Determining brain states with pupil size and eye movements in sleeping mice and humans

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DETERMINING BRAIN STATES WITH PUPIL SIZE AND EYE MOVEMENTS IN SLEEPING MICE AND HUMANS

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Summary

Brain states, defined by global activity changes across brain regions are arguably the most important determinant of behavioral output in mammals. Sleep is the longest state that most animals spend their lives in and comprises distinct sub-states crucial for different physiological and behavioral processes. Classification of different sleep stages is therefore essential for understanding and studying processes taking place throughout sleep in health and disease in both mice, the most common animal model in neuroscience, and humans.

Sleep stage classification has typically been done through analysing multiple physiological measures including electroencephalography, electromyography and electrooculography. While these techniques have enabled numerous studies, they remained complex, expensive and tedious to implement. Other auxiliary measures such as heartbeat, breathing and skin conductance revealed body-wide changes across sleep and their correlation to the autonomic rhythms and neuromodulator levels. However, these signals have a too low time resolution to derive significant results from. We have taken inspiration from studies involving eye pupil tracking in wakefulness to predict brain states and translated them into sleep settings by merging them with novel infrared illumination methods for both mice and humans.

In this thesis, I present findings on pupil tracking during sleep in mice and humans. We discovered a robust correlation between brain states and fluctuations in pupil size as well as eye movements. Our findings suggest a strong parasympathetic control of pupil size during sleep in both species and the involvement of sympathetic pathway in humans, indicating fragility and arousal periods in sleep. We furthermore show that pupil constrictions during sleep might serve a protective function to preserve the stability of deep sleep. Taken together, our research revealed a reliable relationship between pupillary dynamics in sleep and brain states, which has so far been hidden behind closed eyelids. It underlines the importance of developing novel methods for physiological tracking in sleep that can be used for assessments in clinical or research settings.

L'étude du mouvement des yeux et de la dilatation des pupilles pour la caractérisation des différentes phases du sommeil chez l'Homme et la souris

Résumé

Les rythmes cérébraux résultent de l'activité simultanée d'un grand nombre de neurones à travers différentes régions du cerveau et sont étroitement liés à l'état d'activité des mammifères. La majorité des animaux passent une grande partie de leur vie à dormir. il est admis que le sommeil peut être subdivisé en différentes phases, chacune ayant des caractéristiques physiologiques et comportementales propres. Classer les différentes phases du sommeil est une étape essentielle à la compréhension de ces mécanismes sous-jacents.

De nos jours, la classification des différentes phases du sommeil est permise grâce aux donnés fournies par différents examens tels que l'électroencéphalographie, l'électromyographie et l'électro-oculographie. Même si un grand nombre d'études ont pu être menées grâce à ces examens, ils n'en restent pas moins difficiles à mettre en place du fait de leur inconfort et de leur coût. Le rythme cardiaque ainsi que la conductance de la peau sont contrôlées par le système nerveux autonome. Leurs mesures évoluent au cours des différentes phases du sommeil, mais leurs faibles résolutions temporelles ne permettent pas une identification précise de chaque changement de phase. De nombreuses études ont montré qu'il était possible de suivre notre état d'éveil en fonction de la dilatation de nos pupilles. Nous nous sommes inspirés de celles-ci, en association avec une méthode d'illumination infrarouge de l'œil, afin de suivre les phases du sommeil chez l'Homme et chez la souris.

Cette thèse est le résultat de nos découvertes sur le suivi des pupilles lors du sommeil humain ou chez la souris. Nos analyses révèlent une forte corrélation entre mouvement de l'œil, taille de la pupille et rythme cérébral. Nos recherches ont permis de mettre en évidence que le contrôle des pupilles se fait majoritairement par le système parasympathique chez ces deux espèces. Ceci indique une fragilité, voire des périodes de micro-éveil dans le sommeil. De plus, il ressort également de ces expériences que la constriction de la pupille a un rôle protecteur du sommeil, garantissant une phase de sommeil profond stable. L'ensemble de ces données permet de démontrer la relation entre la dynamique des pupilles lors du sommeil et l'état de l'activité cérébrale, information qui, jusqu'à présent était caché derrière nos paupières. Cela souligne l'importance de développer de nouvelles méthodes de mesure du sommeil pour une application en clinique ou en recherche.

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List of Abbreviations (in the order of occurrence)

EEG: electroencephalography
REM: rapid eye movement
NREM: non-rapid eye movement
EOG: electrooculography
EMG: electromyography
ACh: acetylcholine
NA: noradrenaline
LC: locus coeruleus
5-TH: serotonin
DRN: dorsal raphe nucleus
TMN: tuberomammillary nucleus
PPT: pedunculopontine tegmental
LDT: lateral dorsal tegmental
VLPO: ventrolateral preoptic area
MnPO: median preoptic nucleus
SCN: suprachiasmatic nucleus
ANS: Autonomic nervous system
CAP: cyclic alternating patterns
IGL: intergeniculate leaflet
OPN: olivary pretectal nuclei
EW: edinger-westphal
VOR: vestibulo-ocular reflex
OKN: optokinetic reflex
SC: superior colliculus
FEF: frontal eye fields
PFC: prefrontal cortex
BG: basal ganglia
MT: medial temporal
MST: medial superior temporal area
SEF: supplementary eye field
VR: vestibular nerve
IML: intermediolateral cell column
ipRGCs: intrinsically photosensitive retinal ganglion cells
SEM: slow eye movement
IR: infrared
iBIP: infrared back-illumination pupillometry
PSG: Polysomnography

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1.Introduction

Description and Importance of Brain States

In order to survive, adapt and respond to constant environmental changes, animals need to switch between different behavioral states. Broad activity in the compartmentalized mammalian brain is the main determinant of behavioral output. The state of large scale cortical networks, measured by optical, magnetic or electrical methods, has been shown to impact learning and memory performance, sensory processes, vigilance and attention. In most cases, a brain-wide activity state can be described for each ongoing process mentioned above. Disturbances or irregularities in the transitions or maintenance of these states can predict failures in sensory, attentional, learning or memory processes; critically affecting fitness and survival of the animal.

In a more technical sense, brain states can be described as the pattern of activity observed in a neural network, measured by electrophysiological or imaging methods, at a defined period of time, discrete or continuous (David A. McCormick, Nestvogel, and He 2020; Zagha and McCormick 2014). The pattern of neural activity is considered to be multidimensional, taking place across time and space, including variable changes in the brain's physical constituents: action potentials, neurotransmitters, synaptic activity. While brain states mostly correspond to specific behavioral context and other externally observable physiological markers, they could also take place without a clear external indication (Stringer et al. 2019; Poulet and Crochet 2018; Damoiseaux et al. 2006). Brain states can include substates, and changes between them do not take place randomly; with the help of external markers and computational modeling they could be predicted (Vidaurre, Smith, and Woolrich 2017). Together with experimental findings, theoretical studies have extensively modeled various brain states in relation to sensory processing, resting, attention, arousal, sleep and many more (Kringelbach and Deco 2020).

One of the most conspicuous state changes happens during the transition between wakefulness and sleep. Sleep itself also consists of functionally and physiologically distinct sub-states, namely rapid eye movement (REM) and non-rapid eye movement (NREM) states (Fig. 1). Healthy maintenance of sleep states is crucial for consolidating and balancing information acquired during wakefulness and for basic physiological regulation. Therefore, it is essential to quantify, detect and predict various brain states and the transitions between them, from mice, the most commonly used scientific model of neuroscience, to humans in order to optimally utilize brain states in basic, clinical research and practical applications.

1.1. Characterization and function of sleep states

Throughout sleep, cellular dynamics, network connectivity and biochemical activity undergo significant changes. Synchronized neural activity and major alterations in the balance of neurotransmitters are some of the changes that take place in the central nervous system. Minimized skeletal muscle activity, slowed down smooth muscle rhythm and changes in eye movement patterns are some examples of peripheral changes. Behaviorally, mammals have varying responsiveness and postures depending on the ongoing physiological changes. Based on these changes, sleep states are traditionally classified into two parts: REM and NREM sleep. While in humans the NREM stage is divided into three sub-states, in mice it is considered as a single state.

Numerous studies have shown that different sleep states are important for distinct biological processes. Disturbance of certain sleep states has been shown to severely impact life quality as well as health and performance in wakefulness (Van Someren et al. 2015; Michael H. Bonnet and Arand 2010). Moreover, disappearance of sleep states can signal neurological disorders (Benca 1996; Benca et al. 1997). Therefore, it is essential to clearly define and classify cellular, biochemical and physiological changes across sleep states.

1.1.1. Physiological characteristics of sleep states

In healthy mammals, the start of NREM stage is mostly viewed as a gradual transition. As the NREM stage starts and progresses, the heart rate, respiratory rate and body temperature gradually decrease, muscle tone reduces. This stage can be characterized mainly by global changes in the electroencephalographic (EEG) activity (Fig. 1), transitioning from a low amplitude - high frequency to a high amplitude - low frequency progressively. In human sleep, this stage is divided into three progressive substates with distinct electrophysiological markers (Fig. 1). NREM1 (or N1) is characterized by low amplitude theta frequency (4-7 Hz) activity and vertex waves (negative sharp wave followed by a slower positive component). Electrooculograms (EOG) show that the eyes roll slowly. NREM2 (or N2) is characterized by distinctive sleep spindles (0.5-2.0 s long bursting waves at a peak amplitude around 100uV, at 12-14 Hz frequency), K-complexes (negative sharp wave followed by immediate slow positive component) and appearance of delta (1-4 Hz, or slow wave) activity. NREM3 (or N3) sleep is characterized by high amplitude (150-250 uV) slow oscillations (0.1-1Hz) present most of the time (at least 50%), increased delta activity.

