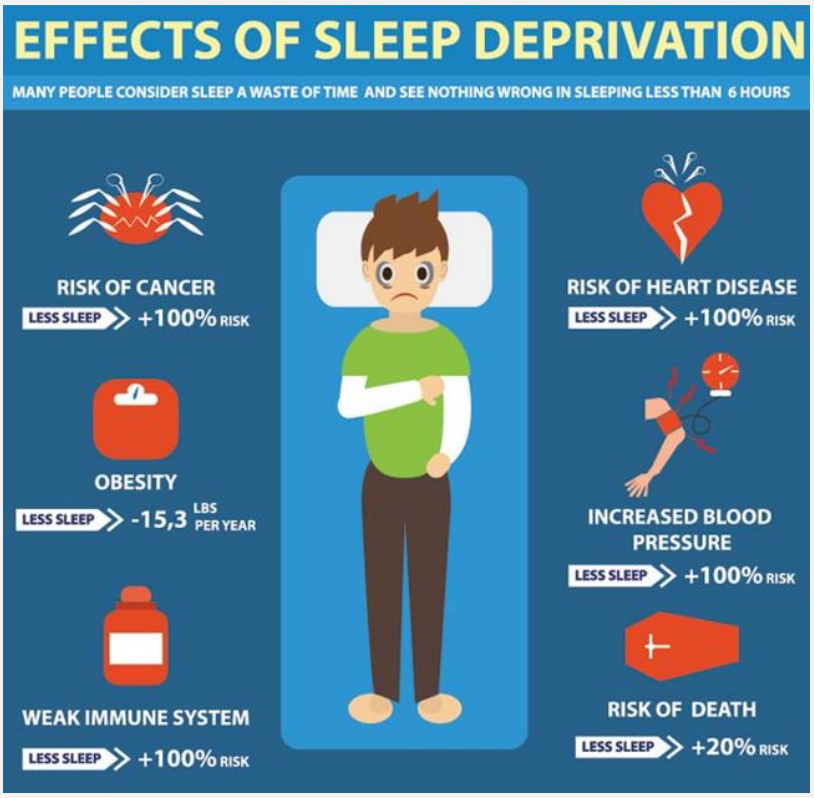

Figure 7 Effects of sleep deprivation. *Sleep deprivation has several physiological and neurological consequences: it was implicated in cancer formation, obesity, the weakness of the immune system, it is considered a risk factor in developing heart diseases, loss of sleep increases blood pressure and chronic sleep deprivation leads to death. Source: Lecture by Charlotte Boccara*

According to the definition of sleep (see Chapter: **Error! Reference source not found.**) the following arguments would prove the necessity of sleep in nature (Cirelli & Tononi, 2008):

(1) All animals should perform some sort of sleep state.

(2) Sleep is homeostatically regulated, therefore all animals need recovery sleep when they stay awake longer.

(3) The lack of sleep occurs with serious consequences.

Sleep has been mostly studied in birds and mammals (Siegel, 2008), species which were the basis for identification of sleep features. Sleep has thus been harder to identify in certain species, e.g., reptiles, amphibians, fish, and invertebrates and some authors have even question the ubiquity of sleep across the animal kingdom(Cirelli & Tononi, 2008; Siegel, 2008). Recent studies have resolve this debate to some extent, showing some form of sleep (or sleep-like stage) has been observed in all species studied so far, for example in fruit-flies (Hendricks et al., 2000), zebrafish (Zhdanova et al., 2001), *C. elegans* (Raizen et al., 2008) or teleosts (Goldshmid et al., 2004). Recently, researchers have identified sleep stages resembling REM and SWS sleep in lizards, that were only observed in mammals

and birds before (Shein-Idelson et al., 2016). These findings are pointing towards the notion that there are no clear evidence of a species that does not perform sleep (Cirelli & Tononi, 2008).

As discussed in Chapter 2.1.4, sleep deprivation does not always result in an increase in sleep time in all animals, as sleep has quantitative (duration) and also qualitative (intensity) dimensions (Tobler, 1995). Sleep can therefore be recovered in several ways: either with sleeping for longer periods; or sleeping in a more consolidated manner – meaning that sleep is less frequently interrupted by brief awakenings; or sleeping more deeply – meaning exhibiting higher synchronisation in the delta frequency (Cirelli & Tononi, 2008). Sleep intensity, measured by SWA, is used to identify sleep rebound in animals. In humans, sleep rebound is clearly present and usually made up of a mixture of the phenomena mentioned above (Siegel, 2008). Sleep rebound after sleep deprivation was also observed in zebrafish and dolphins in both intensity and duration (Cirelli & Tononi, 2008). Although the same results could not be observed in experiments with pigeons (Berger & Phillips, 1994), a phenomena was revealed where SWA levels remained the same despite the elimination of sleep, suggesting that slow waves might leak into the wakeful states, maintaining a constant amount of SWA (a phenomenon called sleep intrusion (Franken et al., 1991)) (Cirelli & Tononi, 2008). Similar phenomenon was observed in sleep deprived humans, with slow wave activity and "microsleep" episodes appearing during wakeful states (Cajochen et al., 1999).Conclusively some form of sleep seems to be present and homeostatically regulated in most animal species observed so far.

Prolonged sleep deprivation leads to serious consequences, eventually even death. Sleep-deprivation experiments conducted on rats with the disk-over-water method led to the development of a behavioural and physiological syndrome with increased metabolic rate, decreased body weight and death after 2 or 3 weeks (Allan Rechtschaffen & Bergmann, 1995, 2002). In accordance with this, other studies showed that that sleep-deprivation has fatal consequences in dogs (Bentivoglio & Grassi-Zucconi, 1997), cockroaches (Stephenson et al., 2007), fruit-flies (Shaw et al., 2002) and also in humans (Cirelli & Tononi, 2008; Siegel, 2008). However, later studies refuted the fatal effects of sleep deprivation on fruit flies, initiating a debate among researchers (Geissmann et al., 2019). Sleep deprivation also leads to considerable deterioration of performance and cognitive impairment in humans, flies, birds, and rodents (Cirelli & Tononi, 2008). According to human studies, sleep restriction leads to performance decline in psychomotor vigilance tasks (Belenky et al., 2003) and the impairment of waking neurobehavioral functions, which the subjects are usually unaware of (Chu et al., 2011). A cumulative effect of even mild sleep loss for consecutive days was observed on the cognitive abilities (Chu et al., 2011). Although it was also shown, that sleep loss affects individuals differently in terms of the extent of the impairment and of what tasks it concern (Van Dongen et al., 2004). Sleep deprivation was also linked with increased food intake, gain weight and eventually obesity in rodents (Mavanji et

al., 2013; Parrish & Teske, 2017). In conclusion, sleep loss and deprivation have serious consequences including death for certain species, which suggests that sleep must serve fundamental functions – even vital for many species including humans.

2.2.2. Learning and memory

There are increasing amount of experimental evidence that reveals important role of sleep in physiological functions such as homeostatic regulation, thermoregulation, immunity, tissue repair, and memory processing (Walker, 2008). Special emphasis has been given for uncovering the beneficial effects of sleep on learning and memory (Born et al., 2006; Diekelmann & Born, 2010). Sleep has been considered to have an important function in optimizing the consolidation of new memories. Consolidation refers to an active process, during which newly acquired information get processed, reactivated, and reorganized (Born et al., 2006; Maquet, 2001). It is important to mention here that there are two very distinct forms of sleep – NREM and REM – which might serve different functions regarding memory formation.

Sleep is important both before and after learning and sleep quality is in close correlation with the memory processing (Walker, 2009). For example, the quality and amount of NREM sleep before learning enhances the efficiency of hippocampal-dependent memory encoding during the next day (Mander et al., 2014; Van Der Werf et al., 2009). Moreover, NREM quality after encoding (with special emphasis on slow waves) has also shown to be implicated in the consolidation of the hippocampal-dependent representations, thus acquiring more stable, long-term memories (Walker, 2008). Three types of sleep (NREM) oscillations contribute to this long-term consolidation: hippocampal sharp-wave ripples(100-300 Hz), cortical slow oscillations (<1 Hz) and sleep spindles (10-16 Hz) (Diekelmann & Born, 2010). It was proposed, that during slow-wave sleep, newly acquired memory are being reactivated and replayed during sharp-wave ripple (SWR) oscillations in the hippocampus (Buzsáki, 1998). Sharp wave ripples originate in the hippocampal CA3 area (Csicsvari et al., 2000) and often occur coinciding with spindles in the thalamo-cortical circuitry, suggesting and interaction between hippocampal and neocortical networks (Buzsáki, 1998). These reactivations (replays) – supposedly driven by the slow oscillations – activate the transfer of memory representations to a cortical-dependent form by synchronizing with spindles and thus leading to long-term potentiation of synapses (Born et al., 2006; Diekelmann & Born, 2010; Walker, 2009). Slow oscillations play an important role in synchronizing the neuron activity in the cortex, the hippocampus or the thalamus (Born et al., 2006). These oscillations during NREM, together provide the transfer of newly acquired information and strengthening memories (Born et al., 2006).

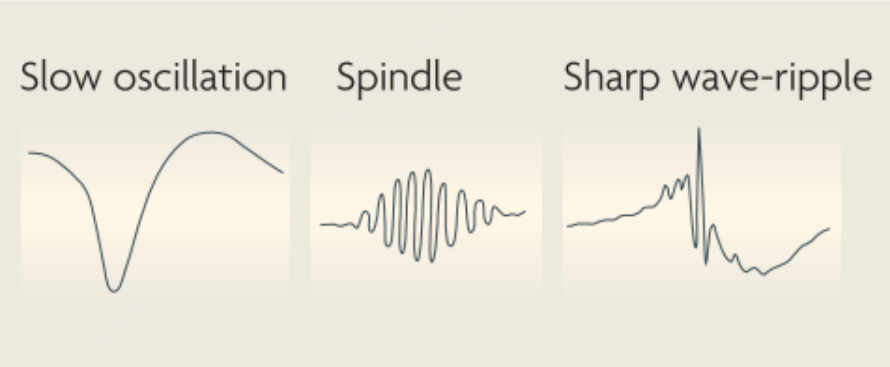

Figure 8 NREM related oscillations implicated in learning and memory consolidation. *Slow wave oscillations are continuous oscillations often tied to the process of memory consolidation. Two transient oscillatory phenomena are also implicated in learning, these are spindles and sharp wave ripples. (Diekelmann & Born, 2010)*

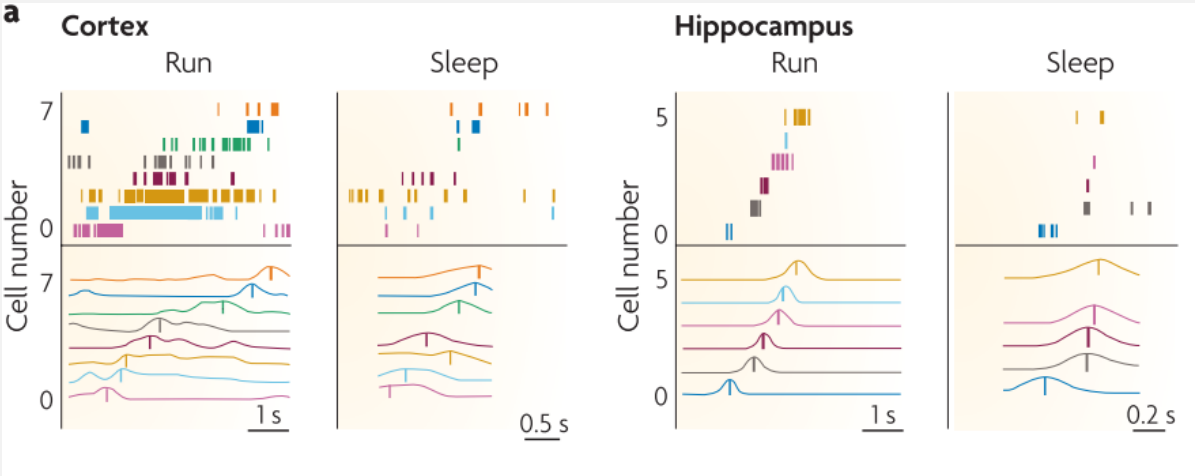

Figure 9 Memory re-activation during slow wave sleep in the sensory cortex and the hippocampus. *During the activity (Run), certain cells fire in a specific sequence for a specific duration. After this activity during sleep, the same cells repeat this sequence (thereby replaying the memory of the event) both in the sensory cortex and the hippocampus. (Diekelmann & Born, 2010)*

REM sleep is also implicated in memory consolidation; however, its exact mechanism of function is much less well described. While declarative memories seem to be more reliant on NREM sleep, REM sleep seems to be beneficial for the strengthening of procedural memories, nondeclarative memory and amygdala-dependent emotional memories (Plihal & Born, 1997, 1999; Wagner et al., 2001, 2003). It was later also suggested that REM acts as to stabilize the memories that had been consolidated during NREM sleep (Diekelmann & Born, 2010). Nevertheless, it was revealed, that REM sleep has a crucial role in maintaining the regulation of the motor nervous system (see Chapter 2.5) and sleep twitching contributes to neural maturation (see Chapter 2.3.1) – issues revisited in the following chapters.

The synaptic homeostasis hypothesis (SHY) – proposed by the lab of Cirelli and Tononi – claims, that sleep is necessary for the maintenance of the synaptic homeostasis and the regulation of synaptic plasticity. According to SHY, NREM sleep supports the renormalization of synaptic strength by the depotentiation of certain synapses, based on the synaptic activations occurring during the day and thus increasing the signal to noise ratio of acquired information about the environment. (Tononi & Cirelli, 2014).

In any case, it is clear, that both NREM and REM have important role in maintaining the healthy functions of the nervous system.

2.3. The development of sleep architecture and sleep homeostasis

It has been long observed that we spend more time asleep when we are young. Humans sleep approximately 16 hours when they are born, but it decreases to only 8 hours as they grow up and reach adulthood. Sleep quality also changes – around birth sleep is characterized by a prevalence of active sleep (REM around 50% of all sleep, approximately 8 hours/day), and less NREM sleep in total. These proportions change quickly during development: adults spend most time sleeping in SWS/NREM sleep, and the amount of REM drops considerably, to only 2 hours/day. Similar results have been observed in other mammals as well. REM sleep continues to fall steadily with aging in line with the total sleep amount and with a less pronounced decrease in NREM sleep. (Roffwarg et al., 1966).