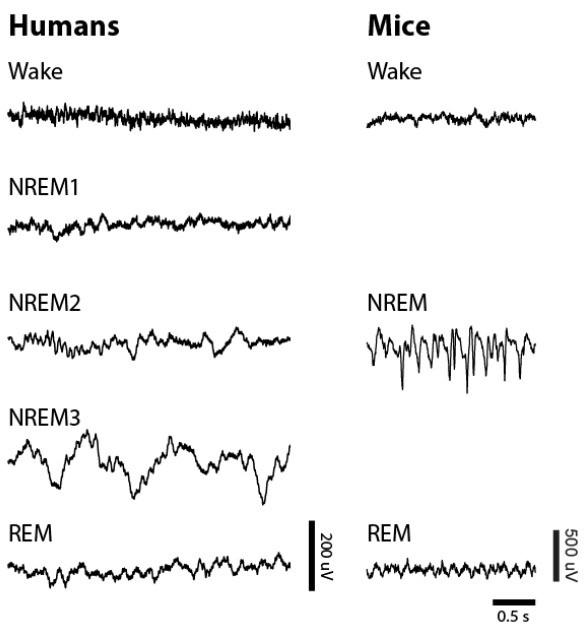

Figure 1: Wakefulness, NREM and REM sleep characteristics in human and rodent EEGs Note the increase in signal amplitude from wake to NREM sleep in both species. Higher frequency activity disappears from wake to NREM1, spindles and K-complexes emerge in NREM2 and large amplitude slow waves become prominent in NREM3 in human sleep. In both species rhythmic theta waves are observed in REM sleep.

After varying amounts of time, relative to the brain and body size of the animal, the progressively deepening NREM stages are followed by REM sleep. As the name suggests, this stage is marked primarily by the fast, tonic movements of the eyeballs detectable by EOGs, while the EEG activity is quite like wakefulness (high frequency, low amplitude); however, the muscle tone is significantly decreased in skeletal muscles. In addition, twitches in facial, hand and in proximal muscles as well as erections can be observed through Electromyograms (EMG). Body temperature, heart and breathing rates fluctuate similar to wakefulness. While in mice and humans described sleep related physiological changes mostly occur at a global scale in each stage, upon sleep deprivation, exhaustive experience or disease conditions, local sleep or local wakefulness could take place (Huber et al. 2004; Vladyslav V. Vyazovskiy et al. 2011). In other species including avians and dolphins local and hemispheric sleep is frequently observed (Rattenborg, Lima, and Amlaner 1999; Gole 1999).

1.1.2. Distinct functional roles of physiological changes across sleep stages

A question that has been approached by numerous studies in the field of sleep is whether there is a functional role associated with different sleep stages. A very well known consequence of sleep deprivation is debilitation of cognitive capacities and memory impairment (Alhola and Polo-Kantola 2007; Killgore 2010; Curcio, Ferrara, and De Gennaro 2006). Many studies have shown that sleep facilitates consolidation of hippocampal and cortical-based newly acquired memories and improves memory-based cognitive capacities (Diekelmann and Born 2010; Ficca and Salzarulo 2004; Rasch and Born 2013). A closer look

into how different stages of sleep and specific EEG markers contribute to the memory consolidation process revealed distinct roles for light, deep and REM sleep as well as for different frequency components of the EEG signal.

Recordings of cortical EEG and hippocampal neuronal recordings demonstrated that sleep spindles in the cortex during NREM2 sleep (i.e. “light” sleep) were correlated with cortical and hippocampal reactivations of previously acquired information underlining a role on episodic memory consolidation (Siapas and Wilson 1998; Miyawaki and Diba 2016). On the other hand, slow waves in NREM sleep were reported to organize the global excitability across brain regions (Battaglia, Sutherland, and McNaughton 2004; Levenstein, Buzsáki, and Rinzel 2019) via up and down states. While UP states correspond to depolarized membrane potentials, during DOWN states, cortical neurons are silent. Slow waves synchronize up and down states across large populations of neurons, organizing spindles and hippocampal ripples (fast bursting activity of ~200 Hz) into a window of activity during the rising phase of “silenced” DOWN state (Staresina et al. 2015; Genzel et al. 2014). With the increased percentage of slow waves in deeper parts of sleep (NREM3) the brain is believed to enter into a scaling down mode, where potentiated synapses are pruned (Tononi and Cirelli 2006, 2014). Besides the slow rhythms, the network activity in cortical and hippocampal regions in REM sleep has been shown to be regulated around rhythmic theta activity (Grosmark et al. 2012). This activity is similar to awake exploration or locomotion mode in the hippocampus and is suggested to facilitate and regulate the timing of long term potentiation and depression at the synaptic level (Hölscher, Anwyl, and Rowan 1997; Tononi and Cirelli 2014). During wakefulness the firing of hippocampal place cells at the peak of theta oscillations lead to potentiation, while in REM sleep they mostly fire in throughs, which leads to depression. This is considered to be important for recycling hippocampal memories and transfer into cortical space (Gina R. Poe, Walsh, and Bjorness 2010).

In addition to its role in memory, other homeostatic functions have been ascribed to sleep. One of such being a balancing role of firing rates in different brain substrates (Vladyslav V. Vyazovskiy et al. 2009; Miyawaki, Watson, and Diba 2019; Levenstein et al. 2017; Watson et al. 2016) . Through distinct stages of sleep, firing rate, synaptic activity and connectivity is differentially modulated. This changing modulation is considered to complement systems memory consolidation in neural networks. (Genzel et al. 2014; Tononi and Cirelli 2006, 2014; Klinzing, Niethard, and Born 2019). Sensory deprivation paradigms throughout the sleep-wake cycle have shown the differentiation of homeostatic plasticity between sleep and wakefulness, promoting learning and memory (Hengen et al. 2016). During wakefulness, cortical activity is high and increases throughout sustained wakefulness. During NREM sleep, neurons synchronize into highly depolarized up and hyperpolarized down states through slow

waves. After a cycle of sustained sleep, the levels of neural activity decrease to homeostatic levels. On the other hand, in the hippocampus, activity increases in NREM and decreases in REM sleep, a process crucial for memory and learning (Grosmark et al. 2012; Miyawaki and Diba 2016). In terms of the distribution of firing rates, REM sleep is suggested to diversify firing rates by increasing variability while NREM sleep homogenizes through narrowing the distribution of firing rates. (Miyawaki, Watson, and Diba 2019). The precisely timed and coordinated cellular activity between cortical and hippocampal regions therefore seems to be distinct through sleep stages, and is of importance to distinguish them clearly.

Other lines of evidence indicate that distinct sleep states would strengthen different types of memories (Rasch and Born 2013; Diekelmann and Born 2010). For instance, REM sleep seems to regulate emotional memories due to heightened amygdala activation (Palmer and Alfano 2017; van der Helm et al. 2011). REM sleep also improves non-declarative types of memories (Plihal and Born 1997). Numerous studies have shown that procedural memories benefit from NREM sleep (Plihal and Born 1999). These studies have confirmed that it is not only one type of sleep that helps the consolidation of memories but a regulated sequence of all stages that result in robust memories. Meaning, while NREM sleep can contribute to consolidation of non-declarative memories, REM sleep can also strengthen declarative-like memories, suggesting that these two stages of sleep might complement each other in strengthening of acquired information (Giuditta et al. 1995; Ficca and Salzarulo 2004). It is therefore important to not only detect sleep states, but also correctly identify the transitions between them.

1.1.3. Substrates and neuromodulators regulating brain states

Brainstem, hypothalamus, basal forebrain, thalamus and cortex are the main regions implicated in the regulation of brain states during sleep (S.-H. Lee and Dan 2012). While many of these pathways play redundant roles, distinct permutations of them lead to switching into specific brain states. In other words, sleep states can be identified based on the primarily active brain regions and neuromodulator release (Fig. 2).

Wakefulness is promoted by several interacting arousal-related networks (Clifford B. Saper et al. 2010). While the ascending projections of the reticular activating system in the brainstem influence all the other neuromodulatory systems (thalamus, hypothalamus, basal forebrain, and neocortex) and is important for cortical activation, the descending pathway to the spinal cord is critical for maintaining muscle tone. The two pathways need to coordinate to perform voluntary movement in wakefulness (Holstege and Kuypers 1987; Jones and Yang 1985).

The ascending wake-promoting pathways act upon their target nuclei via various neuromodulators: acetylcholine (ACh), noradrenaline (NA) from the locus coeruleus (LC), serotonin (5-HT) from dorsal raphe nucleus (DRN), and histamine from tuberomammillary nucleus (TMN). LC projects widely to almost any part of the nervous system (Sara 2009) and its activation results in a sudden shift from sleep to wakefulness (Carter et al. 2010). Pharmacological blockage of NA in the cortex also results in sleep-like synchronized states, suggesting that NA is important for desynchronization in wakefulness (Constantinople and Bruno 2011). Although exact role of LC activity and NA is unclear in sleep regulation, activity of noradrenergic LC cells have been shown to take part in regulating windows of NREM sleep bouts through arousals and promoting plasticity in rodents (Eschenko et al. 2012; Takahashi et al. 2010; Aston-Jones and Bloom 1981). GABAergic LC cells have also been shown to contribute to switching into REM sleep (Nitz and Siegel 1997; Weber et al. 2018).

Additionally, histamine and serotonin release have been shown to induce wakefulness through promoting electrocorticographic desynchronization and behavioral arousal (J. M. Monti 1993; Jaime M. Monti and Jantos 2008). ACh neurons in the brainstem located mainly in pedunculopontine tegmental (PPT) and lateral dorsal tegmental (LDT) nuclei, on the other hand, is shown to promote cortical desynchronization but not necessarily behavioral arousal. They are reported to be active also during REM sleep and lesioning of the ACh neurons in these regions reduces REM sleep (Metherate, Cox, and Ashe 1992).

Other brain regions that regulate the wake state are lateral hypothalamus containing orexin/hypocretin neurons and basal forebrain containing cholinergic neurons. Orexin neurons have very wide range excitatory connections to arousal systems in the cortex, basal forebrain and brainstem (Sutcliffe and de Lecea 2002). They show high activity during wakefulness and low activity during sleep (M. G. Lee, Hassani, and Jones 2005). Optogenetic activation of these neurons induce wakefulness; their loss results in extreme sleepiness or sleep attacks (Adamantidis et al. 2007). Similar to orexin neurons, cholinergic neurons in the basal forebrain, making up the majority of cholinergic input to the cortex, are also active during wakefulness and REM sleep (M. G. Lee et al. 2005; Buzsaki et al. 1988). Their lesions cause a significant increase in EEG delta waves during wakefulness. Blockage of orexin neurons together with serotonin neurons lead to a complete synchronization in the cortex and stimulation induces cortical desynchronization (Metherate, Cox, and Ashe 1992).

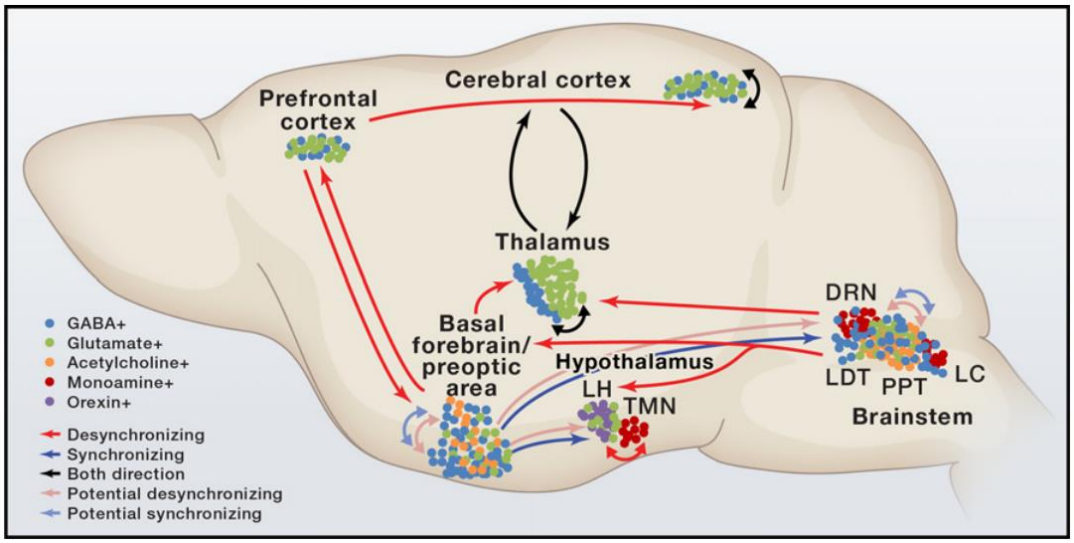

Figure 2: Key brain regions and neurotransmitters regulating sleep in the mouse brain (S.-H. Lee and Dan 2012)**.** Main pathways connecting these major brain areas are outlined with colored arrows, red indicating desynchronizing (light red for potential desynchronizing), blue synchronizing (light blue potential synchronizing), black both synchronizing and desynchronizing. Cell types are illustrated by colored dots, blue for GABA+, green for Glutamate+, orange for Acetylcholine+, red for Monoamine+ and purple for Orexin+.

In addition to the neuromodulators mentioned above, GABAergic neurons also play a key role in the regulation of sleep. The role of GABAergic neurons present in various nuclei seem a bit less straightforward than the types of the neuromodulatory neurons. In the basal forebrain about 60% neurons are GABAergic and different groups are active across all sleep stages. On the clear side, GABAergic neurons in ventrolateral preoptic area (VLPO) and median preoptic nucleus (MnPO) have been shown to promote sleep (Clifford B. Saper et al. 2010; Sherin et al. 1996). Their projections onto the arousal systems allow them to shut down the wake-promoting neurons and their loss results in insomnia (Lu et al. 2000; Sherin et al. 1998). The projected regions can also mutually act upon VLPO and inhibit sleep (Clifford B. Saper et al. 2010). On the more unclear side, the GABA populations elsewhere in the basal forebrain/preoptic area seem to have mixed roles by both modulating synchronization in the cortex and inducing insomnia (Buzsaki et al. 1988; Szymusiak and McGinty 1989). This is suggested to be possible due to local reciprocal inhibition between sleep versus wake active GABA neurons, their interactions between the cholinergic neurons in the basal forebrain/preoptic area and the vast variety of molecular markers in GABAergic neurons.

Thalamus, being the sensory gateway into the cortex, is a crucial player in shifting global brain states, in reciprocal connections with the cortex (D. A. McCormick and Bal 1997).

It is also innervated vastly by ascending arousal pathways as well as the basal forebrain, regulating the cortical state through neuromodulatory inputs. Cortical activity is directly altered by the thalamic activity into a sleep-like state, through spindles and delta oscillations, even during wakefulness (D. A. McCormick and Pape 1990). Activation of thalamocortical neurons can also induce arousal-like states in the cortex through desynchronization, although thalamic lesioning does not necessarily prevent cortex from being desynchronized (Buzsaki et al. 1988). To sum up, thalamus can induce distinct sleep and wake like states in the cortex.

Cortex, within itself is also able to self-induce sleep states through slow waves and can influence the global brain states (Sanchez-Vives and McCormick 2000). These oscillations have been shown to organize other synchronized activity across brain regions. Cortex also projects back on to thalamus and neuromodulatory circuits in the basal forebrain as well as brainstem (Zaborszky et al. 1997). In addition to the listed brain regions, suprachiasmatic nucleus (SCN) modulates sleep and stage transitions through circadian rhythms.

In coordination with the part of the central nervous system which regulates brain states during sleep, the autonomic nervous system (ANS) also plays a significant role in orchestrating physiological dynamics during sleep. ANS cell bodies lie in the spinal cord and brainstem. Branching throughout the body, they project onto the smooth muscle organs and tissues such as lungs, blood vessels, heart and the eye. (Fig. 3) (Squire et al. 2008). ANS is made up of two counteracting parts. The activating “fight or flight” sympathetic nervous system increases blood pressure, heart and breathing rate, dilates pupils, while the dampening “rest and digest” parasympathetic nervous system slows down the cardiovascular system and constricts the pupils. Both divisions are organized into two-neuron (pre- and post- ganglia) efferent pathways before synapsing onto the target organ. While all preganglionic neurons use acetylcholine as a neurotransmitter, sympathetic and parasympathetic postganglionic neurons use different ones: catecholaminergic and cholinergic, respectively. Together with the neuroendocrine mechanisms, ANS coordinates adjustments in respiration, circulation, digestion during sleep and is responsible for homeostasis. Disturbance to ANS elements are not only implicated in sleep disorders such as apnea and insomnia but also in cardiovascular and systemic diseases (Squire et al. 2008).

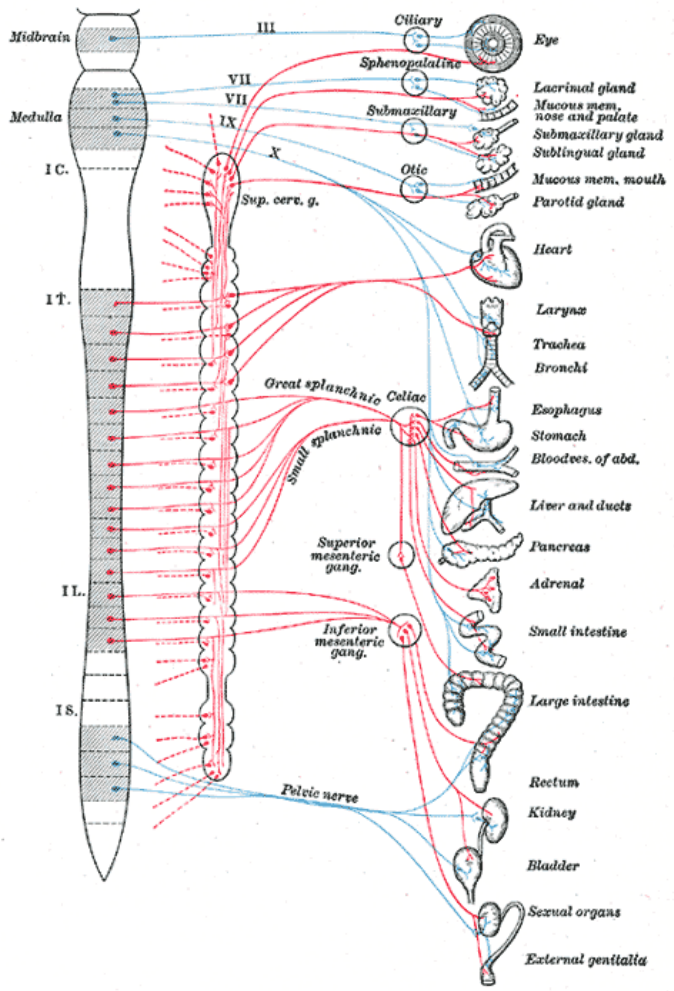

Figure 3: Autonomic innervation of the organs through the parasympathetic (blue) and sympathetic (magenta) pathways (Gray 1918). Left: brainstem and spinal cord, center: splanchnic (paired visceral nerves) nerves, right: ANS ganglia and the target organs. Note the dual innervation of the pupil on the top right, parasympathetic input through the ciliary ganglion marked in blue, and the sympathetic input from the superior cervical ganglion marked in magenta.

Having looked at the distinct sleep stages and the regulatory regions, one question still remains: how do sleep stages transition between one another and how the order of sleep stages is maintained across nights? In order to define healthy or normal transitions between sleep stages, it is important to consider how these switches could be regulated in the brain. Currently there are alternating views on this subject with several proposed mechanisms that are not necessarily mutually exclusive.