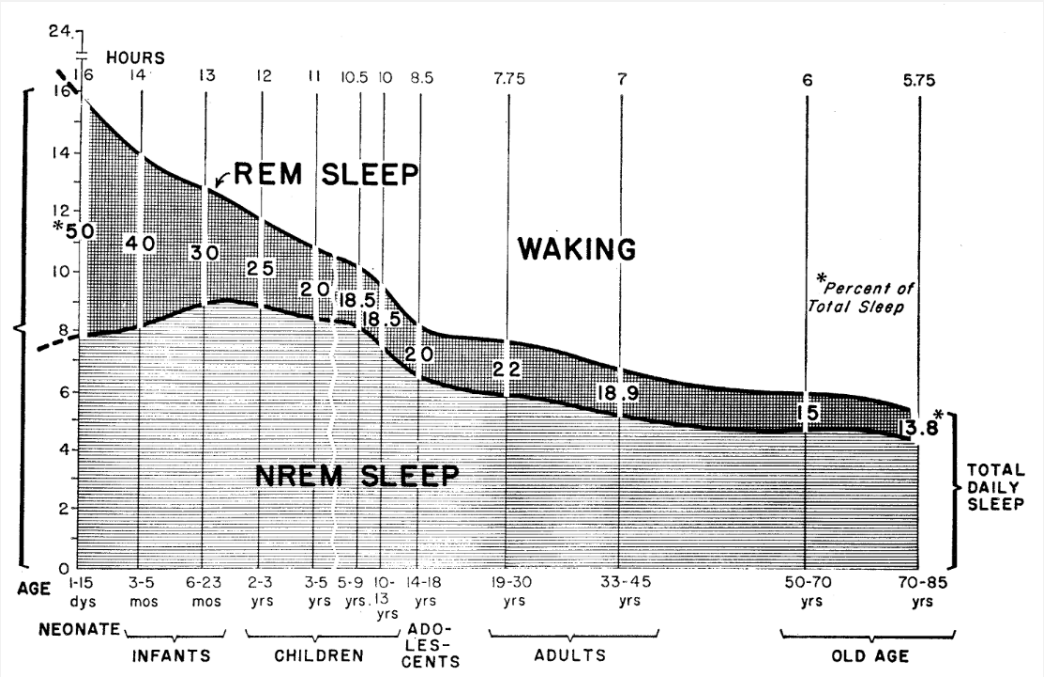

Figure 10 Changes in sleep distribution across the lifespan in humans. *Overall sleep amount drops rapidly during development and continues to decrease during the lifespan and aging. Significant change in REM sleep amount occurs during early development with slight decrease during later life stages. In contrast with this, NREM sleep increases slowly, then decreases also moderately across life. (Roffwarg et al., 1966)*

Observations of sleep patterns in mice during adolescence to adulthood (P19-P111) shed light to this transition more closely. The study by Cirelli and Tononi showed a sharp decrease in REM sleep amount from adolescence to adulthood, while sleep amount remained steady and NREM increased slightly. The drop of REM duration happened quickly around P40 and then stayed stable throughout the lifespan. In contrast with this, NREM duration increased to adult levels during early adolescence According to the same study, sleep duration did not change remarkable during the observed ages. (Nelson et al., 2013). Our study aims similar purposes, although it offers a more accurate tool for sleep scoring by considering data from intracerebral (local LFP) electrodes besides the ECoG channels. Furthermore, the analysis methods include the analysis of oscillations across lifespan, specific to sleep stages.

Mentioned observations about the changes in sleep stages lead to the following question: what essential function does REM fulfil during development that would explain its extreme prevalence in developing mammals and subsequent decrease in adulthood?

2.3.1. REM amount correlates with brain level of maturation

Roffwarg and colleagues were pioneers in addressing this question in 1966, coming up with the "*ontogenetic hypothesis*", that claimed that active sleep promotes brain maturation and neuromuscular development via twitches, stimulations produced by the brainstem, by sending ascending impulses to the motor areas and ascending impulses to sensory areas in the forebrain (M. Blumberg, 2010; Roffwarg et al., 1966). Twitching was observed to be even more pronounced and intense in infant rats and it was observed that twitches already occur in the womb (M. Blumberg, 2010). Blumberg and colleagues further investigated the question to find out how these sleep-related movements, the twitches, contribute to brain development. They managed to link twitching to neural maturation: they observed that twitches provide sensory feedback that modulates the neural activity of the spinal cord as well as of the cortex and the hippocampus. It is important to mention, that said experiments were performed in considerably unrealistic sleep conditions, as the animals were head-fixed with a stereotaxic apparatus (M. Blumberg, 2010; Mohns & Blumberg, 2008).

2.3.2. Different sleep oscillations and their maturation across development

It is not as straightforward to score sleep in newborn and infant animals. Rats do not perform eye-movements before 1 week of age. Moreover, continuous and state dependent EEG activity that resembles the one observed in adults only emerge over the first couple of months after birth in humans (Jenni et al., 2004), and by the second postnatal week in rats (Blumberg MS, Freeman JH, 2010).

Active sleep (AS) and quiet sleep(QS) are the “immature forms” of sleep and it is heavily debated whether adult REM and NREM emerge from these states, respectively (Frank & Heller, 1997). According to a review by Frank and colleagues, REM (or even classical sleep) might not be present at birth at all – instead, one irregular, dissociated activity, called “presleep” exists, and the organization of this state forms both REM and NREM sleep later during development (Frank & Heller, 2003). This claim led to a debate between said researchers and Blumberg and colleagues, who reasoned and showed evidence in support for the existence of sleep of infants, including a premature form of REM sleep that is already present at P2 in rats. However, evidence whether infant sleep can be divided to two states analogous to adult states, is still in question (M. S. Blumberg, Karlsson, et al., 2005).

According to studies in new-born rats, EMG measurements show oscillation between periods of high muscle tone, low muscle tone and atonia. These oscillations in the muscle tone are aligned with behaviour (Seelke & Blumberg, 2010). Developed and differentiated EEG activity is absent until P11 (Frank & Heller, 1997), therefore QS is recognised by atonia coupled with behavioural quiescence, whereas AS is identified with atonia accompanied by twitches (M. S. Blumberg et al., 2014). Twitches are present from P2 and are detected by brief and sharp spikes on the EMG (M. S. Blumberg et al., 2014). A bout of infant sleep starts with a short period of quiet sleep with atonia and behavioural quiescence, then ends with bursts of twitches. Increased muscle tone and normal movements indicates the end of a cycle (M. S. Blumberg et al., 2014). After P11 and the maturation of the cortical EEG, delta activity line up with the state recognized as QS by the behavioural and EMG data (M. S. Blumberg et al., 2014).

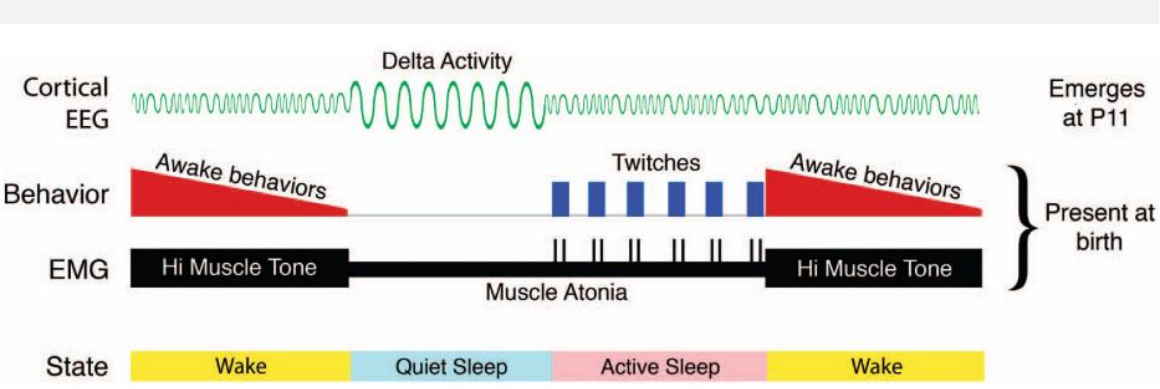

Figure 11 The alternation of the developing sleep stages observed in infant rats. *At birth, wake state is coupled with awake behaviour and high muscle, whereas quiet sleep is characterized by muscle atonia and behavioural quiescence. REM sleep is recognized by muscle atonia interrupted by twitches that can be detected with the EMG as well as observed with bare eyes. After the emergence of the cortical EEG at P11, delta waves align to the stage considered as QS, while it shows fast, irregular activity during the rest of the stages. These observations suggest, that sleep stages can be identified before the emergence of EEG. (M. S. Blumberg et al., 2014)*

Delta activity, 1-4 Hz oscillating waves, are expressed from P11 (Frank & Heller, 1997), and shortly after delta maturation, it gets restricted to only QS (Seelke & Blumberg, 2008). The ultradian sleep cycle also changes considerably. At, and before P9 sleep starts with a QS bout and ends with burst of AS. At P11, when delta develops, it is detected during the first QS episode and during bouts between AS sleep as well. These bouts and the delta power increase at P13, and QS with delta activity also appears during the last third of sleep (Seelke & Blumberg, 2008).

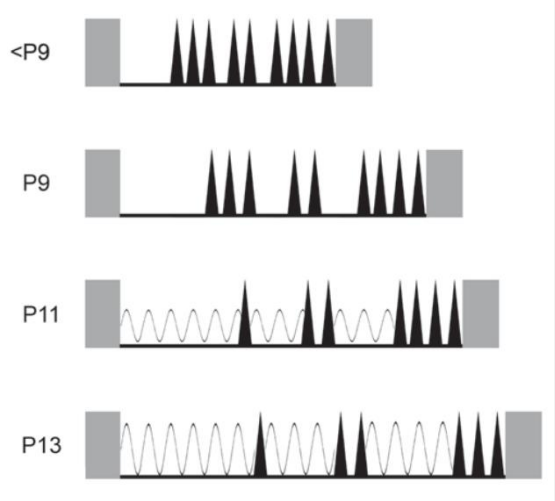

Figure 12 The ultradian cycle of developing rats before and after the emergence of the cortical delta activity. *Grey blocks represent high muscle tone and indicate wakefulness and black lines show muscle atonia, indicative of sleep. Black triangles are phasic burst of twitches, pointing out active sleep. Before P11, bouts of active sleep are frequent and separated by short quiet sleep periods. With the emergence of the cortical activity, periods of quiet sleep increases and quiet sleep episodes get shorter and more pronounced by the end of the sleep. (Seelke & Blumberg, 2008)*

Spindle bursts are present in the developing sensorimotor cortex of new-born rats and occur as responses to limb movements as twitches (Khazipov et al., 2004). These are distinct from sleep spindles and are predominant early in development and disappear relatively soon after birth (Tiriac & Blumberg, 2016). Hippocampal theta (4-7 Hz) appears around P8 (Seelke & Blumberg, 2010), first as brief bouts after the twitches, then starts to form a continuous rhythm during REM around P12 (M. S. Blumberg et al., 2020; Mohns & Blumberg, 2008). Twitches provide stimulation and sensory feedback to the hippocampus, where twitches get coupled with gamma-oscillations at P5, then theta and unit activity arises as well at P8. By P11, high amplitude and theta and gamma oscillations are present generally in the hippocampus, with elevated power during twitches (Mohns & Blumberg, 2008). Sharp wave-like oscillations begin to arise around the end of the second postnatal week and maturate to adult like oscillations at P20 (Buhl & Buzsáki, 2005). It was recently proposed that the highly synchronous activity of the medial entorhinal cortex (MEC) and the hippocampus is mediated by

myoclonic movements (e.g., twitches) and the generation of sharp wave ripples require the synchrony of said two areas (Valeeva et al., 2019).

2.3.3. Sleep and wake cyclicity and sleep homeostasis in development

Fragmentation, meaning the rapid cyclicity of brief sleep and wake bouts are an important feature of infant sleep, and has been observed in rats and humans as well. Sleep and wake bouts consolidate during the first couple months after birth in humans, and increase dramatically (four times to its initial value) over the first two postnatal weeks in rats (M. S. Blumberg, Seelke, et al., 2005).

Sleep homeostasis is usually measured with the SWA in adults; however, this marker can only be used from P11 in rats – after the delta wave activity emerges. Delta power changes only after P24 in rats in response to sleep deprivation (Frank et al., 1998), but sleep pressure already increased after P2 in sleep deprived rat pups, resulting in sleep rebound, characterized by more consolidated sleep bouts (Todd et al., 2010), and P12 rats performed sleep rebound with an increase in the quiet sleep, specifically (Frank et al., 1998).

Sleep pressure and sleep rebound seem to be dissociated early in development (P2) and probably have distinct developmental routes. The brainstem is involved in sleep pressure, while the forebrain seems to be implicated in the regulation of sleep rebound (M. S. Blumberg et al., 2020). The brainstem produces the basic sleep-wake oscillations during early ages, while later during development a bidirectional communication emerges between the brainstem and forebrain to express the critical features of sleep-wake functions in adults, like bout consolidation, sleep rebound and the circadian rhythmicity. It was proposed by Blumberg and colleagues, that the brainstem as a necessary component of the sleep-wake system, stands as a basis and additional components build on it during development (M. S. Blumberg et al., 2014). The homeostatic response matures fully at P25, when it can be detected as changes in NREM sleep delta power (Frank et al., 2017). SWA can be detected as early as P24 in mice (in accordance with mentioned observations in rats), with an increase during the wake period and a gradual decrease during the sleep period. SWS was also found to increase rapidly in response to sleep deprivation even at P24 and the effect was even more pronounced at P30 (Nelson et al., 2013).

The suprachiasmatic nucleus is called a “circadian pacemaker” that creates a 24-hour cycle by regulating both physiological and behavioural events. SCN develops approximately around the 22nd embryonic day (E22) in rats. Circadian rhythms are first get synchronized throughout the mother’s circadian system, and only after P8 will light take over this role of entrainment (M. S. Blumberg et al.,

2014). Day-night differences are first detected at P2 and daytime wake bouts increase considerably between P8 and P15 (Todd et al., 2012).

A 24-hour rhythm of wake-sleep cyclicity emerge around P17. This means when light/dark differences in the amount of time spent sleep and wake (sleep-wake bouts), and in the EEG-activity periods arise indicating the coupling of the EEG/EMG measured states with the SCN. Frank and colleagues also observed the appearance of another, 12-hour component of the waking period, that takes place over the 3 days after the establishment of the 24-hour cycle. 24-hour organization of sleep-wake bouts are present at P15-P17 and adult like at P20. Therefore, the establishment of the circadian cyclicity regulation seems to precede the development of the adult forms of sleep homeostasis. (Frank et al., 2017)

2.4. Qualitative and quantitative changes of sleep during aging

The quality and quantity of sleep declines dramatically in aging humans. The negative correlation of age with the sleep quality raises many questions, most importantly whether this decline is the result or the cause of the aging brain? The following chapter revises current studies conducted with regards to sleep changes due to aging in humans.

Aging is associated with several changes in sleep architecture, which usually begin to manifest from the middle-ages (50 -) and become more pronounced onwards (Mander et al., 2017). The quantitative changes in sleep architecture include a shift in sleep timing to earlier hours of the day (the shift is mediated by the change in the circadian rhythm), as well as difficulties falling asleep and maintaining sleep during the night (Mander et al., 2017). More importantly, a steady decrease can be observed in the duration of sleep and an increase in the percentage of time spent awake during the night after sleep onset (Feinberg & Carlson, 1968). Furthermore, sleep in older individuals is characterized by the fragmentation of sleep (sleep is more often disrupted by wake bouts, resulting in shorter sleep bouts), the decreased stability of sleep (the sleeper is more easily awakened by external stimuli) and a more frequent transition to lighter sleep stages (Conte et al., 2014). There are also alterations concerning the quality of sleep. The time spent in deeper sleep stages in humans, namely slow-wave sleep (including NREM3 and 4) decreases from early adulthood to older ages and is paralleled by an increase in the time spent with lighter sleep (NREM 1 and 2). According to certain studies, this is followed by a decrease in REM sleep as well (Plat et al., 2001). Sleep periods in older individuals are also characterized by shorter cycles than in younger adults (see figure) (Conte et al., 2014).