According to the flip-flop switch model, mutual inhibition allows rapid and discrete changes between states. It has been hypothesized that brain states are maintained through bistable feedback loop and intermediate states are avoided (Clifford B. Saper et al. 2010; C. B. Saper, Chou, and Scammell 2001). As the neuronal groups put more than enough equilibrating inhibition on each other, the sleep stage changes to another one. This model also accounts for circadian and homeostatic changes. Sleep disorders such as narcolepsy are considered to be the result of a faulty switch between sleep-wake states. The reciprocal

interaction model suggests that the interaction between excitatory and inhibitory groups of cells in the brainstem may generate alternation between sleep stages (Peter Achermann and Borbély 2003). While in earlier studies this was proposed for the switch between wakefulness and NREM (McCarley and Hobson 1975), later the same principle was suggested for REM-NREM cycle alternation (Luppi et al. 2006). Alternatively, as per the thalamocortical loop model, sleep is initiated at the cellular level based on previous activity and then propagated to neural networks (Krueger et al. 2008). Sleep is coordinated across brain regions through oscillating activity, regulated by thalamocortical networks (Llinás and Steriade 2006; Gent et al. 2018). In addition to these models, recent theories on sleep propose a sleep vs. arousal-action circuit. According to this theory sleep's primary function is to simultaneously suppress motor activity through distributed and redundant networks (D. Liu and Dan 2019). Further research is necessary to establish the experimental validity of these models across species and conditions. Nevertheless, these hypotheses make it even more important to work towards new and reliable ways of determining sleep stage transitions and what substrates might be involved in those transitions.

1.1.4. Macro-architecture and ultraslow rhythms in mice and human sleep

In human sleep, a cycle of slumber starting with NREM1 deepening into NREM3 and transitioning to REM sleep (in some cases transition from NREM1 or NREM2 to REM) takes about 90 min (Fig. 4). Depending on the size of their brains, this duration changes for different animals and is around 10 minutes for mice. Names of sleep stages and their compositions may also change across species. For humans, sleep is classified into NREM1, NREM2 and NREM3, REM sleep while for mice there are only NREM and REM stages although many scientists argue otherwise (Lacroix et al. 2018). The classification of sleep stages is traditionally done manually for humans and algorithmically for rodents. To score human sleep, a trained expert scans through the sleep session in 30 s windows, looking for the key time-frequency characteristics of each stage in EEG, EMG, ECG and EOG electrodes. If a stage of sleep comprises more than 50% of that window, the window is marked as that stage. For mice the gold standard of sleep classification is done by setting activity thresholds on EMG and EEG electrode signals.

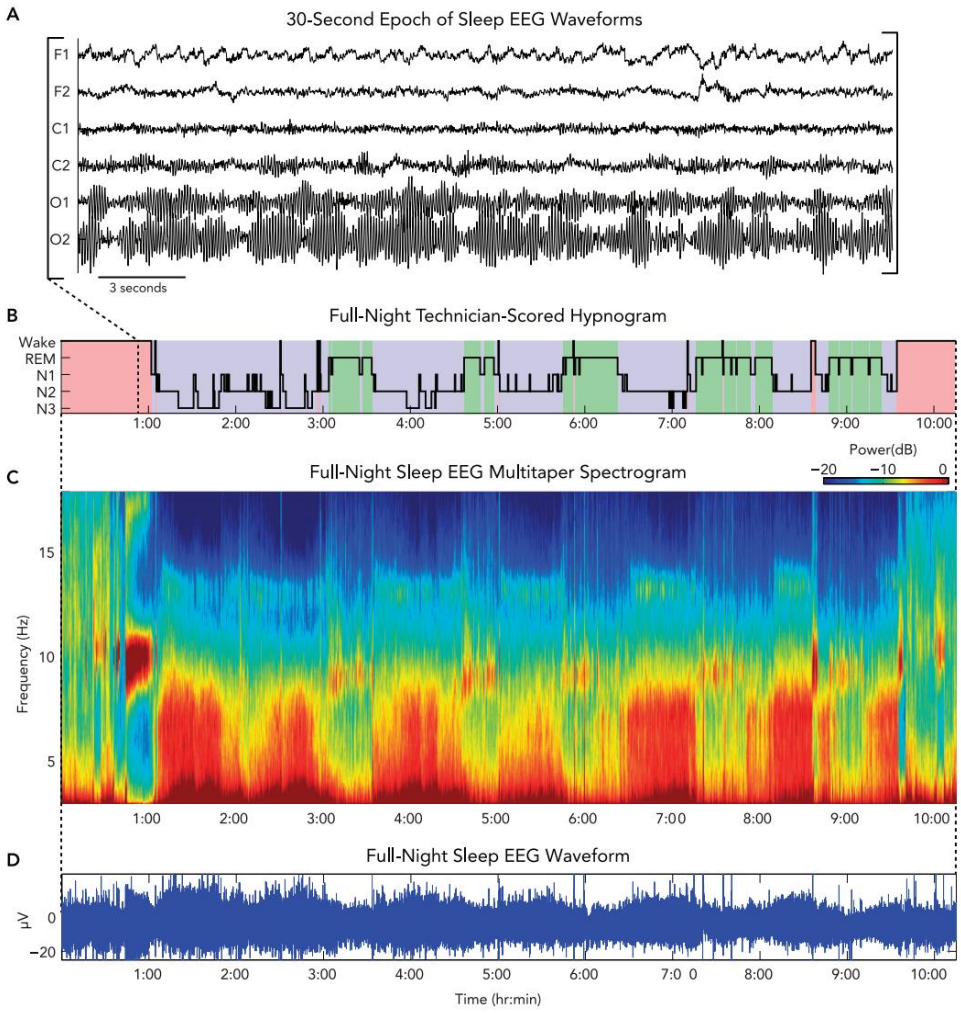

Figure 4: Macroarchitecture of sleep defined by EEG characteristics through the night (Prerau et al. 2017)**.** A. Brain activity during wakefulness across different EEG electrodes, from frontal (F1-F2) to central (C1-C2) and to occipital (O1-O2) regions. B. Hypogram of full night sleep, displaying the periodic occurrence of NREM and REM sleep stages. C. EEG Spectrogram shows the frequency composition of different sleep stages more in detail, emphasizing the density in lower frequencies in NREM, clear presence of theta band in REM and high frequency activity in wakefulness. D. Broadband EEG signal of full night's sleep from frontal cortex.

In humans, NREM1 is marked by a decrease in muscle tone, rolling eye movements, disappearance of alpha and higher frequencies of activity, occurrence of K-complexes. Occurrence of sleep spindles, increased delta activity, occasional appearance of slow waves signal NREM2 sleep. When the brain activity/EEG is composed of more than 50% of slow waves and very low muscle tone, it is marked as NREM3 sleep. REM sleep is characterized by saccadic eye movement, high frequency cortical activity, complete disappearance of

muscle tone with twitches. Within each state, “arousals” can also be observed, defined by brief (5-10s) sudden shift in EEG slow sleep frequencies into higher frequencies, small activation in the muscles, preceded or followed by K-complexes (Berry et al. 2017). In mice, the decrease in EMG activity by multiple levels of standard deviation with an increase in delta activity of above two standard deviations indicates NREM sleep. Disappearance of muscle tone except for twitches in the EMG activity together with an increase in theta/delta ratio signals REM sleep. High frequency EEG and EMG activity, low delta activity is classified as wakefulness.

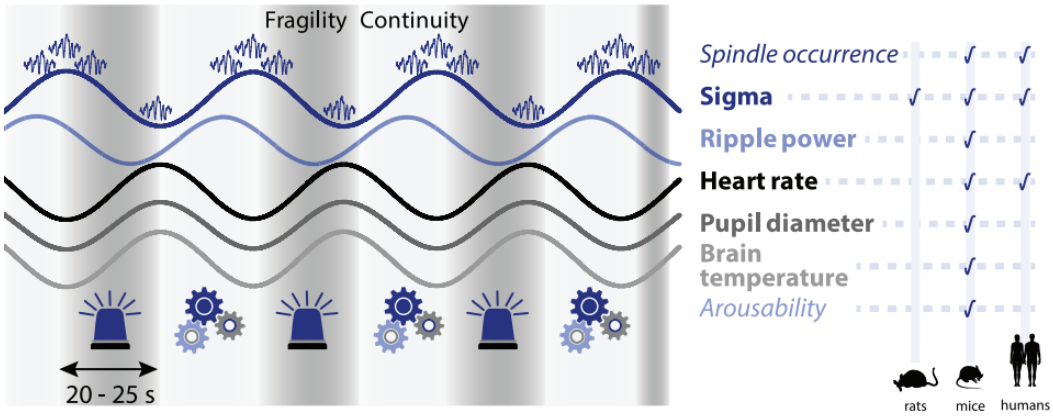

Figure 5: Infraslow alternation of brain rhythms, physiological markers and arousability throughout sleep in different species (Fernandez and Lüthi 2020)**.** From mice to rats to humans spindle power and spindle occurence fluctuates at an infraslow rhythm, negatively correlated with autonomic correlates such as heart rate, pupil diameter and brain temperature as well as arousability.

At a larger time window, sleep frequencies and architecture are also arranged by ultraslow oscillations at the scale of minutes or hours (P. Achermann and Borbély 1997). These fluctuations also include fractal rhythms or cyclic alternating patterns impacting the responsiveness to sensory stimulation during sleep, modulating sleep stability, duration, distribution (Pittman-Polletta et al. 2013; Curie et al. 2013; Dement and Kleitman 1957b). Years ago they have been shown to correlate with eye movements, body motility and sleep stages and have been used as markers of health and disorder (Dement and Kleitman 1957b). Many years after their discovery, the emergence of new technologies and advances in data analysis allowed researchers to look at these rhythms more in detail.

The power of various EEG bands during sleep fluctuate rhythmically. For the alpha (10-15Hz) or spindle (12-15 Hz) bands the power of the fluctuations is defined as the sigma

band and has been shown to vary across sleep stages and along the circadian rhythm (Uchida et al. 1991) (Fernandez and Lüthi 2020)(Fig. 5). Increased sigma power is suggested to be a marker of continuous, consolidating sleep. The power of delta waves have also been shown to fluctuate throughout sleep and across the day depending on the circadian time (Uchida et al. 1991; Borbély et al. 2016). It has furthermore been shown that the power of sigma band oscillates in a counteracting fashion to the fluctuations in the delta band in NREM sleep, potentially associated with the distinct activity patterns in the thalamic substrates (Uchida et al. 1991; V. V. Vyazovskiy et al. 2004; Dijk 1995; Dijk, Brunner, and Borbély 1990).

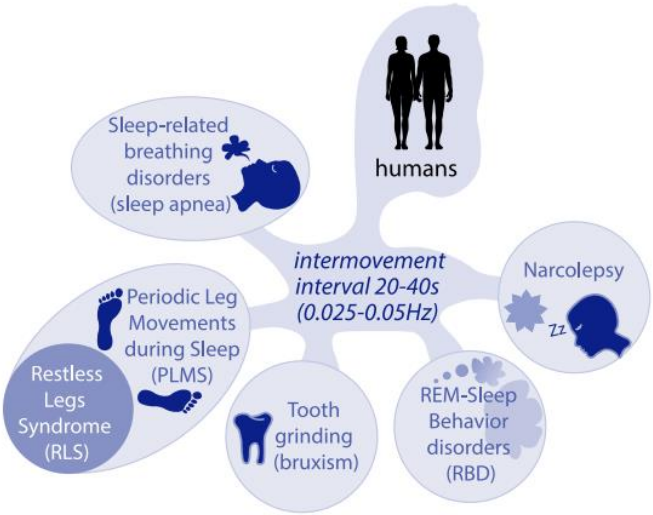

Figure 6: Sleep disorders display infraslow rhythms in NREM sleep humans (Fernandez and Lüthi 2020)**.** Polysomnographic recordings show that various rhythmic muscle activity related sleep disorders such as periodic leg movements, tooth grinding, rem-sleep behavior and sleep apnea come about at regular intervals corresponding to the sleep infraslow rhythm. Narcolepsy, the sudden pathological shift into REM sleep from wakefulness is also likely to occur at infraslow intervals.

Intraslow (<1 Hz) components in the EEG have been shown to correlate with breathing and heart rate as well as fluctuations in pupil size (P. Achermann and Borbély 1997; Lecci et al. 2017; Yuzgec et al. 2018). These oscillations not only seem to organize faster network activity across different regions, but also modulate excitability, epileptic activity in the cortex and other rhythmic sleep disorders during the increased infraslow oscillation (0.02-0.2 Hz) periods (Steriade 2006; Fernandez and Lüthi 2020) (Vanhatalo et al. 2004)(Fig. 6). Earlier studies report changes in general arousability of sleeping humans, namely the Cyclic Alternating Patterns (CAP), 10-60s period comprising 2 phases, in which the sleeping subjects are more or less likely to wake up from external stimulation (Terzano et al. 1985). Although CAPs losely carry similarities to infraslow oscillations, their physiological relationship is not clear (Manconi, Silvani, and Ferri 2017).

In mammals, one of the major influences on brain states is from the circadian system, mainly driven by the SCN located in the hypothalamus. SCN is a sturdy clock maker by its intrinsic properties, impacting the timing of many physiological events (Reppert and Weaver 2002). It has been suggested that the cell intrinsic and population dynamic properties result in the generation of a reliable 24-hour rhythm in the body, regulating diurnal and nocturnal processes. SCN receives the majority of its inputs from the retina, mainly from the melanopsin ganglion cell projections, limbic system, hypothalamus, raphe nuclei, thalamus and extraretinal visual system (intergeniculate leaflet (IGL), pretectum) (Morin and Allen 2006). The neurons in SCN have intrinsic rhythms that propagate through its immediate projections and beyond (Miller and Fuller 1992; Aton et al. 2005). SCN projects on various autonomic circuits such as olivary pretectal nuclei (OPN), IGL, and Edinger Westphal (EW) nuclei (Smeraski et al. 2004) and is believed to have an impact on autonomic rhythms through these projections. Additionally, SCN's indirect projections on arousal and motivation related brain areas have been shown to adjust the impulse activity according to circadian time (Luo and Aston-Jones 2009). When studying rhythms across brain regions and body it is of utmost importance to consider all these micro and macro architectural timing-structures that are embedded into each other for reliable detection of brain states.

1.2. Pupil size and eye movement tracking in determining brain states

Since decades, eye tracking has attracted not only neuroscientists but many other disciplines from market research to automobile technologies to game development. Due to ease of implementation and inexpensiveness it has been implemented in numerous research studies and practical applications, providing a reliable readout for intentions, choice, emotional or arousal states. Furthermore, pupil size monitoring as a part of eye tracking has further enabled researchers and engineers to investigate the physiological changes through a fast and non-invasive biomarker.

Recent studies on primates and rodents have underlined the control mechanisms of the tight correlation between pupil size and brain states in more detail. Many studies have shown that pupil size is not only a reliable indicator of optimal cortical processing but also gives robust clues on the autonomic and neuromodulatory dynamics in the brain. The fast movement range in pupil size and its close relationship to cortical, neuromodulatory and autonomic states together with large autonomic and cortical rhythm fluctuations during sleep makes pupil tracking a prime candidate for utilization in sleep research. In this section, we will tap into the background of pupil size and eye movement control, their domain of usage, and in-depth relationship to brain states.

1.2.1. Functional circuits that control pupil size

Pupil size, the ever changing opening of the iris, is determined by the activity of two smooth muscle groups that work like a seesaw: the circular constrictor (sphincter) muscles and the radial dilator muscles (Fig. 7). These muscles are under the control of opposing autonomic nervous systems, namely parasympathetic and sympathetic nervous systems. Pupil muscles are innervated by postganglionic projections from EW nucleus for the parasympathetic input and from intermediolateral cell column (IML) for the sympathetic inputs, respectively. Numerous central nervous system inputs act upon EW and IML, depending on factors such as the amount of light that reaches to the retina, distance from the target visual object, cognitive load and vigilance (McDougal and Gamlin 2015). The pupil separates the part of the eye with the retina and the lens (posterior chamber) from the part with cornea (anterior chamber) and modulates the amount of light that reaches to the retina as well as facilitating visual accommodation through constrictions and dilations.

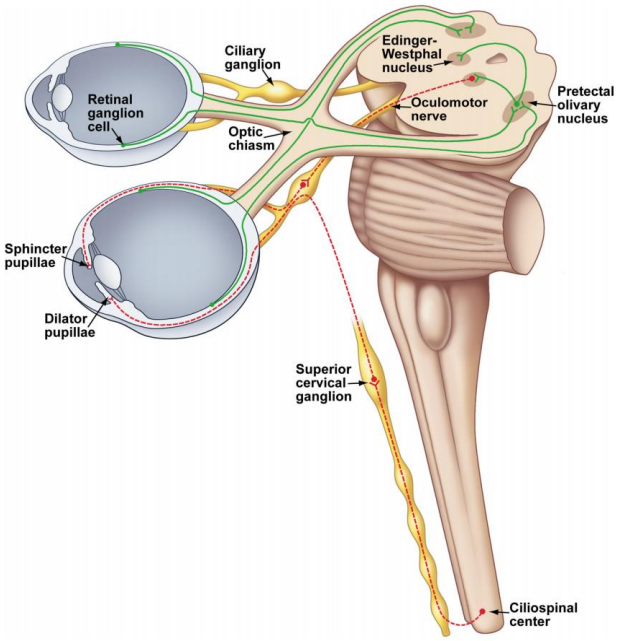

Figure 7: Parasympathetic and sympathetic innervation of the iris in primates (McDougal and Gamlin 2015)**.** The retina bilaterally projects to the pretectum, as shown in green. The pretectal olivary nucleus projects bilaterally to the parasympathetic Edinger-Westphal nucleus. The axons of EW neurons travel in the third cranial nerve to postganglionic pupilloconstriction neurons in the ciliary ganglion. The axons of these postganglionic neurons enter the eye via the short ciliary nerves, and innervate the sphincter muscle of the iris. The sympathetic preganglionic pupillodilation neurons in the spinal cord project rostrally to the superior cervical ganglion postganglionic neurons, then travelling through the neck and carotid plexus, and into the orbit of the eye. These fibers pass through the ciliary ganglion and enter in the short ciliary nerves, innervating the dilator muscle of the iris.

At the biochemical level, pupil muscles are controlled by distinct neuromodulators. The sphincter muscle receives cholinergic input from ciliary nerves. These postganglionic nerves are parasympathetic in nature and arise from the ciliary ganglion (McDougal and Gamlin 2015). Binding of acetylcholine to the muscarinic receptors on the smooth muscle cells lead to a synchronized contraction of the sphincter through increased intracellular Ca2+ concentration. The application of muscarinic antagonists such as atropine or tropicamide

results in the full dilation of the pupil (McDougal and Gamlin 2015) mydriasis (McDougal and Gamlin 2015) while agonists produce extensive constriction (McDougal and Gamlin 2015)miosis(McDougal and Gamlin 2015). Dilator muscle on the other hand, is innervated by sympathetic, postganglionic adrenergic inputs from the ciliary nerves arising from the superior cervical ganglion. Binding of norepinephrine to the predominant adrenoreceptors on the smooth muscle cells of the dilator results in the contraction of this muscle and pulling the pupillary margin outwards (McDougal and Gamlin 2015). The application of alpha-adrenoceptor antagonists such as dapiprazole lead to miosis, while amphetamine derived drugs and general adrenoreceptor agonists produce mydriasis. It has been shown that the sympathetic innervation can be antagonized by the parasympathetic input through the activation of muscarinic receptors and inhibition of noradrenaline release. Also, trigeminal nerve stimulation is shown to produce miosis (McDougal and Gamlin 2015).