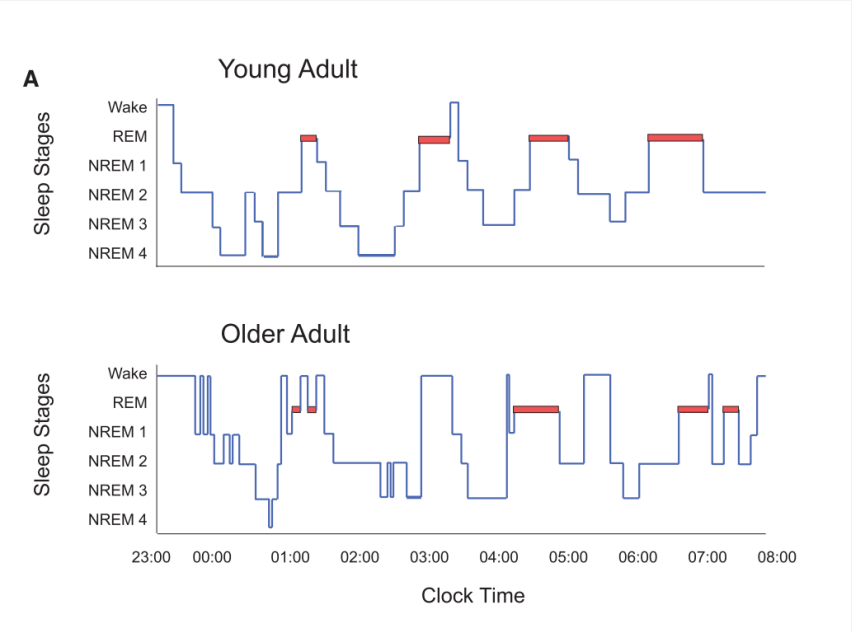

Figure 13 Age-related changes in sleep architecture in humans. *As age progresses the sleep architecture changes markedly. Deep sleep is getting more scarce during older ages and sleep becomes more and more frequently interrupted by wake bouts. The most significant change is the reduction in the amount of the deeper NREM stages (NREM-3 and NREM-4). (Mander et al., 2017)*

Alterations also affect the electrical oscillations of sleep. Slow-wave activity (SWA) during NREM sleep drops in middle-aged adults and continues to decrease with aging (Landolt et al., 1996; Landolt & Borbély, 2001), resulting from a decline in both the amplitude and the density of the slow waves (Carrier et al., 2011). SWA, functioning as a marker of sleep pressure, exponentially falls during sleep in young adults, but is shown to decrease to a much lesser extent in older people (Landolt et al., 1996; Landolt & Borbély, 2001), suggesting a decline in sleep homeostasis regulation during aging. The motion is further supported by the observation, that SWA fails to increase with response to sleep deprivation (Landolt & Borbély, 2001). Sleep spindle density and frequency range declines with age as well, where both spindle duration, mean amplitude and peak drops (De Gennaro & Ferrara, 2003; Landolt et al., 1996)

As sleep supports countless physiological and cognitive processes (see chapter: Role of sleep) it is clear why sleep decline in aging leads to ponderous consequences. Sleep disturbances in older individuals are tightly linked with cognitive decline and the inability to form or recall new memories (with emphasis on hippocampal-dependent memory encoding and consolidation) (Mander et al., 2017). According to a study with self-reported sleep of older adults, poor sleep leads to weaker performance in cognitive tests, and seem to be affected in verbal memory encoding (Lo et al., 2016). Changes in oscillations might explain this impairment of the sleep-dependent memory encoding: NREM sleep spindles reduce in older individuals, the reduction being the most pronounced in the

prefrontal cortex (PFC). It was shown that the density of sleep spindles in this area predicts the encoding capacity of the hippocampus and therefore the learning efficiency (Mander et al., 2014). This aligns with the finding, that disruption of NREM sleep reduces the hippocampal encoding activity, thus the learning ability in older adults (Van Der Werf et al., 2009). Sleep disruption was also linked with the impairment of long-term memory consolidation (Mary et al., 2013) and slow-wave sleep time reduction in middle-age adults showed a positive correlation with the performance degradation of memory retention (Backhaus et al., 2007). Sleep dependent consolidation impairment can be explained with the oscillatory alterations due to aging as well. The ratio of the reduction in the SWA predicts the efficacy of overnight memory consolidation and thus the memory recall the next day in older adults –the SWA decline observed at the PFC showed remarkable correlation with memory recall deficits (Mander et al., 2013).

Therefore, evidence points to the fact that cognitive decline during aging could be at least partially due to the decline of sleep quality and quantity, but more analyses and invasive animal experiments are necessary to pin-point specific mechanisms supporting these effects.

2.5. Sleep disturbances and neurodegeneration

Chronic sleep restriction and extended wakefulness are often implicated as risk factors for neurodegeneration (Owen & Veasey, 2020). It was found that even a couple days of sleep disruption can lead to significant (~ 30%) cell loss in certain brain regions including the locus coeruleus and orexinergic neurons (Zhu et al., 2016) or neurons in the medial prefrontal cortex and even the glial cells of this region (Noorafshan, Karimi, Karbalay-Doust, et al., 2017). More excessive sleep restrictions of 21 days led to 25% cell loss of neurons in the dentate gyrus, as well as an even more notable 48% reduction of CA1 pyramidal cells (Noorafshan, Karimi, Kamali, et al., 2017). Acute sleep loss was observed to markedly reduce the number of glial cells, which fulfil important function in neuroprotection (Owen & Veasey, 2020).

Sleep disturbances are severely implicated in the development of neurodegenerative diseases, such as Alzheimer disease (AD), dementia or Parkinson's Disease (AD) (Guarnieri et al., 2012). AD is a common neurodegenerative disease linked with the deposition of amyloid-β plaques and the aggregation of intracellular tau protein resulting in severe neuronal damage, thus cognitive impairment and dementia (Lucey, 2020). 25-44% of AD patients report sleep disturbances (Guarnieri et al., 2012) and sleep loss has been linked to the increase of Aβ and tau aggregation (Lucey, 2020). Recent studies have yet to reveal whether sleep disturbances are risk factors in the development of

AD or are symptoms of the disease itself, many findings pointing towards a possible bi-directional relationship (Brown et al., 2016; Lucey, 2020; Mander et al., 2016).

REM behaviour disorder (RBD) is a degenerative disorder resulting from the impairment of the brainstem networks, which fail to inhibit the activation of skeletal muscles and therefore fails to produce atonia during active sleep (M. Blumberg, 2010). RBD was observed to have a positive correlation with the development of Parkinson's Disease (PD) and similar neurodegenerative diseases, in fact RBD often precedes the development of the disease (Claassen et al., 2010). Therefore, the degeneration of the motor neuron system in PD patients seems to be linked to or even arise from the impaired control and regulation of motor activity during REM (Bliwise et al., 2010; M. Blumberg, 2010).

Sleep is severely implicated in neurodegeneration, in fact, recent studies point out, that malfunctions in sleep regulation might be a major risk factor in developing neurodegenerative diseases. Thus, studying sleep, appears to be a great tool for understanding the mechanisms behind the development of such pathologies.

3. Methodology and refinement of methods

In this chapter I will present the methods performed during our experiments and while doing that, I will also introduce the refinements that we applied for improving our experimental results. The methodology refinement is in itself a very important result of this study; therefore, I prefer to show these together with the methods themselves, in this section.

3.1. Developing recording devices to measure brain oscillations during sleep

3.1.1. Electrode preparation

Preparation of tetrode electrodes for intracerebral recording

The tetrode electrodes for the intracerebral recording were made by 17 µm diameter Platinum-Iridium wires (*California wire)* – these wires provide the best features regarding to signal conductance. The wires were twisted together to form a four-part tetrode electrode, where twisting was done using magnetic stirrer device. After twisting, the electrodes were fixed together by heating and partial melting of the outer insulation (polyimide) of the four wires at 220 C° degrees. Each tetrode consisted of a twisted part and separated by the end with four branches of single electrodes. These single electrode parts were used to wire the pins of the recording device.

Preparation of electrodes for EEG

EEG electrodes were made in two parts from 0.1 mm diameter copper wire (Rs online). The first part – hereinafter the "device-part", as it was later attached to the device – was an approximately 4-5 cm long wire, was de-insulated in both ends. One of the ends was twisted to form a loop that was filled out with solder (providing means for the attachment to the second part).

The second part, hereinafter the "head-part", of the EEG electrode was of similar size, also de-insulated at both ends. One end was attached to a M1.2x2.4 mm (1.2 mm thread size and 2.4 mm thread length, small parts) screw to record EOG signals from the cortical surface. The other end of the wire was twisted and filled with solder similarly to the device-part.

The impedances (Z) for both wires ranged from 0.6-0.8 1/Ω, depending on the wire length, the thickness of the solder layer and the quality of connection.

Preparation of electrodes for EMG

The EMG electrodes were first prepared in a similar fashion to the EEG electrodes, except that the "head-part" was fashioned into a hook on one end (instead of attached to a screw). This hook was intended to be hooked into the neck muscle of the animal in order to record muscle activity.

Refinement: I used this prototype of EMG wire for the first two devices and two following experiments (rat #1 and #2). However, we noticed that such a configuration did not provide good quality signal for recording the activity of the neck muscle. Indeed, EMG signals turned noisier and more unreliable with time, suggesting that the connection between the muscle and the wire spoiled (see **Figure 14**). We hypothesized that this could be due to the oxidation of the uninsulated wire end in the muscle, resulting in higher resistance and lower signal to noise ratio (S/N).

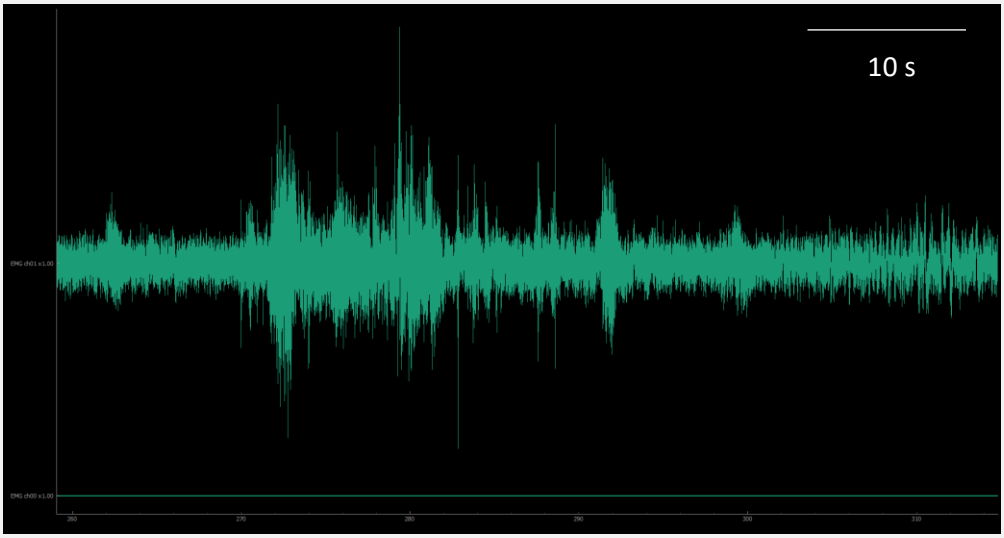

***Figure 14 Signal from the copper EMG wire**. Copper wires were used for the first two experiments (rat#1 and rat #2) to measure the EMG signal. The figure shows that one of the EMG electrodes are disconnected (see the one at the bottom), probably due to the animal movements. Therefore, the method for fixing the electrode to the muscle needed to be revised. The other EMG channel (the one at the top) had considerably noisy signal.*

For that reason, two novel EMG electrode prototypes were tried out and used for the next two experiments (rat #3 and #4):

One EMG electrode (hereinafter referred to as T50) was made by a 0.05 mm diameter 4 cm long Tungsten wire with PTFE insulation. The two parts – "head-part" and "device-part" – were uninsulated in both ends and designed to be attached with a silver pin instead of solder bubbles – as the Tungsten wire is resistant to soldering. The impedance for each part were measured around approximately 1.7 1/Ω for the 4 cm wire.

For the second EMG electrode (S50) a 0.05 mm diameter, PTFE insulated stainless steel wire was used. It was noted that the impedance increases dramatically with the wire length, therefore in favour of the desired values approximately 4-6 cm long wires were used. Impedances were measured around 14.5 1/Ω. Steel wires were made of two parts for the third experiment (attached by a silver pin, similar to T50 wires) and of one part for the experiment of Rat#4.

Tungsten and steel wires provided better signal to noise ratio and sensitivity than the copper wires. Tungsten wires are rigid; therefore, their use is more complicated than the steel wires. However, both electrodes were fixed well into the muscle and provided stable and clear signal throughout the experiments for both Rat#3 and Rat#4. Tungsten wire provided a slightly more sensitive but also noisier signal than the steel wire during the experiments with rat#3 (see Figure 15). During that experiment, the wires were installed in two pieces. For rat#4, both Tungsten and steel wires were installed in one piece and the resulting signals provided consistently good EMG recordings, with good sensitivity and high S/N ratio (see Figure 16). It was observed that the signal from the steel wire corresponded more accurately to the movements of the animal that were observed by eye during the recordings. Conclusively, steel wires of one part proved to be the best for acquiring EMG signal. However, it is important to mention, that the incorporation of the electrodes of one part turned out to be more complicated and required considerably more time during the surgery. For both experiments (rat#3 and rat#4) signal from the steel wire was used for the analysis and sleep scoring.