From a mechanistic perspective, the light reflex, the near response (the constriction of the pupil after the shift of viewing an object in the far distance to a near one,), cortical and neuromodulatory substrates are the main modulators of the constrictions and dilations of the pupil. The roleplayers that are involved in the generation and maintenance of the pupillary light reflex are: EW, pretectum (more precisely, olivary pretectal nucleus - OPN) and intrinsically photosensitive retinal ganglion cells (ipRGCs) (Fig. 8). Melanopsin carrying ipRGCs are slow acting photoreceptors that make up 1% of the retina. They have projections to SC, EW, OPN and SCN not only regulating the size of the pupil but also contributing to the synchronization of the circadian rhythm to the 24 hr light/dark cycle. The increase of illumination on the retina has been shown to proportionally increase the firing rate of cells in OPN. OPN projects onto EW, which in turn adjusts the size of the pupil through the ciliary ganglion, contracting the sphincter depending on the illumination levels. Sympathetic nervous system has been suggested to tonically adjust the pupillary light reflex but not impact the dynamics of it (McDougal and Gamlin 2015). Pupillary near response is also considered to be controlled by the same circuitry that mediates the pupillary light reflex.

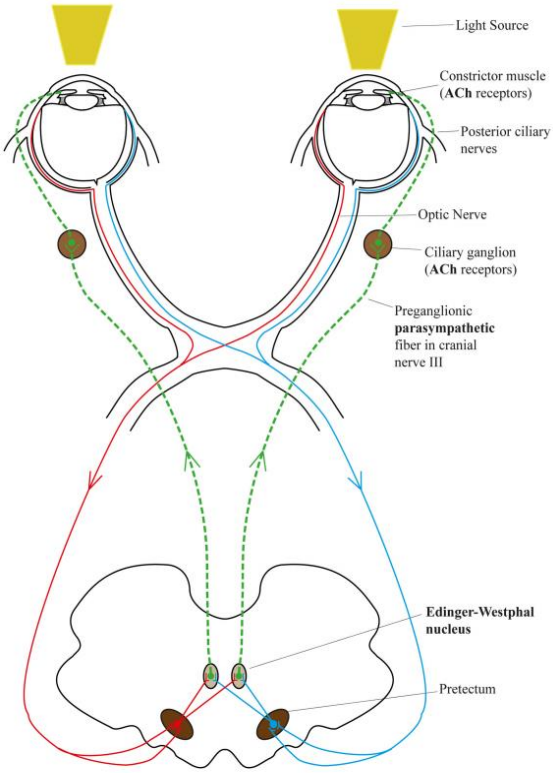

Figure 8: Pupillary light reflex pathway. (Wang et al. 2016). Red and blue lines indicate afferent, green lines indicate efferent pathways. Retinal ganglion cells project to pretectum in the midbrain, which then projects onto EW. Constriction signal is transmitted to the iris through preganglionic parasympathetic fibers, ciliary ganglion and ciliary nerves.

Even in the absence of change in retinal illuminance or viewing distance of a target, pupil size is reported to change. These changes are mainly regulated by cortical substrates and neuromodulatory systems such as midbrain and pons. Regulating sleep, arousal, autonomic rhythms and plasticity, these systems have been shown to mainly act on the EW, increasing or inhibiting pupil constrictions. In the case of pupil dilations, LC has been shown to mediate EW activity and inhibit constrictions. Dopamine, serotonin and noradrenaline afferents have been shown to cause pupil dilation (McDougal and Gamlin 2015). Additionally, locomotion, surprise and fear response have been shown to increase pupil size, while opioids and anesthesia decrease it (Larsen and Waters 2018).

1.2.2. Functional circuits that control eye movements

Voluntary and involuntary movements of the eyes enable animals to visually fixate and track objects. Pupil size changes during wakefulness enhance the visual processing further, adjusting the aperture size depending on the light and distance conditions. Eye movements and pupil size also show changes depending on the autonomic input related to arousal and vigilance. They have both been reported to continue to vary throughout sleep (Pizza et al. 2011; Fuchs and Ron 1968; Berlucchi et al. 1964; Dement and Kleitman 1957b).

Eye movement control is executed through 6 different sets of extraocular muscles that hold the eyeball in place in the eye socket (Fig. 9). These muscles are innervated by 3 different

cranial nerves. Two of these extraocular muscles work antagonistically to enable horizontal movements: adduction (medial rectus) and abduction (lateral rectus). The other four work together (superior rectus, superior oblique, inferior rectus, inferior oblique) to execute vertical movements: elevation (superior rectus, inferior oblique) and depression (inferior rectus, superior oblique) as well as eye rotation (mainly by oblique muscles) : intorsion (towards the nose) and extortion (away from the nose). These muscles are innervated by the oculomotor nerve, superior oblique is innervated by trochlear nerve and lateral rectus is innervated by abducens nerve (Fig. 9). The commands for the eye movements originate at the premotor neurons in different parts of the brainstem, pons, medulla, cortex, cerebellum during wakefulness. These neurons display a variety of discharge patterns including tonic firing, phasic activity, high and low frequency bursting (Sparks 2002).

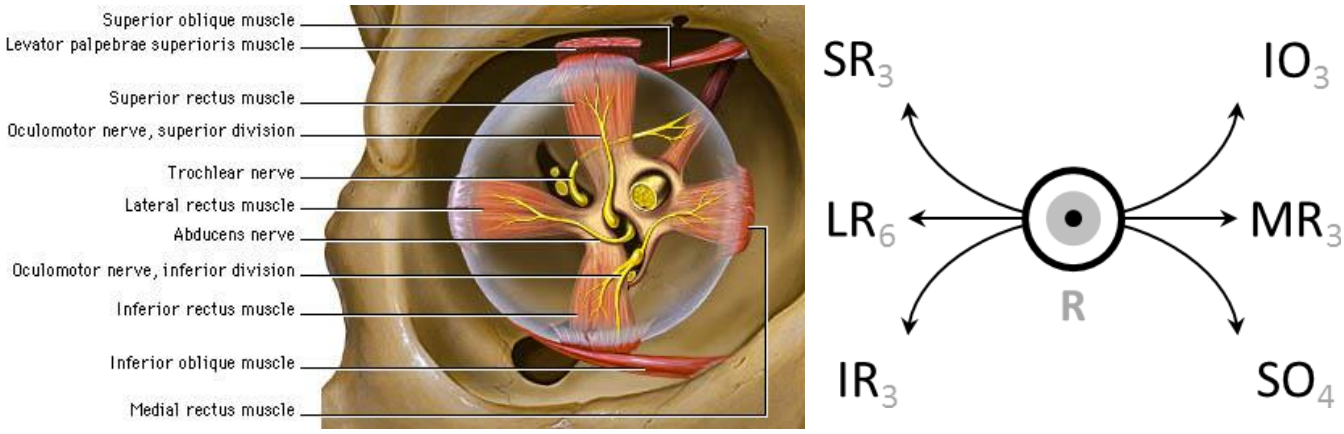

Figure 9: Anatomy, actions and innervation of extraocular muscles (Kerdels and Peters 2016)**.** Left panel: Oculomotor nerves (from cranial nerve-III) carry movement signals to superior rectus (SR), inferior rectus (IR), inferior oblique (IO) muscles, abducens nerve (from cranial nerve-VI) to lateral rectus (LR) and trochlear nerve (from cranial nerve-IV) to superior oblique (SO) muscles. Levator palpebrae superioris muscle lifts up the upper eyelid. Right panel: Activation of IO rotates the eyes in and upwards, SR rotates up and outwards, SO in and downwards, IR out and downwards, LR laterally and MR medially.

Eye movements can be classified by a couple of different systems. The movement of a single eye is called duction while the movement of two eyes is called version. The motion of two eyes traveling to opposite directions is called vergence while the movement towards each other is called convergence. Depending on their functional roles during conscious wakefulness, they are classified as gaze-stabilization or gaze-shifting movements (Cullen and Van Horn 2011). Vestibulo-ocular (VOR) and optokinetic reflexes (OKN) are the main

involuntary gaze-stabilization movements. These movements compensate for the head movements or the movements of the object, respectively. Fixations, vergence, smooth pursuit and saccade enable maintenance of an object on the retina by adjusting the position of the eyes.

From a speed of movement perspective, saccades are the fastest of all of the eye movements. These short, rapid, ballistic movements direct the eyes toward a visual target at a reaction time of 200 ms and with the velocity of 400-800°/second (Sparks 2002). These movements could take place in the horizontal, vertical or the oblique planes. They are controlled by the pulse (bursting) and step (tonic) commands that are sent from the motor neurons in the brainstem. Superior colliculus (SC) is reported to be the main brainstem region that integrates visual and goal directed signals from many cortical (frontal eye fields [FEF], prefrontal cortex [PFC], striate visual cortex) and subcortical areas, providing the commands into the pontine and midbrain pulse -step generator circuits controlling eye and head movements (Sparks 2002). Small or large, upward or downward saccades are represented topographically in SC. It has also been shown that basal ganglia (BG) has an inhibitory role in the control of saccadic eye movements (Vokoun, Mahamed, and Basso 2011). Thalamus and cerebellum are considered to have monitoring and correcting roles in the control of saccadic but also smooth pursuit eye movements (Thier 2011; Tanaka 2012).

Smooth pursuit movements, as the name suggests are rather slower, coordinated motions of the eyes while tracking moving targets at the speed of 0-30°/second with response time at about 130 ms (Sparks 2002). These movements are only present when following a moving object and cannot be self-generated. Information from descending pathways in cortical regions involved in visual motion (medial temporal MT, medial superior temporal [MST], FEF areas) merge in brainstem circuitry and then are projected to pons and cerebellum (Cullen and Van Horn 2011). Cerebellum, being a key area in controlling smooth pursuit movements encodes eye velocity, position and acceleration. Other regions involved in the control of pursuits are: supplementary eye field (SEF), BG and pretectal nucleus. Recent studies show that, although saccades and smooth pursuit movements are regarded differently their control circuitry are very similar (Krauzlis 2004)(Fig. 10).