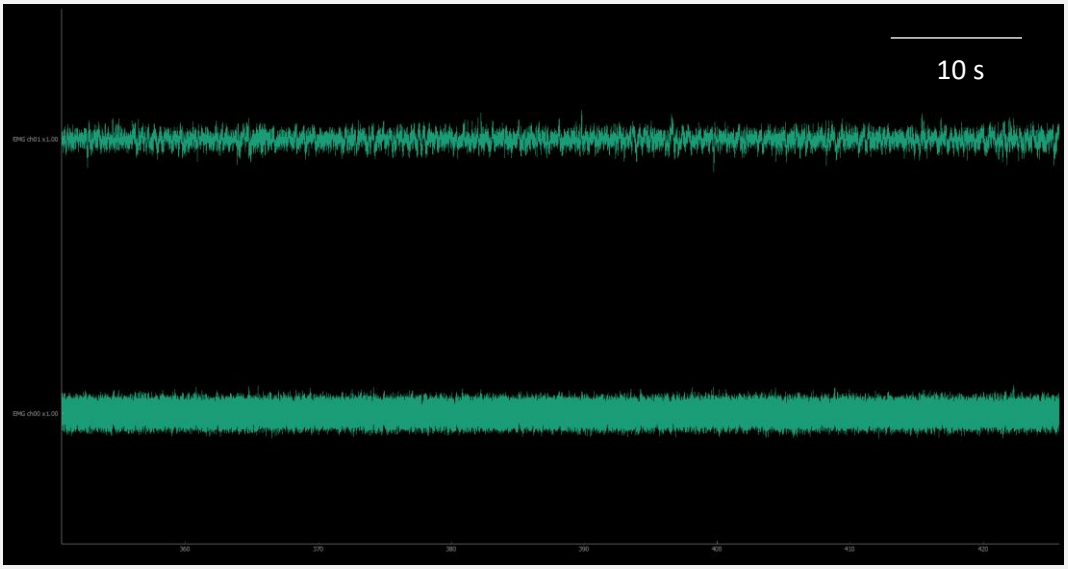

Figure 15 EMG signal of Rat#3. *The EMG signal was acquired by a Tungsten wire (see below) and a steel wire (see above). Both wires were installed in two pieces and were soldered together during the surgery. Both channels picked up considerably less noise. The Tungsten provided a more sensitive signal, however this included in somewhat higher noise levels. The steel wire was less noisy but also less sensitive. For that reason, the signal acquired by the steel wire was used for the analysis.*

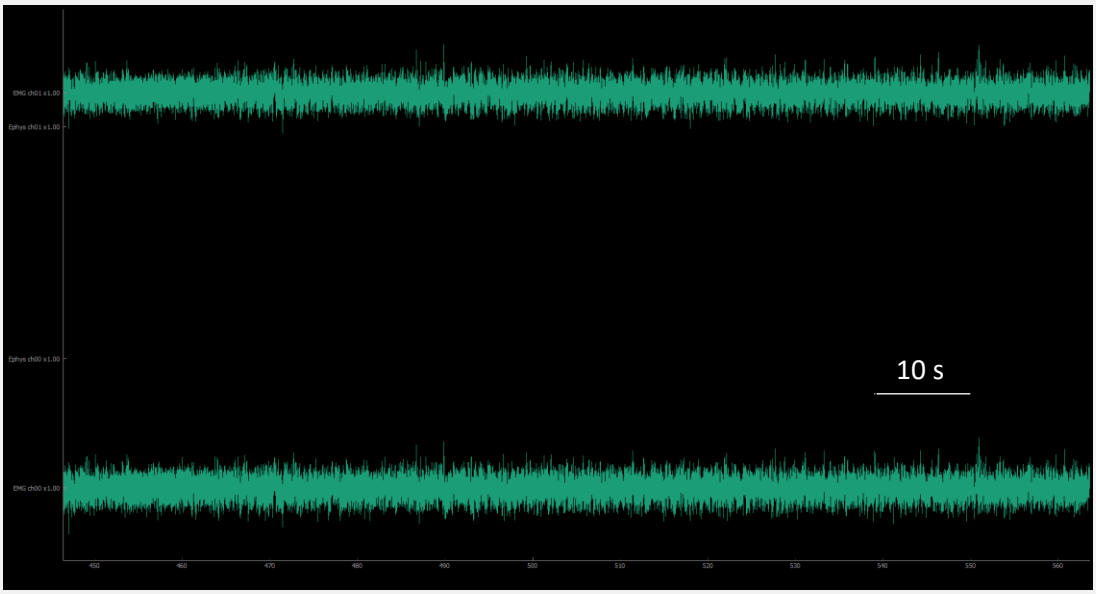

Figure 16 EMG recordings of Rat#4. *The trace below corresponds to the signal from the Tungsten wire and the trace above to the steel wire. These wires were applied as one piece, which resulted in consistent and stable signal. The two signals were similar in their signal to noise ratio and sensitivity.*

3.1.2. Assembling of micro-mechanics

The recording device was built from a 16-channel custom Microdrive (Axona Ltd., Herts, UK; Ax-17; see Figure 17). The device has four rows of pins labelled with different colours, each row consisting of four pins. The base also includes a separate ground wire that is labelled black. The pins join in the connector, where the signal is transmitted. As the preparation of the recording device for the wiring, first, the pins of the microdrive were uninsulated partially, as well as the ground wire. Then, the inner and outer canula for holding the tetrode electrodes were fixed with dental cement in the middle of the drive.

Figure 17 Base of microdrive recording device from Axona Ltd. *The base is a re-usable drive with 16 channels with an additional fixed ground wire, attached to a steel frame and completed with a screw that makes it possible to move all tetrodes simultaneously. See: http://www.axona.com/products/microdrives.*

Such drives have been successfully used for tetrode recordings since over 20 years (Hollup et al., 2001). Our aim was to refine sleep scoring techniques on such a robust device before moving on to a smaller custom-device based on omnetics.

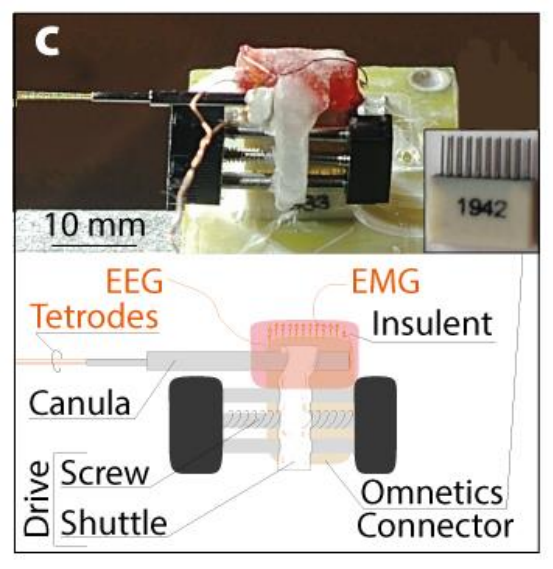

Figure 18 The omnetics drive. *The omnetics drive would provide the same features as the bigger Ax-17 drives but in a smaller, more compact body. Therefore, it would allow recording from younger animals (even pups).*

3.1.3. Wiring and insulation

Two EEG and two EMG electrodes were wired to the first row of pins (labelled with red colour) on the microdrive (starting from the bottom to the top, respectively). Each electrode was wired around one of the pins, so that the de-insulated part was twisted tightly around the pin, providing strong signal transduction. The other end with the solder bubble (and later without) was left freely until the implantation (see **Figure 19** and **Figure 21**).

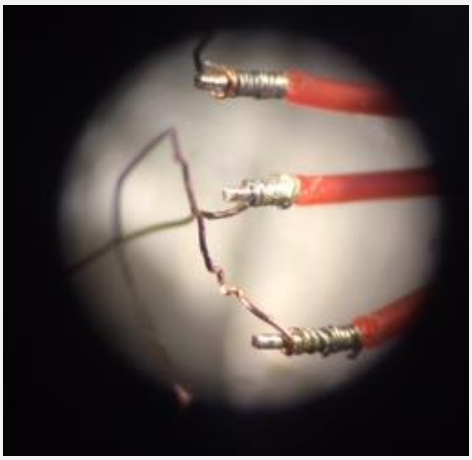

Figure 19 The EEG and EMG electrodes are wired on the Ax-17 drive pins. *The figure shows how the de-insulated ends of the EEG and EMG wires are wrapped around the pins of the drive. This tight connection provides good signal transduction.*

The other three row of pins were wired by the tetrode electrodes, each row belonging to one tetrode. The tetrodes were installed and wired one at a time. First, the branches of the tetrode were uninsulated using flames. Then the tetrode was slid into the canula, and each branch was wired – twisted tightly – around one pin of appropriate same row/colour.

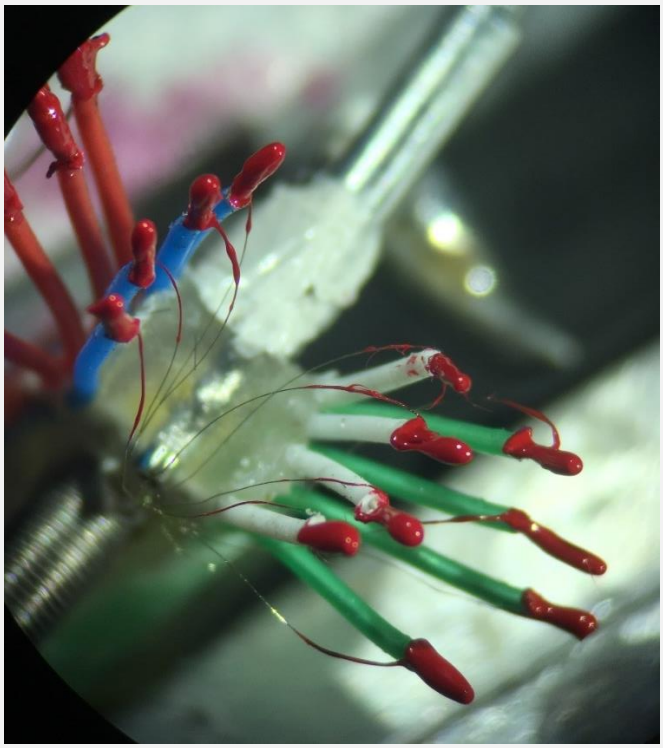

Figure 20 The pins of the device are wired and covered with insulation. *The figure indicates the stage, when all the pins of the device are wired with either the EEG and EMG wires (red pins) or the tetrodes (blue, white, and green pins). The pins are also covered with a layer of silver paint and then a layer of nail polish – visible on the picture.*

After the installation of the EEG and EMG wires and all three tetrodes, the contact points (of pins and electrodes) were covered with silver paint in order to increase the surface of connection and strengthen the signal transduction. After drying, the silver layer was covered with nail polish for insulation as well as the “naked” parts of the tetrodes (see Figure 20). Further, thin, protective nail polish layers were applied until the pins and the formed a cohesive strong and solid nail polish bubble. This structure was acting as insulation and protection of the electronics from the environment, including the impact of the animal and its movements.

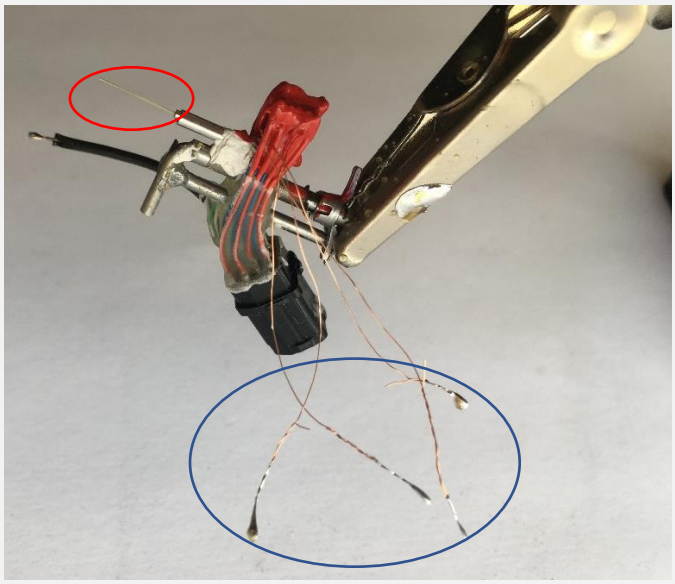

Figure 21 Picture of the prepared drive, ready for implantation. *The prepared drive is wired with the EEG and EMG electrodes, with the solder bubbles attached to their ends (indicated by the blue circle). The drive is also installed with the tetrodes, which will be inserted inside the brain (see the red circle). The top of the structure is covered with nail polish to protect the tetrodes and secure their connections.*

3.1.4. Maximization of signal conductance

To further increase the conductance of the signal and maximize signal to noise ratio, two techniques were applied.

First, for increasing the surface of contact, the three tetrode ends were cut with special wire scissors horizontally, so that their surfaces had become flushed and flat. This was done under microscope and the best results were identified based on the intensity of light reflection from the endings. The length of the tetrodes from outside of the canula was aimed to become around 6 mm (approximately the size of the outer canula plus the depth of the implantation).

After cutting, the conductance of the tetrodes was increased further with electroplating. Electroplating is a method used for increasing the conductive surface by covering the surface with a metal. In this case, the electrode ends were covered with platinum. The NanoZ™ Software was used for the plating procedure, where the plating current was set to -0,875 µA and target impedance was set to 350 kΩ in order to attain the ideal values of 180-220 kΩ in saline. The explanation for this is that the Platinum solution has higher conductance than the brain and the saline solution.

3.2. Refinement of surgical and recording procedures to analyse sleep in adolescent and adult animals

All experiments were approved by the National Animal Research Authority of Norway (FOT SkjemaID 27229 and 11325). Four Long Evans rats (*Rattus Norvegicus*) were used for the experiments conducted in this study. The subject rats for the four experiments were 1 month old (rat#1), 3 months old (rat#2), 6 months old (rat#3) and one year old (rat#4).

3.2.1. Animal handling and refining the environments

The animals used for the experiments were pre-handled before the performance of any procedures in order to minimize the stress. This included getting them used to handling, introducing them to treats, introducing them to the arena used as the environment for the exploration periods (during wake) as well as introducing them to the sleeping environments. It was observed that longer periods of habituation to any environment leads to less stress of the animal and more efficient data acquisition, therefore the animals were habituated to the sleeping environment for a week before starting the experiments.

Moreover, different sleep environments were tested during the experiments of the first two rats (3 months old and one month old; rat#1 and rat#2, respectively). In the case of the first animal (Rat#1) no additional environment was introduced and sleeping occurred in the same environment as the active exploration (after thorough cleaning, to get rid of the scent as distractions). This paradigm did not allow us to train the animal to certain activities – sleep or wake – based on the environment, therefore it was considerably time consuming to wait for the animal to fall asleep. Not to mention, that it might have caused differences in the quality of the sleep for the animal. During subsequent experiments a square shaped plastic architecture piece was introduced, that allowed the division of the running arena to smaller areas – small enough for the animal to get bored, but big enough to feel comfortable in it. The area was also covered with cloths for more comfort, because rats like to hide or cover themselves when sleeping. This improvement allowed us to habituate the animals to perform different “activities” in the different environments. Experiments of Rat#2, Rat #3 and Rat#4 were conducted with these settings. In order to provide an even more successful sleep environment for the animals many more small details were taken into consideration, e.g., the silent keyboards, chairs, decluttering the environment.

Even after extensive habituation, animals were getting better and better at falling asleep when placed in the sleeping environment – the latency to sleep improved markedly. Therefore, the

uniformization of the habituation periods and the sleep environment are important for future experiments.

3.2.2. Surgical implantation

Surgical implantation happened in accordance with the rules and regulations. The surgery was performed by my supervisor, Dr. Charlotte Boccara who I was assisting. I was also involved with the refinement of the surgical procedures.

Animal were kept anaesthetised during the whole procedures using varying concentrations of isoflurane (0.8-3%) in oxygen kept at a flow of 1–1,3 L/min). The anaesthesia was induced in a compressed air chamber (isoflurane concentration at 4–5%). When the animal showed signs of slowing of breathing, it was transferred to a stereotaxic mask with lower (0.8-3%) isoflurane concentration and 1–1,3 L/min oxygen flow. Isoflurane concentrations were adjusted depending on depth of anaesthesia (animal breathing) and painfulness of the procedure performed at the time by the surgeon. The animal was placed on a heating pad, its head was fixed with ear bars in a stereotaxic apparatus, eye cream was applied to avoid dehydration of the eyes and the area of interest was shaved. Local anaesthetics were applied (lidocaine, xylocaine). Anaesthesia was monitored regularly by inspection of the blood oxygen saturation, as well as the heart and respiration rate. Several analgesics and pain killers were used (Zolazepam, Tiletamine, Xylazine) and the animal was hydrated with saline every 1-2 hours.

First, the skin on top of the skull and the neck muscle was incised with a scalpel. The skull was thoroughly cleaned, and groves were marked in the bone to help with later adherence of the cement. Second, the coordinates of the right hippocampus were marked on the skull for intracerebral implantation (AP: 3.5 to 5 mm; ML: -3 to -4 mm; DV: 0.8 to 1.3 mm). The corresponding coordinates of the hippocampus CA1 in rats of a given age were refined with the progression of the experiments. Consequently, the hippocampus was not reached during the first two experiments (rat_01 and rat_02) and intracerebral data was extracted from surrounding brain areas.

Third, 8 holes were drilled and 7 screws were fixed in the skull. Two of the screws were the previously prepared “head-parts” of the EEG wires, inserted to aquire the ECoG signal, among which one was placed above the left prefrontal area and one above the left hippocampal area in order to get a more general and a more area-specific ECoG signal. One ground screw wasfixed above the cerebellum and later fixed to the 17th channel of the AX17 device to filter out noise. The three remaining screws were fixed for stabilizing and anchoring the implant to the skull (see Figure 22).

The eighth hole was drilled at calculated coordinates for the hippocampus and was assigned for the intracerebral tetrodes. One the hole drilled, the deviced was placed above, the dura was removed

and the tetrodes were carefully lowered . The tetrodes were implented approximately 1 mm deep below the cortical surface. Once the tetrode had reached their position, the outer canula was lowered, protecting the tetrodes from any physical impact, mostly to avoid them to be stucked with cement so that they can move later.

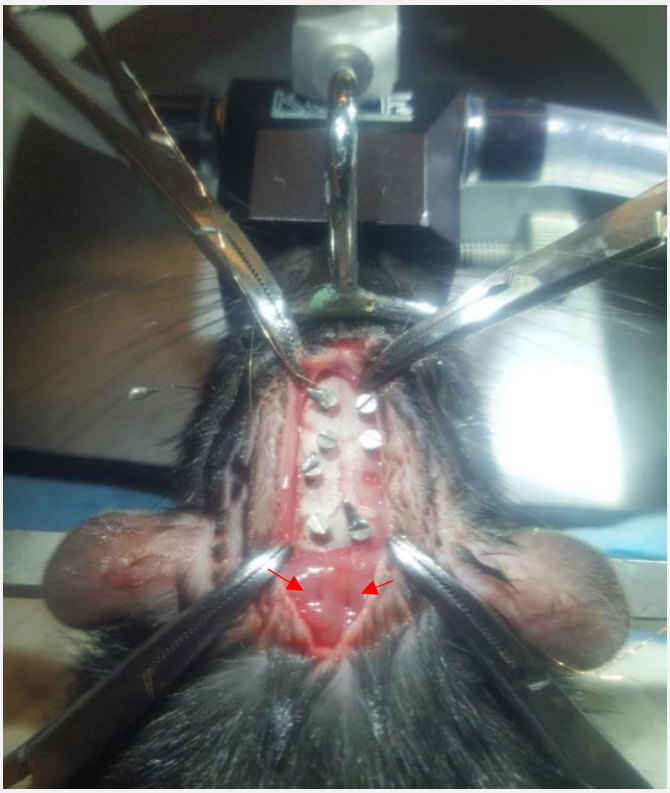

Figure 22 Surgical implantation of the recording device*. the picture shows the skull of the animal after installing the screws. The whole on the right corresponds to the opening above the hippocampus, where the tetrodes were later inserted. The arrows indicate the cite of insertion of the EMG electrodes that can be seen as shadows on the picture.*

The "head part" of the EMG wires were inserted into the neck muscles of the animal. The EMG wire was hooked into a needle, which was then inserted into the muscle. After that the needle could be taken out without the wire itself. For the first two experiments (rat#1 and rat#2) the EMG wires were stabilized with vet bond, which did not result in the desired stability. For that reason, during later experiments (rat#3 and rat#4), a knot was formed from the wires after inserting into the muscle.

Eventually, all four wires (two ECoG and two EMG) were connected ("drive-parts" to "head-parts") with soldering. The structure was stabilised and covered with cement.

3.2.3. Data acquisition

Animal and behavioural paradigm

Recording of the data happened in a grounded environment inside the KPM Animal Laboratory Facility. The electromagnetic noise was very high in our recording room. We therefore had to shield all electric outlets and use a pseudo faraday cage as a recording environment. This the reason, why the sleep session had to take place in that shielded environment. The software used for the recording was dacqUSB (axona). Animals were tested in a round or squared shaped arena during freely behaving states and the environment was supplemented with additional borders and clothes to provide a smaller and more comfortable (and more boring) environment for the sleep sessions (see Chapter 3.2.1.).

Four types of data were recorded during the experiments: ECoG (electrocorticogram) signal, EMG (electromyogram) signal, as well as LFP (local field potential) and multiunit signal from the intracerebral tetrodes. In addition to that, video signal and tracking data were also acquired by a camera fixed on the roof, above the recording environment and tuned to recognize the two infrared LEDs fixed on the animal head stage.

The first paradigm included the recording of freely behaving rats, when they were exploring the open field (the arena) while motivated by biscuit crumbs. The rats were placed in either a circle shaped or a square shaped environment. Sleep was recorded in the same environment for the first rat (rat#2). A new sleeping environment was introduced for the rest of the animals which was a smaller square shaped architecture, providing borders, and therefore creating a small environment for sleep. The environment was also lined with clothes for the comfort of the animal.

As already mentioned in Chapter 3.2.1, assigning a specific environment for sleep proved to be effective in getting better sleep recordings. The animals (especially rat#3 and rat#4, which were extensively habituated to this environment) learned to fall asleep in shorter time and were less disturbed during the recording sessions.

Electrophysiological signal acquisition

Minimal filtering and pre-processing were applied already during data acquisition. Electrophysiologic signal was amplified by a pre-amplifier and filtered with a bandpass filter of 0.5 Hz to 7000 Hz, to filter out the noise. Video signal was also pre-processed by adjusting the parameters of the two LED lights: contrast and brightness was set to obtain clear signal and to track the animal movements precisely.

Acquisition rate was either 24 or 48 kHz, which then was down sampled to an integrated 24 kHz rate. Sleep was recorded in "raw" system setup, where no additional filters were applied on the signal.

Multiunit recordings of extracellular single cells were obtained via the intracerebral tetrodes while the animal was freely exploring. In order to access where the intracerebral electrodes are a putative estimation was based on the LFP, the high theta power, sharp wave ripple power and the presence of pyramidal cells. To be able to visualize and isolate the activity of individual neurons a band pass filter of 300 Hz to 7000 Hz were used as well as referential mode, where activity from one electrode channel was subtracted from the other, to see only individual spikes coming from isolated neurons. Multiunit recordings were obtained from the last two experiments (rat#3 and rat#4).

3.2.4. Data pre-processing:

Data was pre-processed to fit the format necessary to proceed to the analyses. Each recording session was converted to nbw format. After acquisition, the data were converted back to units of Volts and then stored as NWB files. Neurodata Without Borders (NWB) is a standard for storing neurophysiology data and metadata aimed at providing a common framework for sharing and analyses. Data is stored in HDF5 format, together with all relevant metadata, in a self-explanatory and easily accessible fashion. Data was downsampled to 24 Hz and pre-processing also included the smoothing and interpolation of the animal path obtained from the tracking. Speed was extracted and computed from the animal path.

For one of the rats (rat#1) we observed the presence of a significant quantity of artifacts, possibly due to an implant grounding problem, and resulting in the frequent saturation of the signal. To address the issue, we identified the intervals where the signal was saturating and we filled them with zeroes and lowpass filtered the signal at 250Hz (forward and backward filtering with a 4^{th} order Butterworth filter), proceeding then to carry on the rest of the analyses as for the other rats.

3.3. Development of new computational tools to analyse sleep architecture across lifespan

3.3.1. Sleep scoring:

The analysis program for sleep scoring was developed by my colleague Eis Annavini, and further refined and improved by us during this study. The program was written in Python programming language and allowed the comprehensive analytics of the data acquired during the sleep sessions. The program was refined to measure the quantity of all sleep as well as the different sleep stages. Moreover, it allowed

us to detect and measure the different oscillations during these sleep stages and also during whole sessions.

We computed EMG power by computing the signal root mean square (RMS) of the EMG signal over 5 s overlapping windows (4s overlap). Then the theta and delta power were computed by filtering the ECoG signal, from the steel screw implanted above the hippocampus, in the appropriate bands (theta: 4 -12 and delta: 0.5-4.0) using a 4th order Butterworth filter. From that we computed the normalized theta/delta ratio, defined as $\frac{\theta}{\theta+\delta}$.

We estimated the overall vigilance state (WAKE vs. SLEEP) via speed thresholding; to consider twitches and small movements commonly found during sleep, epochs where the animal was moving faster than 2cm/s were scored as WAKE, while the rest was scored as SLEEP.

Micro awakenings were scored by thresholding the z-scored RMS power of the EMG signal, recorded by the EMG electrode implanted in the neck muscle during surgery.

Remaining sleep was further scored using both EMG and normalized theta/delta ratio; we defined REM as having a normalized theta to delta ratio higher than 0.5 (Bojarskaite et al., 2020) during muscle atonia, SWS as having a normalized theta to delta ratio lower than 0.5 with low muscle tone. IS was defined with either high theta/delta ratio coupled with medium muscle activity or low theta/delta ratio with atonia. The theta/delta ratio was set to 0.5 based on thorough examination of previous studies (Bojarskaite et al., 2020) and seemed in alignment with our needs. Two EMG RMS power thresholds were established: a high threshold set to a value of 1.3, above which value, the epochs were scored as awake; and a low threshold set to -0.8, above which the animal was either in NREM sleep (paired with low theta/delta ratio) or IS (high theta/delta ratio) and below this threshold the epochs were considered either REM sleep (with high theta/delta ratio) or IS (low theta/delta ratio).

Finally, all bouts shorter than 10 seconds (De Vivo et al., 2016) were scored the same as the preceding state, and any wake at the beginning of the sleep session was considered an artifact from the process of the animal falling asleep and was consequently dropped.

Recorded data was visualized, and manual scoring was performed by the Graphical User Interface (GUI) of the program (see Figure 23 and Figure 24). The GUI allowed to visualize the traces of EMG, ECoG and LFP signals either together or individually while the time scale could be adjusted freely. The GUI also provided a hypnogram window (Figure 24), where the EMG RMS power, theta/delta ratio, speed along with thresholds could be visualized and the corresponding sleep stages were indicated.

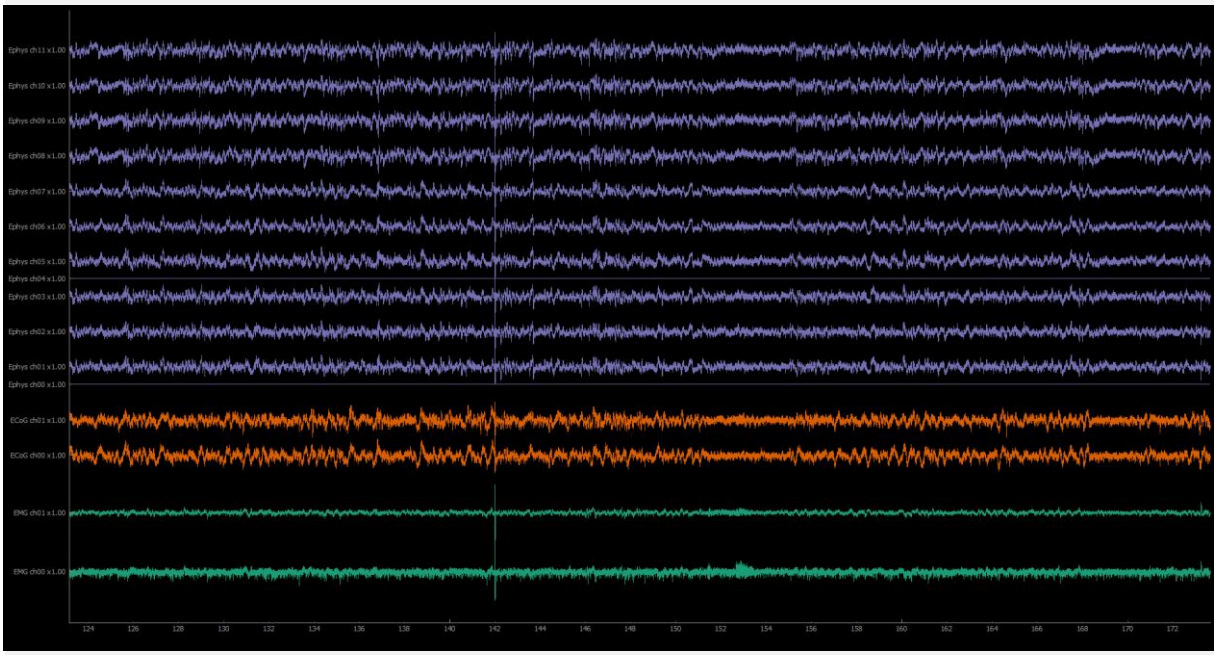

Figure 23 Example of the traces from the recordings represented with the graphical user interface (GUI) of the analysis program. *The x axis represents the time scale, and on y axis the labels for the channels are represented. EMG signals are indicated with green colour, ECoG with orange colour and the purple traces are the LFP signal from the intracerebral electrodes.*

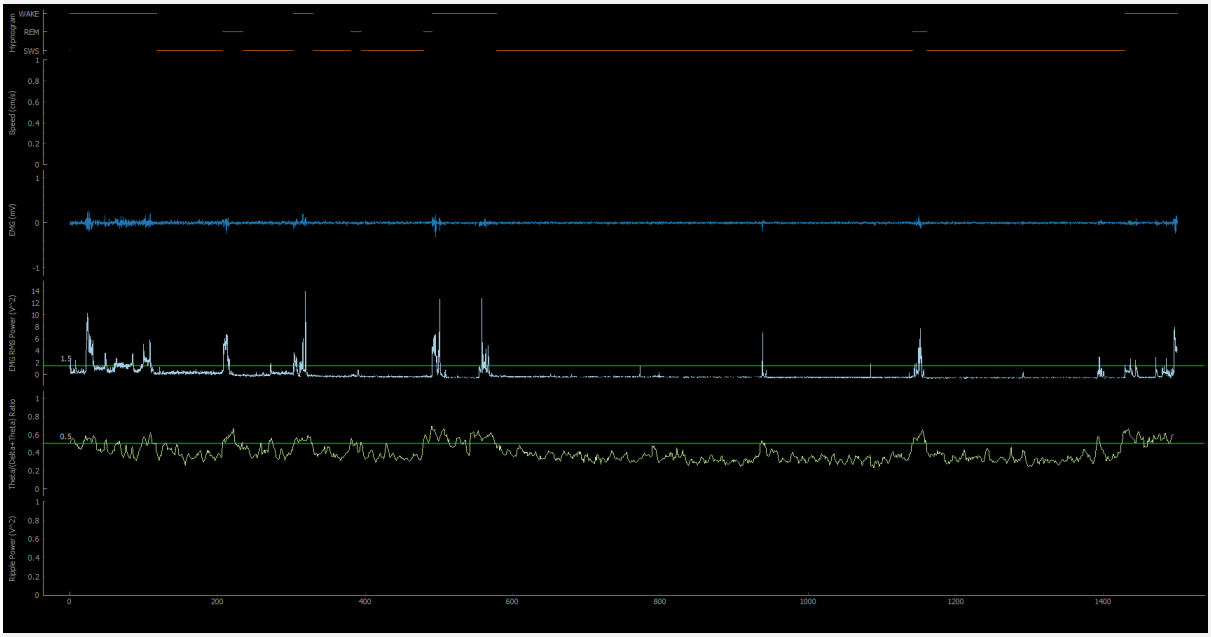

Figure 24 Hypnogram visualized by the GUI. *The trace at the bottom (yellow) is the theta/delta ratio across the session along with the threshold (indicated with a green horizontal line and set to 0.5). The white trace is the EMG RMS power calculated from the EMG (blue trace). The high threshold for the EMG RMS power is also visualized (green horizontal line) and set to 1.5 for this example. The figure on the top visualizes the corresponding sleep stages: NREM is represented with orange, wake with purple and REM with green colour.*

The appropriate channels needed to be chosen for the automatic sleep analysis. One ECoG channel and one EMG channel was used for the sleep scoring and for the analysis of the recorded data. Choosing the appropriate EMG channel happened as discussed before in 3.1.1 and also by thorough examination of the corresponding traces and hypnograms in the GUI. The ECoG channels were chosen similarly (see Figure 25). Traces were observed in the GUI and the channel with the highest theta oscillation (characteristic of the ECoG above the hippocampus) was chosen; the corresponding hypnogram was loaded and evaluated to check the if the generated results are appropriate. The chosen channels were loaded into and used by the program for automatic scoring.