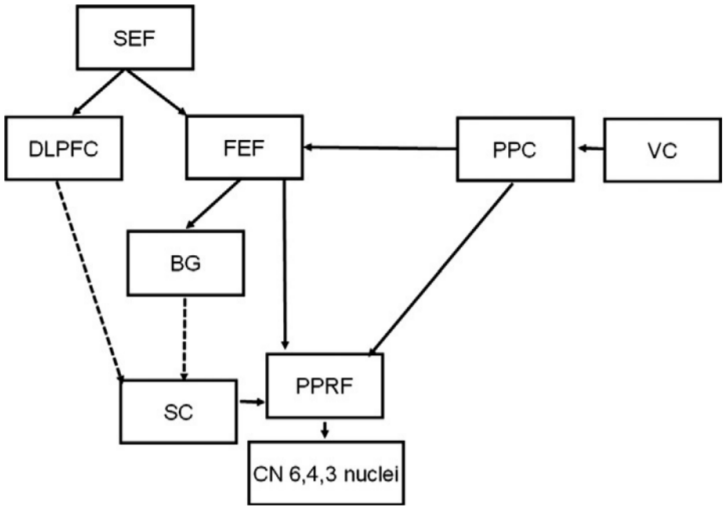

Figure 10: Neural basis of voluntary eye movement control during wakefulness (Kennard 2011)**.** Various cortical and subcortical regions work in coordination to generate eye movements. DLPFC: dorsolateral prefrontal cortex PPRF: paramedian pontine reticular formation PPC: posterior parietal cortex VC: visual cortex CN: cranial nerve. Solid lines mark excitatory, dashed lines inhibitory connections.

Vergence is a depth adaptation mechanism to fixate on a target, through the rotation of the eyes in opposite directions, at the velocity between 30-150°/s and with around 160 ms reaction time (Cullen and Van Horn 2011; Sparks 2002). Somewhere in between saccades and smooth pursuit, the control of this movement is shown to take place also through the brainstem nuclei, by a subpopulation independent of the saccade neurons. SC has also been shown to be involved in the vergence movement (White and Munoz 2011).

Despite its stationary-sounding name, fixations are essential dynamic processes that are actively controlled by many brain regions to improve visual acuity by placing the target image in the retina. Fixation velocity is similar to saccades but is at a much smaller amplitude, up to 1° changes. SC, cerebellum and reticular formation are some substrates that are involved in the control of fixations (Krauzlis, Goffart, and Hafed 2017). During fixations, although the eyes are rather 'fixed' on a target, as the retinal ganglion cells saturate over time, the eyes are moved slightly and in a controlled manner in the form of microsaccades and drifts. These adjustments improve visual accuracy and compensate for the distortions caused by larger eye movements described above.

The remaining two eye movement types, VOR and OKN are the reflexive movements that are complementary to each other. While the VOR compensates for the rotation or translation of the head of the body and adjusts the position of the eyes to stabilize the image, OKN readjusts the eyes according to the speed and direction of full-field image motion. They are both rather fast movements, VOR reaching to 800°/s and OKN covering more low-frequency range (Sparks 2002). VOR merges information from semicircular and otolith organs in response to head motion. This information is then sent to the brainstem through the

vestibular nerve (VR) then to pons and medulla. OKN, on the other hand uses visual instead of vestibular information to stabilize the gaze (Cullen and Van Horn 2011). These movements are mediated by subcortical pathways. Pretectal and mesencephalic neurons send the retinal information into vestibular nuclei in medulla, which directly project onto the extraocular motor nuclei. Vestibulocerebellum has also been shown to contribute to generation of OKN (Cullen and Van Horn 2011).

1.2.3. Pupil size and eye movement tracking in healthy subjects

Providing a non-invasive proxy for vigilance states, pupil tracking or pupillometry has long attracted neuroscientists but also market researchers, athletes and engineers. In human studies, pupil dynamics have been shown to indicate arousal, vigilance levels (Bradley et al. 2008), emotional responses (Ebitz, Pearson, and Platt 2014; Partala and Surakka 2003) and decision parameters (de Gee, Knapen, and Donner 2014). Pupillometry has allowed practical applications such as drowsiness tracking while driving (Nishiyama et al. 2007), fatigue (Morad et al. 2000) and mental health assessments (Steidtmann, Ingram, and Siegle 2010) and affective state classification (Onorati et al. 2013). While these studies have underlined where and how the pupil size might be used as a proxy, functional mechanisms of how pupil size indicates underlying brain states remained unsolved. Studies in rodents and non-human primates have shown that a close coupling between brain states and pupil size exists during quiet wakefulness (Reimer et al. 2014). It has been shown that pupil size is a reliable indicator of cortical gain and optimal window for sensory processing (McGinley, David, and McCormick 2015; Vinck et al. 2015). This relationship is mediated through noradrenergic and cholinergic systems (Reimer et al. 2016).

As the saying ‘eyes are the window into the soul’ suggests, what the control and kinematics of eye movements indicate regarding one’s physiological and intellectual processes has been of great interest. In research, eye movements have been tracked in many animals across different physiological conditions, revealing that eye movements are indeed reliable handles to many mental and biological processes.

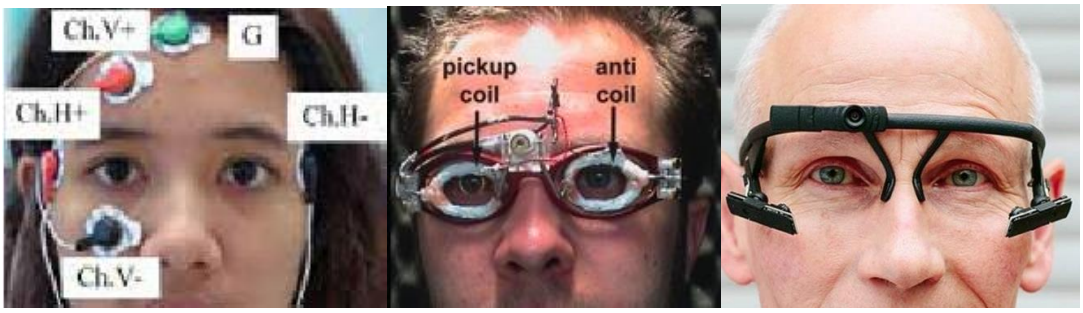

Figure 11: Human eye tracking. EOG electrodes (Aungsakul et al. 2012), magnetic coil (Bremen, Van der Willigen, and Van Opstal 2007) and video-based (pupil labs) eye tracking systems, shown from left to right. Video-based eye trackers provide ease of use and reduced complexity for both the researcher and experimental participants.

Eye tracking has traditionally been done with 3 different methods: magnetic wire coils (Robinson 1963), video tracking (Young and Sheena 1975); (Kimmel, Mammo, and Newsome 2012) and EOG electrodes (Arden and Constable 2006)(Fig. 11). While coils and EOG electrodes have provided the researchers with the initial and important findings about eye movements, the improvements in the optical imaging methods together with machine learning tracking algorithms (Mathis et al. 2018) make video tracking the next best method for eye tracking. Eye tracking has been vastly utilized in psychological and cognitive research through the analysis of gaze, blinking and fixation patterns, revealing personality traits (Rauthmann et al. 2012), signaling attention dynamics and choice inclinations (Orquin and Mueller Loose 2013) (Higgins, Leinenger, and Rayner 2014). Due to ease of use, eye tracking has made its way into practical applications. Many automated driving aids, computer and gaming technologies, identification procedures include eye trackers.

1.2.4. Abnormal pupil size and eye movements in disorders

While in healthy human and non-human subjects pupil size and eye movements reveal dynamics about attentional, arousal, vigilance and emotional states, in disease, trauma and lesion conditions pupil size and eye movement dynamics might be different. It has been shown that the abnormalities in eye movements, pupil size and reactivity are not only a result of the disorders of the extraocular or pupillary muscles themselves but can also be due to the damage or malfunctioning of the upstream substrates. Using eye motility and pupil reactivity tests, neurologists have been able to address what afferent or efferent mechanisms might be affected in the nervous system (Kennard 2011; Wilhelm 2011). Here we will briefly review some of these problems.

There are a limited number of parameters required to define integrity and functionality of pupil and eye movements. Light responsiveness, symmetry in size and coordination in responsiveness are examined to establish that the pupils are working normally (Wilhelm 2011). To test the health of the pupillary efferent systems, subjects are firstly presented with a flashlight on one eye at a time to check that there is rapid, consensual light responses and full return to dilation. Then, under dim and bright light conditions the size of both pupils are controlled and compared to rule out asymmetry (anisocoria). Lastly, to detect differences between the visual afferent pathways, the swinging flashlight test is performed. Bright, indirect light source illuminates one of the eyes for 2-4 seconds then rapidly moves to the other eye, swinging back and forth for several times. While one is illuminated the other eye starts dark adapting and becomes more sensitive to light. While the light is switching to the adapted eye, the pupil first dilates slightly but rapidly constricts with the light flash on it. If one of the pupils does not constrict following this pattern of light stimulation a potential damage in the optic nerves is considered. Testing the near reaction, bringing an object of focus from far away to near the subject, leads to the vergence of eyes and constriction of pupils. Absence of this reaction could signal damage to midbrain or parasympathetic denervation. Pharmacological testing can be carried out to establish the integrity of parasympathetic and sympathetic innervation of pupils and to rule out disorders of iris such as tonic pupil, Horner's syndrome or receptor blockades. By targeting noradrenergic, cholinergic or muscarinic receptors one could block or amplify the signals coming from specific neurons and ganglions into the smooth muscles of the pupil.