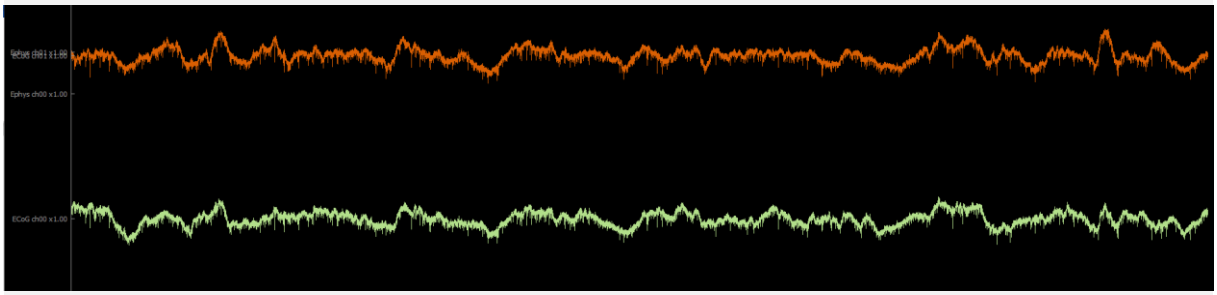

Figure 25 Example of ECoG traces. *The orange trace corresponds to the signal coming from the electrode above the hippocampus, and the yellow trace shows the ECoG signal acquired from the prefrontal area.*

The automatic analysis of the data required extensive refinement. This included setting the variables such as thresholds for the theta/delta ratio, the speed and the EMG RMS power. In order to do so, initial values of the variables were first adopted from previous studies (Bojarskaite et al., 2020), then these were adjusted to our experimental conditions via the manual scoring of the signal traces. Manual scoring included thorough examination of the behaviour of the animal observed during the recording sessions. Sleep was “scored” based on the quiescent state of the animal, the lack of movements and stereotypical posture. These observations were considered and when examining the signal traces in the GUI and setting thresholds manually - eventually, sleep stages were concluded in a manual manner and corresponding thresholds were kept and loaded for the automatic analysis. The results of the automatic sleep scoring were then checked with manual evaluations and variables were adjusted further if needed.

Finally, when all variables were set all sleep sessions were analysed automatically with the program.

4. Preliminary results on the evolution of sleep architecture across lifespan

Preliminary results were acquired during the refinement of the experiments with data from rats at different life stages. These results offer indication of further needs in refinement and might also provide insight to the changes in the sleep architecture and oscillations of rodents across the lifespan. When examining these results, one needs to be critical, and comparisons must be done with careful consideration that the methodology continuously evolved during their acquisition resulting in variable conditions. However, I still estimate that they can be a good tool to indicate which parameters future studies conducted in the BoccaraLab should prioritise to focus on.

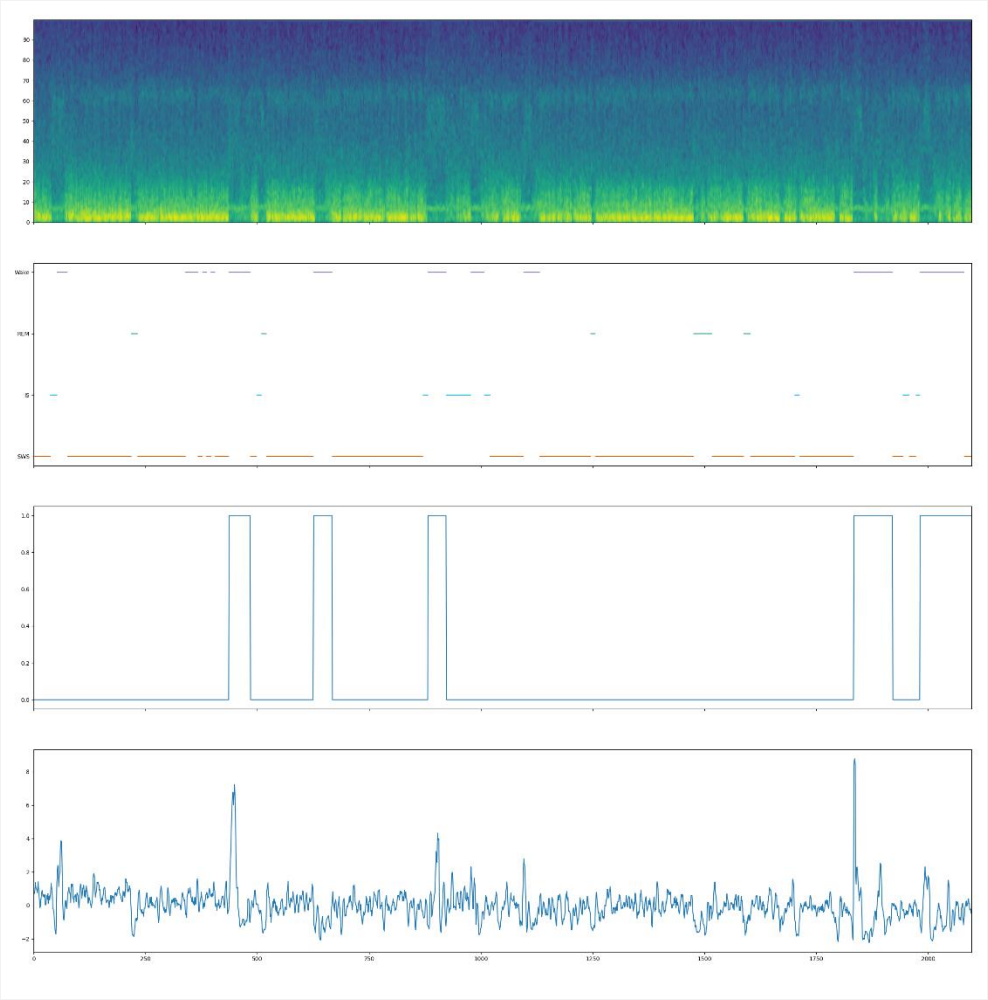

Figure 26 Example of a hypnogram acquired during the analysis. *Charts are as follows: a power spectrum showing the density of oscillation frequencies; corresponding sleep stages (NREM indicated with orange, IS as blue, REM as green and wake as purple); graph showing the occurrence of movements corresponding to wakefulness; theta/delta ratio.*

4.1. Sleep architecture organisation across lifespan

Based on previous research – extensively discussed in the Introduction (see Chapter 0) – it was observed that sleep varies widely across life stages in humans. This points to the different needs of sleep during development, adolescence, adult life, and aging. We attempted to find such changes in the sleep architecture with measuring sleep in four life stages spread across the lifespan (1, 3, 6 and 12 months old).

Sleep recordings were analysed based on one and then the other ECoG channel and then also based on the LFP measured by intracerebral tetrodes. Analysis with ECoG1 (hippocampal) and ECoG2 (prefrontal) showed very similar results in terms of all categories. I chose to present the results based on ECoG1 in this chapter and compare it with results acquired by the LFP signal, if significant.

It is important to mention here, that data for the 1-month-old rat (rat#1) was processed differently from the rest of the data, because of the amount of saturation and artifacts observed during the recording sessions. Consequently, many data points have been lost while cleaning and this probably influenced the results. Thus, while presenting the results, I will often disregard this data point when providing interpretations and observing trends.

4.1.1. Sleep amount across life stages

First, we looked at the amount of time spent asleep during each session for the 1-month-old, 3 months old, 6 months old and 12 months old animal. Sleep percentages correspond to the average percentage of time spent asleep in each session for each animal. Results acquired from the ECoG1 channel (see **Figure 27 Changes in the average percentage of sleep/session across rats in different life stages based on the ECoG**. The blue columns represent the percentages, and the error bars show the SEM values. Sleep percentages declined with) were comparable with results from the LFP (see **Figure 28**).

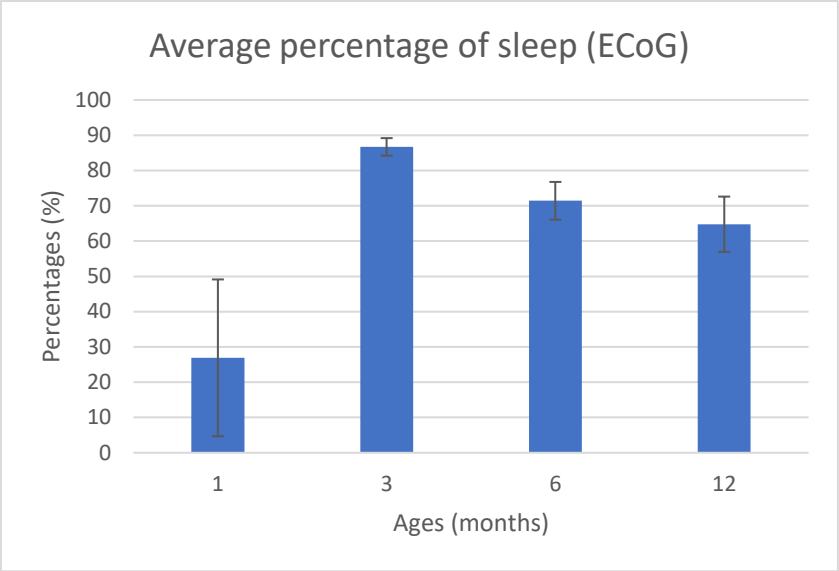

Figure 27 Changes in the average percentage of sleep/session across rats in different life stages based on the ECoG*. The blue columns represent the percentages, and the error bars show the SEM values. Sleep percentages declined with age. T-test showed significance (p>0.05) in sleep amount changes between 3 months and 6 months.*

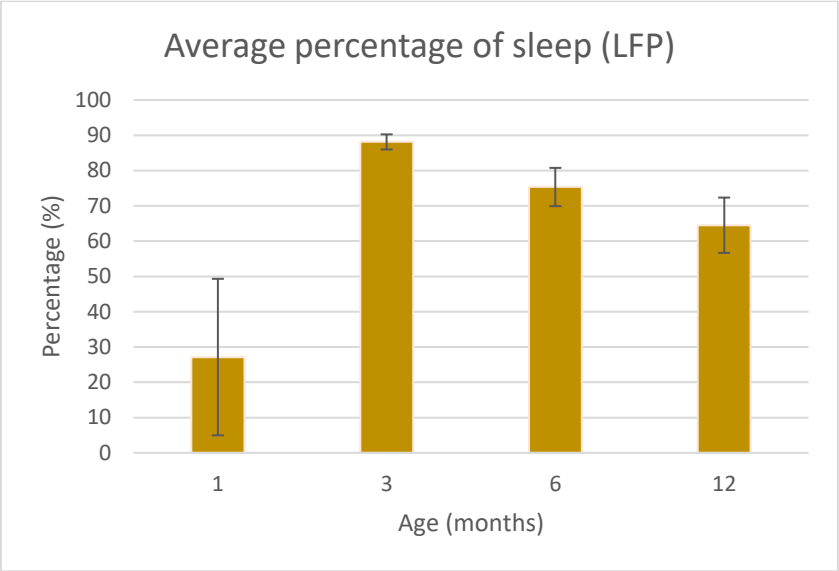

Figure 28 Changes in the average percentage of sleep/session across rats in different life stages based on the LFP*. The blue columns represent the percentages, and the error bars show the SEM values. Sleep percentages declined with age. T-test showed no significance of the changes between any age groups (p-values were above 0.05).*

After excluding the data from the 1-month-old rat, a trend in sleep amounts was observed: The amount of time spent asleep declined steadily with the age of the animals. These results are in accordance with sleep changes observed in human studies.

4.1.2. The percentage of different sleep stages (REM, NREM, IS)

After looking at the overall sleep amount, changes in the amount of the different sleep stages were examined. Results acquired from ECoG1 (see **Figure 29**) looked very much in alignment with results acquired from the LFP (see **Figure 30**).

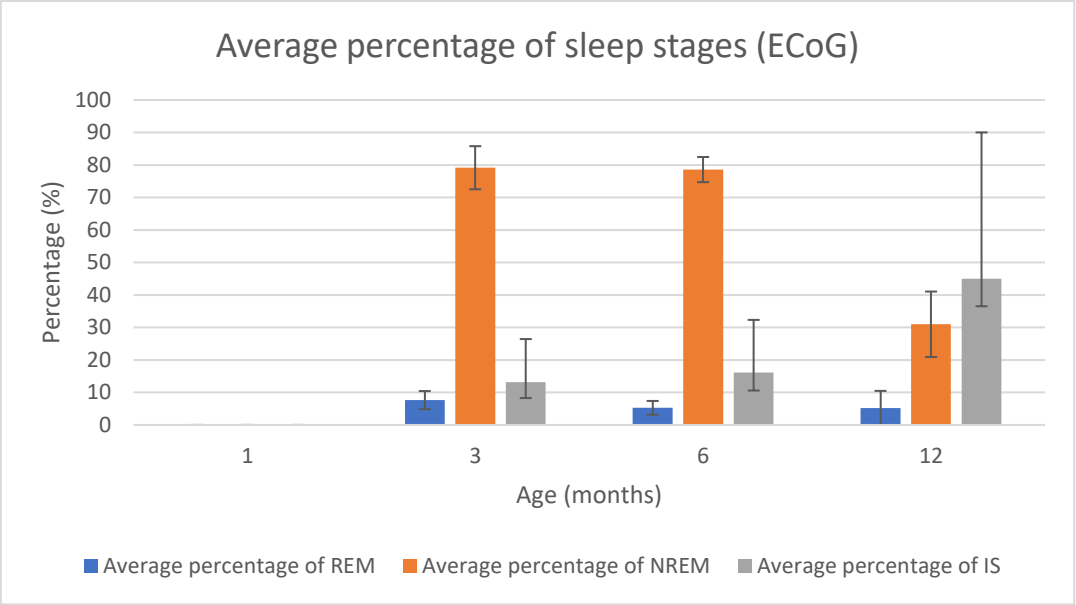

Figure 29 The three sleep stages and their changes across rats in different life stages based on the ECoG. *NREM sleep (indicated by orange) reduces markedly by 12 months, REM (blue) reduces slightly. The amount of IS (grey) increases. T-test showed significance ($p < 0.05$) for NREM and IS between 3 months and 12 months, and in IS between 6 months and 12 months.*

NREM levels were identified as the most prominent sleep stage for all ages and fell markedly between 6 and 12 months. Very little REM stages were identified across animals -a slight reduction in its amount can be observed. IS (intermediate sleep) stages increased with the with age – this change could be an indication for the disintegration of sleep stages, as they become less and less characteristic with age and are getting more difficult to recognise.

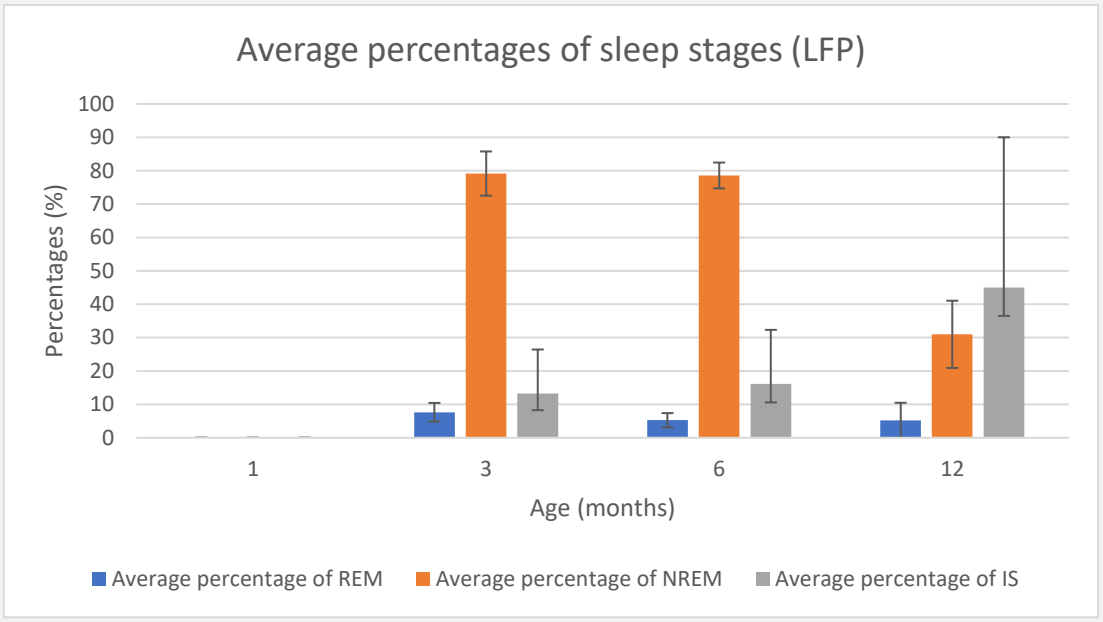

Figure 30 The three sleep stages and their changes across rats in different life stages based on the LFP. *NREM sleep (indicated by orange) reduces markedly by 12 months, REM (blue) reduces slightly. The amount of IS (grey) increases. T-test showed significance ($p < 0.05$) for NREM and IS between 3 months and 12 months, and in IS between 6 months and 12 months.*

4.1.3. Time passed before the first REM bout

The time passed before the first REM bout was measured across life stages. The ECoG (see Figure 31) and the LFP channels provided the same results.

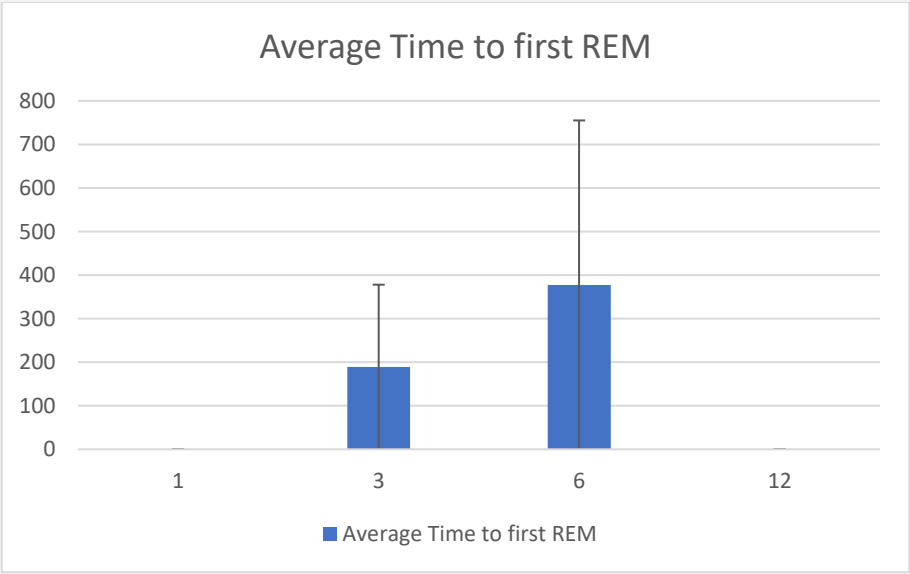

Figure 31 The average time passed before the first REM bout based on ECoG. According to the t-test, changes were not significant between the ages.

Latency to first REM increased slightly between 3 and 6 months, but this increase could not be observed later between 6 months and 12 months.

4.1.4. Average sleep bout duration and frequency

The duration of sleep bouts was measured and averaged between the sessions. Results acquired by the ECoG1 channel are shown in Figure 32. A decreasing trend in the sleep bout duration was observed and the analysis based on the LFP showed a similar trend. These results fit well with the observations from previous studies.

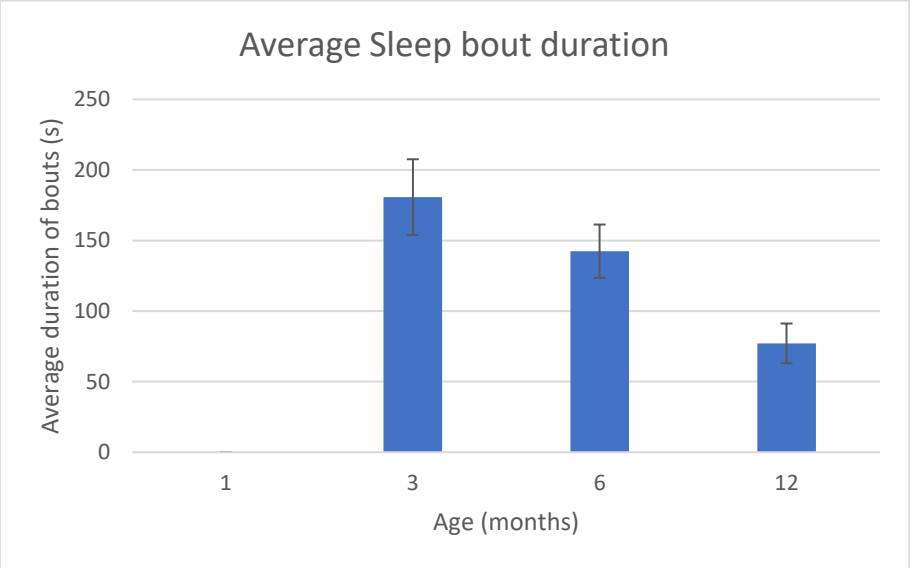

Figure 32 Average sleep bout durations based on the ECoG. *Length of sleep bouts decreased with age. According to the t-test, these changes were not significant.*

The average frequency of sleep bouts (bouts/minute) was also analysed across life stages (see Figure 33). Results showed a trend with a steady increase with age, similar observations were made from the results from the LFP. The increase in sleep bout frequency interpreted with the decrease observed in the sleep bout durations could represent the fragmentation in the sleep bouts occurring during aging (as previously observed in humans).

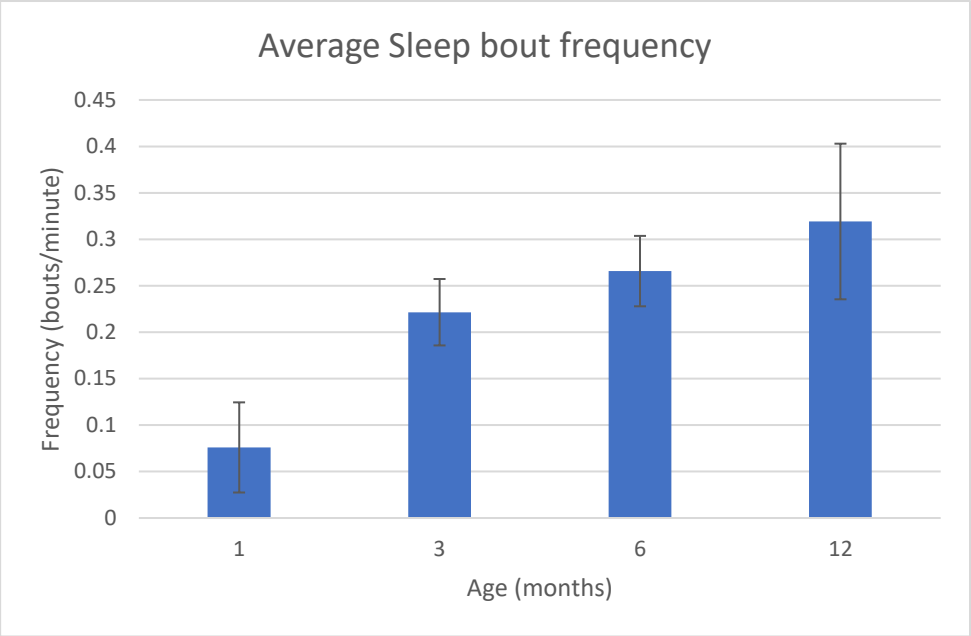

Figure 33 Average sleep bout frequencies based on the ECoG. *Sleep bout frequency increased markedly with age. Changes were not considered significant based on the t-test.*

4.1.5. Average wake bout duration and wake bout frequency

Average wake bout duration (see **Figure 34**) and frequency **Figure 35** were measured similarly to the sleep bouts.

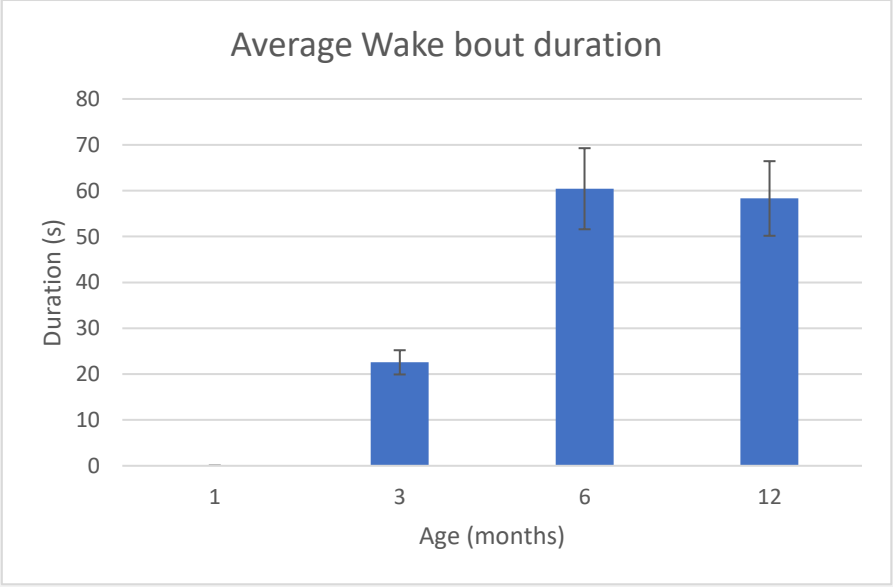

Figure 34 Average wake bout duration based on ECoG*. Wake bout duration increased between 3 and 6 months old. These changes were not significant according to the T-test.*

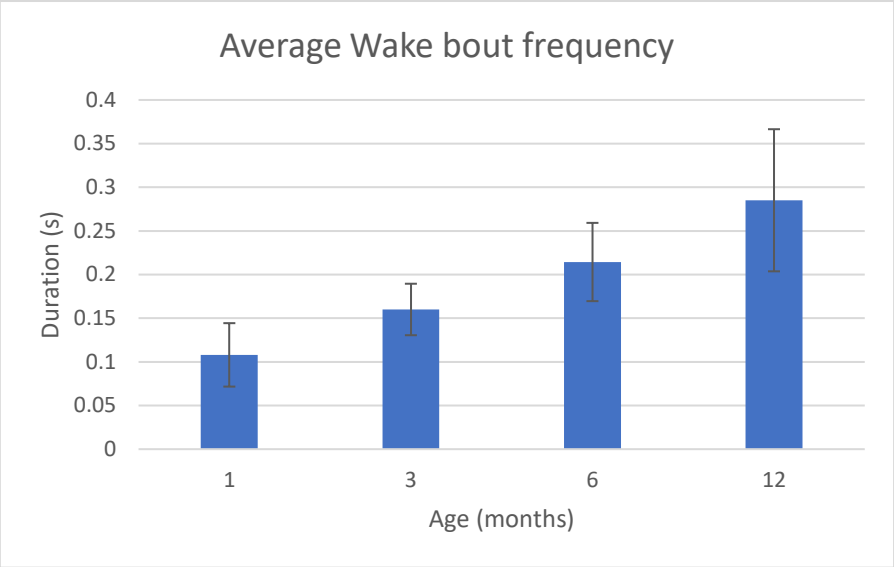

Figure 35 Average wake bout frequencies based on the ECoG. *Frequency of wake bouts increased with age. These changes were not considered significant based on the t-test.*

Wake bout duration increased markedly between the 3 months old and 6 months old animal. Meanwhile, wake bout frequencies increased steadily throughout all life stages. These results are in accordance with the interpretation made in the previous chapter (see Chapter 4.1.4) regarding the fragmentation of sleep with aging.

4.2. Oscillations across life stages

Some of the significant oscillations were measured and their power was computed within their characteristic sleep stages. Delta power was measured during slow wave sleep, while theta and gamma power were observed during REM sleep.

4.2.1. Delta power in SWS

Delta power is an important measure when detecting NREM/slow wave sleep, therefore the power was measured specifically during the NREM/SWS sleep stages. The ECoG1 channel and the LFP provided very similar results.

According to the results delta power showed a decreasing tendency with age.

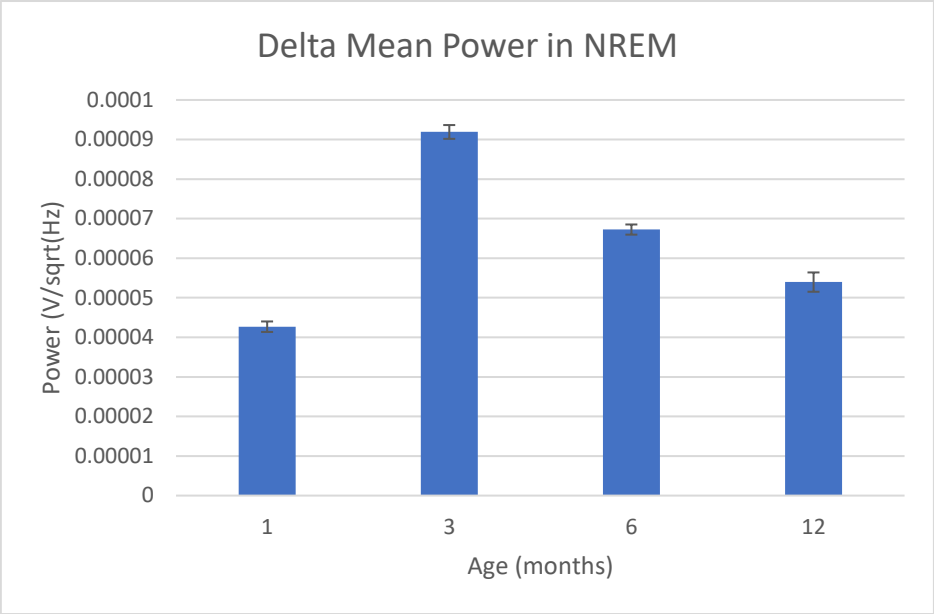

Figure 36 Delta mean power in NREM/slow-wave sleep based on the ECoG. *Delta power decreased from 3 months of age. Changes between 3 and 6 months and changes between 3 and 12 months were considered significant with a p-value below 0.05*

4.2.2. Theta power in REM

Theta rhythms are the characteristic oscillations of REM sleep; therefore their prominence was measured during this specific stage. Theta power showed a similar, decreasing trend both based on the ECoG and the LFP signal.

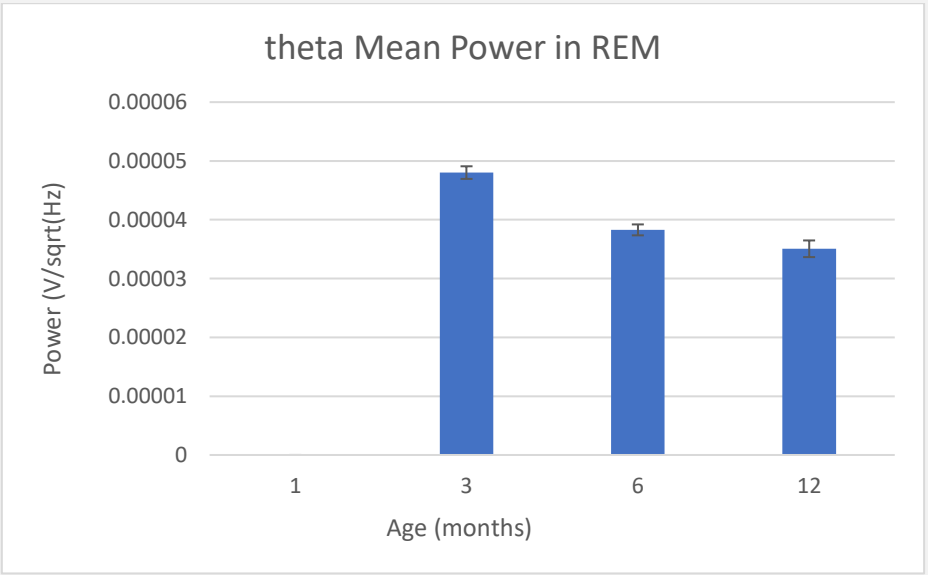

Figure 37 Theta power during REM sleep. *Decreasing tendency is observed. Changes were considered significant according to the t-test between 3 and 3 months old.*

4.2.3. Gamma power in REM

Gamma rhythms are prominent during REM sleep therefore these oscillations were measured during this specific stage. A significant decrease was measured between the 3 months old and the 6 months old animal.

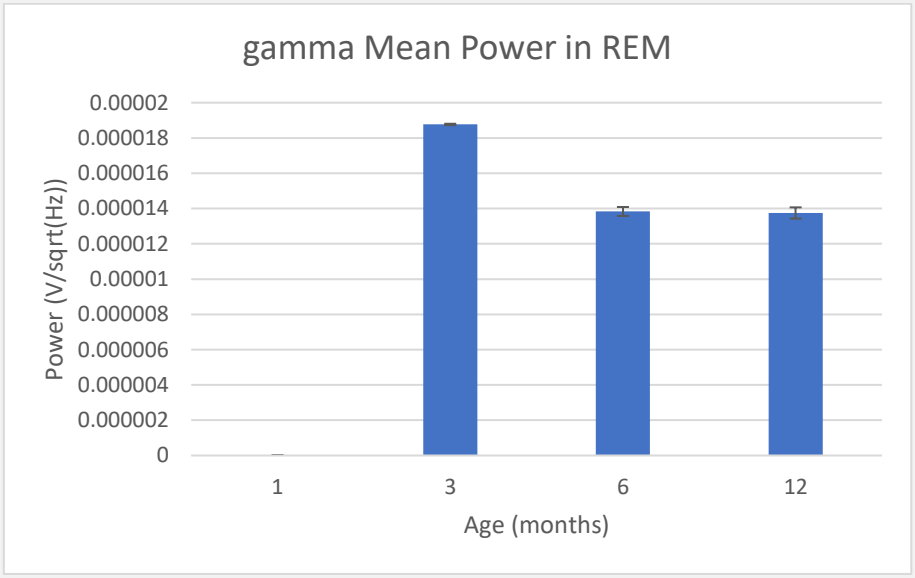

***Figure 38 Gamma power during REM sleep**. Gamma power decreased between 3 months and 6 months. This change was considered significant according to the t-test.*

5. Discussion

5.1. Discussing the preliminary results

According to the preliminary results sleep some marked changes were observed both in the architecture and oscillations of sleep across life stages.

Sleep length was showed to decrease with aging, along with the amount of NREM sleep. Slight reduction of REM sleep was also observed, however very little REM sleep was identified throughout the whole analysis, which could be due to many methodical issues, further discussed in Chapter 5.2.

Wake bouts duration increased along with the amount of wake bouts per minute. The length of sleep bouts decreased, while their frequency also increased across life stages. These results point to the notion, that sleep becomes more fragmented during aging, with sleep more frequently interrupted by wake bouts. These wake bouts seem to even become longer with age. This is in accordance with the increase in sleep bout frequency, which may represent the increasing number of sleep attempts during one session.

Sleep oscillations become less pronounced with age; this observation was the most significant when looking at the delta power in the slow-wave sleep. These results suggest the disintegration of sleep stages during aging, which could also be indicated by the elevated amounts of detected IS in the 12 months old animal.

For the majority of these results a tendency was observed, however changes were not considered to be significant. This could be due to the fact, that the experiments included only one animal per age group. More significant results would be expected with increasing in sample size to 5-10 animals/ age group.

The fact that the LFP channels show very similar results to the results acquired by the ECoG channel, might indicate the reliability of acquired data.

5.2. Sleep scoring and on getting good sleep recordings

During this study I have refined the methods for recording sleep across life stages. These methods are continuously developed and used in the laboratory “sleep and cognitive development” led by Dr Boccara. They have great potential to contribute to the emerging field of developmental system neurosciences.

While my work allowed to refine many of our recording methods, the experimental results revealed that there are still some room for further improvement. I will discuss the mentioned results, as well as the limitations of the experiments and share some ideas for further refinement.

Rats are sensitive animals, and it takes some time for them to habituate to the environment and procedures. The varying stress levels that they experience can turn the procedures more difficult to perform and yield in biased results. Habituation is therefore crucial, and its aim is to reduce stress and eliminate it as a factor in the experiments. In our case, it turned out beneficial to habituate the animals to handling and the environment, as well as to the activities like free exploration, sleep, and recording. Stress levels and the need for habituation varied between animals: younger animals were more energetic and less calm, thus needed more careful restraint and more time to get used to the tasks. Meanwhile, older rats accepted handling and control considerably early. Considering the individual needs of the animal is extremely important, therefore habituation is needed to be practiced until the rat is not stressed about any of the tasks anymore and are comfortable being handled by the experimenter.

Sleeping conditions during the recording need to be refined and made homogenous for further experiments. During my thesis, I have tried different sleeping environments/conditions. I was constrained in my choice by the high electromagnetic noise in the recording room and the need to have the sleep environment placed into a pseudo-faraday cage. We have now a much clearer idea of how sleep environment should be modified to accommodate to long recordings in animal of different ages. The sleeping environment needs to be of a material which is not giving any sounds when the animal is crawling/rubbing its paws and should not be climbable. It must be non-see-through, needs to be adapted to the size of the animal and must be tall enough so that the animal cannot jump out of it. It is required that the sleeping environment is not distracting, not stress inducing and “boring” for the animal. Design and production of such modified environment is in the making in the lab.

Another aspect of the sleep recordings is the starting time and duration of the sessions. The results are comparable when the start of the sessions falls at the same time of day because the exact time of day can influence the sleep quality and the composition of sleep (REM, NREM). While fine-tuning the recording paradigm and because of external constrains (e.g., sharing the recording facilities with other groups), I could not have homogeneous recording time of the day. Rats are nocturnal animals; therefore, the morning hours would be the most efficient starting point to record sleep as that is the beginning of their sleep time (unless reversed light-dark cycles). Ideally, recordings should last throughout the whole day – for 12 hours – so that the whole “night’s” sleep can be evaluated and not only certain fragments of it. Wireless recording devices would make it possible. As some animals have

more trouble falling asleep and wake up regularly, recordings should also include the latency to fall asleep to see if this correlates with age. Present experiments lacked this information, as sleep sessions were started when the animal fell asleep and went on for a couple hours maximum. This may have greatly influenced the preliminary results I am presenting here. It will be taken into considerations for further experiments.

Refining the quality of the EMG signal is of high priority especially when recording from young animals which present less mature EEG oscillations and more frequent twitching. When choosing the appropriate electrode, we needed to consider the impedances of the wire, the contact with the animal and its influence on the material or whether to have one or two pieces (that is a matter of length and flexibility). We compared several materials, as well as methods of attachment to the recording device or to the neck muscle. Out of copper (100 microns), steel (xx microns) and tungsten (xx microns), steel wires had the best results in terms of the signal to noise ratio, although the length had to be controlled strictly, to have acceptable impedances. The method that yielded the best results was to prepare two pieces, each with a metal clamp at one extremity, and then solder the pins together during surgery. This method provided acceptable results; however, the sensitivity of the wire could be further improved as there have been very few REM stages recognized during the current analysis. It is possible to increase sensitivity with reducing the impedance, however one must be careful with picking up also too much noise; an option would be also to filter or reference the signal. With better sensitivity, it would be possible to distinguish minor muscle activity (indicating NREM) from no activity (strictly indicating REM) – which had not been accomplished efficiently with the current method. An essential part of the future analytical work will be to identified twitches and distinguish these brief jerks from movements (probably based on their duration and frequency). It was also concluded that the RMS power of the EMG signal provides better analytical results than the raw signal as the smaller changes in the EMG were detected more easily. Better, more sensitive EMG would be crucial for further experiments to detect nuances in muscle activity, which changes markedly across life stages.

Both ECoG signal and LFP signal has been acquired from the recordings: two ECoG signal were acquired from the prefrontal region and from above the hippocampus, and LFP from the area around the hippocampus. I chose to use the ECoG above the hippocampal region because of several reasons. Even though the LFP provides a better signal, it is greatly area specific and does not provide information about the general brain activity during sleep. This is even a bigger issue when comparing results from different animals – the signal becomes highly dependent on where the electrodes are placed in the individual animal. Theta is considerably strong in the LFP, therefore, when using this measure, the theta/delta threshold needed to be increased to 0.6-0.7. It would be interesting to use in further studies, to measure the emerging hippocampal oscillations in developing animals. In contrast with the

LFP, our multiple ECoG electrodes offers an overview of the oscillations occurring across two separated cortical areas. The ECoG electrode above the hippocampus showed a more pronounced theta activity and the one above the prefrontal cortex a more pronounced delta activity. For future studies, both ECoG signals will be used to map sleep oscillation across the cortex.

During the present analysis all uncategorized stages – that were not recognized as NREM, REM or wake – were considered intermediate sleep (IS). However, in nature, intermediate sleep is a transitional stage from NREM to REM sleep stage, and therefore do not last for long periods. Our methods need to be refined further to identify this stage better. As it is currently used, the amount of IS resembles an estimate for the precision of the algorithm.

More precise definition needs to be determined for wake bouts or awakenings that occur during sleep. Rats are prey animals, and this could explain why their sleep is way less continuous than in humans, frequently interrupted by shorter and longer wake bouts. During current experiments it was indeed observed that longer periods of wakefulness intrude to sleep periods, that are especially pronounced in older animals. In the current study, wake bouts are considered 10-40 second awakenings. Ways to detect these awakenings need to be improved and described better: microawakenings and woken up periods need to be distinguished from each other and their length need to be defined.

During this study, I have helped Dr Eis Anavinni to set up methods to performed automatic sleep scoring. Several thresholds needed to be determined for the sleep scoring: the threshold of the theta-to-delta ratio, high and low threshold of the EMG RMS power and the threshold of the animal speed. These thresholds were inferred from the literature and in some case adjusted to fit short samples of manual sleep scoring, before being used for automatic scoring. While this empiric method is in agreement (or may even be a step above) with what has been used until now, it still has limitations in terms of objectivity, comparisons between animals and on-line scoring. Already in the lab “pipeline”, is a plan to used trained neural network to set thresholds, especially for experiment requiring on-line scoring linked to neural manipulation. Automatized scoring – including threshold setting – is a huge advantage when evaluating sleep.

Here, I have been refining small device to (i) accommodate ECoG, EMG and intracerebral tetrodes and (ii) that can be hold by rats ranging from adolescent to aging. Future work in the lab (already in development, see Figure 18) will be to use even smaller devices to record in younger animals. For this purpose, omnetics recording devices are being developed in the lab. These are smaller, lighter devices that will fulfil the same purposes as the current microdrive but would fit on pre-weaning rats. These devices need to be more stable and protected in order to make sure that the animal and its

environment pose no harm. This is especially important when considering recordings for pre-weaning animals that are kept with their mothers and siblings, subject to continuous interactions from their mother. Eventually, wireless devices would be the ideal solution for recording continuous 12-hour sleep sessions, while the animals are in their cages.

Conclusion

My work has allowed to detect and refine many technical and computational obstacles to record and automatically score sleep across lifespan. I have managed to overcome many of these caveats, and the lab is continuing the process of refining the methodology further based on the limitations that the current study revealed. While doing such technical refinement, I was also able to collect preliminary results that should be taken with a “grain of salt” yet show some interesting trends in sleep architecture across lifespan. These results provide some points of focus for future studies in the lab. Notably, the increased amount of IS during the aging and whether it represents the blurring of sleep stages), or the increased durations and frequency of wake bouts, which could indicate the sleep fragmentation of sleep during aging. In my opinion, with increasing the sample size and refining some of the methodologic caveats mentioned in the previous chapter, this experimental design will allow to gather valuable data regarding sleep changes and their influence in cognitive development.

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