Latency, gain, peak velocity and final eye position are used to describe healthy eye movements (Kennard 2011). Eye motility tests are carried out to determine potential disorders of eye movements and related damages. Subjects focus on a target and are instructed to follow it as the examiner slowly moves the target from left to right and up and down in the center as well as the periphery of the visual field. This examination not only reveals whether extraocular muscles are intact, but also gives clues about potential damages in the cranial nerves and gaze-controlling brain regions. Focal damage to control centers for gaze has been shown to lead to abnormal or involuntary shifts of the globes. Frontal, temporal, parietal and occipital lobe lesions including those that are related to dementia and Alzheimer's disease, impact the latency and amplitude of saccades. These lesions might manifest as inability to inhibit or initiate saccades, slowing down of eye movements, misdirecting eyes, disorganized visual scanning, overshooting visual targets when moving the eyes as well as difficulty to conduct smooth pursuit movements (Kennard 2011). Lesions related to neurodegenerative disorders such as Huntington, Parkinson's diseases or Supranuclear Palsy, or lesions in deeper parts of the brain such as basal ganglia, brainstem and cerebellum also result in

oculomotor deficiencies that could easily be used to diagnose or detect the status and progress of these diseases (Kennard 2011; Gorges, Pinkhardt, and Kassubek 2014). Patients with mood disorders such as depression have been shown to have less slow eye movements (SEM) during sleep and more REMs than healthy counterparts (Benca et al. 1997).

Similar to the modulation of eye movements, damage to autonomic and reticular activating systems may lead to alterations in pupil size. Lesion of ciliary nerves and ganglion has been shown to cause tonic dilated pupils (Wilhelm 2011) with the absence or reduction to the light reflex as well as near response. Numerous systematic disorders from diabetes to arthritis have also been described to lead to tonic pupils in patients. Damage to the third nerve has been shown to cause tonic pupil dilation as well, and usually these cases were reported with other neural complications such as tumors, compressions, aneurysm or damages to other nerves in the face (Wilhelm 2011) as in Horner's syndrome. In brief, eye movement and pupil size dynamics have been frequently used as a window into the integrity of central nervous system structures while their changes have been used for assessment and diagnostic purposes in disease conditions.

1.2.5. Changes in pupil size and eye movements in sleep

Pupil size changes during sleep

What is happening behind the closed eyelids during sleep has long been of interest to many researchers. Since the 19th century, various studies have reported that pupils constrict during sleep (Berlucchi et al. 1964; Rechtschaffen and Foulkes 1965; Plotke 1878). This phenomenon was shown to be present in many mammals from visually impaired and intact cats, to infant and adult humans (Berger, Olley, and Oswald 1962; Krastel, Alexandridis, and Rating 1996). Pupil constriction in sleep was proposed to be mediated by the parasympathetic pathway through increased activity in EW nucleus in humans (Berlucchi et al. 1964; Oswald 1960). Atropine has been reported to reverse pupil constriction in sleeping humans (Plotke 1878; Krastel, Alexandridis, and Rating 1996). Pupil size decreases with sleep onset, gradually becomes very small with the appearance of slow and spindle waves and is completely constricted during REM sleep in cats (Berlucchi et al. 1964). With arousals during sleep the pupils rapidly dilate for brief moments, gradually constricting afterwards. Before the actual behavioral sleep begins, fluctuations in the pupil size and pupillary constrictions have been used as a proxy for drowsiness and sleep onset in humans (Yoss, Moyer, and N. 1970). Several studies debated whether pupil size during the day could be a marker of subjective sleepiness and homeostatic sleep pressure (Daguet, Bouhassira, and Gronfier 2019; Van Egroo et al. 2019).

Many pupil tracking experiments during sleep have been carried out in peculiar ways. Many of them included stitched eyelids or eyeball attachments keeping them open and providing access to the iris. These experiments have the limitation of pupillary light reflex dynamics impacting the sleep-pupil dynamics. To counteract this, the animals were visually deafferented. The described experiments included very invasive procedures and no continuous record of pupil size throughout sleep stages.

Eye movements during sleep

Eye movements during wakefulness have been thoroughly described and studied. Due to the eyes-closed nature of sleep and technical challenges such as lack of precision of search coils, and susceptibility of EOG electrodes to high amplitude brain waves, inability to detect very slow movements through these methods, eye movement and pupil size dynamics during sleep are not explored to the finest detail. In this section, we will review what has so far been described in eye and pupil tracking sleep literature.

Implementation of EOG electrodes and search coils have revealed various eye movements during sleep. Saccades during REM sleep, SEMs during NREM1-2 and REM sleep as well as lack of movement during deep sleep have been reported in studies with humans and other non-human animals (Pizza et al. 2011; Porte 2004; Dement and Kleitman 1957b, [a] 1957; Jacobs, Feldman, and Bender 1971; De Gennaro et al. 2000; Porcu et al. 1998; LaBerge, Baird, and Zimbardo 2018; Aserinsky and Kleitman 1955). SEMs have been described as regular, conjugate, sinusoidal, slow and mainly horizontal movements of the eyes at a medium to large amplitude at periodicity between 60s to 3-4s (Pizza et al. 2011; Aserinsky and Kleitman 1955). They are reported to occur at sleep onset as well as during sleep-wake transitions throughout sleep, decline with spindle activity and are absent with delta activity (De Gennaro et al. 2000; Aserinsky and Kleitman 1955). Special to these periods of sleep, high alpha and theta activity have been shown to correlate with SEMs, and they have been shown as daytime sleepiness (Marzano et al. 2007; Porte 2004). In more detail, SEMs decrease as sleep deepens and throughout sleep cycles overnight with the exception of wakefulness in between cycles, suggesting that they are a marker of sleep homeostasis (Pizza et al. 2011).

Rapid eye movements during sleep have been described as jerky, sudden, ballistic, simultaneous movements of the eyes with velocities between 5-200 degrees/s with both horizontal and vertical components (Porte 2004). Despite the ‘rapid’ connotation, more than 65% of the REMs are below 50 degrees/s. Very rapid saccade-like REMs around 200 degrees/s make up only around 10% of them and less than 1% of the REM sleep time (Fuchs and Ron 1968). REMs occur during the REM sleep, disappearing and reappearing with decreasing intervals across sleep cycles through the night (Aserinsky and Kleitman 1955).

REMs are seen at the low voltage periods without delta activity and with prominent theta (4-7 Hz) and beta activity (15-25 Hz). The presence of REMs correlates with other bursting activity throughout the brain such as the PGO waves in the pons and geniculate nucleus and visual cortex (Bizzi 1966; Nelson, McCarley, and Hobson 1983; Miyauchi et al. 2009; McCarley, Winkelman, and Duffy 1983; Peigneux et al. 2001). The eyes are reported to make looping movements during REM activity in sleep (Herman, Barker, and Roffwarg 1983; Fuchs and Ron 1968).

Besides SEMs and REMs a couple of other eye movements have also been described during sleep. Upon the closure of eyelids, eyes are reported to roll up. During NREM the globes stay upward, and during REM they are described to be looking downwards (Jacobs, Feldman, and Bender 1971). In the same study the eye movements are reported to be up to 15% horizontal, up to 35% vertical and up to 65 % oblique across multiple subjects and nights, suggesting a commonality in oculomotor function during sleep. Vertical REMs were also detected during other parts of sleep, usually co-occurring with K-complexes or with body movements (Jacobs, Feldman, and Bender 1971). It has also been suggested that eye location indicates levels of consciousness, central location being the more 'conscious' space. In one single study lucid dreamers are reported to be able to conduct smooth pursuit movements, different from SEMs or REMs, while dreaming about following their thumbs (LaBerge, Baird, and Zimbardo 2018).

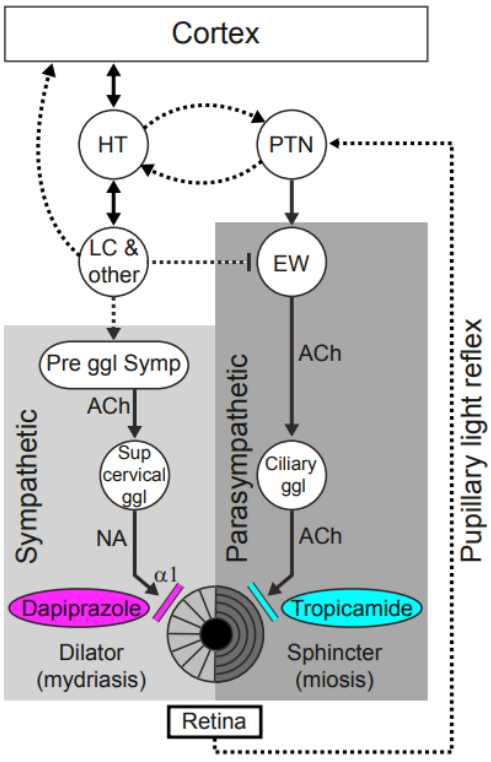

Figure 12: The regulation of pupil size and cortical activity through common parasympathetic and sympathetic pathways. LC modulates the simultaneous activation of cortex and pupil dilation through the sympathetic pathway, while pupil constrictions and synchronization of EEG activity in sleep are carried out through the parasympathetic pathway ((Yuzgec et al. 2018) adapted from (Hou et al. 2007)). Instillation of antimuscarinic Tropicamide eye drops blocks constricting parasympathetic input, resulting in pupil dilation; alpha blocker eye drop Dapiprazole inhibits sympathetic input and reverses pupil dilation.

How eye movements happen to reliably correlate with brain activity during sleep has been a big question since the first discoveries and a few explanations have been put forward (Fig. 12). It has been proposed that the proximity of the substrates in the brainstem for eye movement control and sleep rhythms together with the absence of conscious control might lead to a common waxing and waning of brain rhythms and eye movements (Aserinsky et al. 1985). In other studies it has been further suggested that rhythmic SC activity during sleep might have a role in the generation of SEMs (Porte 2004). It has also been underlined that the oculomotor and reticular activating systems share the same regulation substrates in paramedian pons and the midbrain (Jacobs, Feldman, and Bender 1971). Regarding the SEMs, the firing rate of the oculomotor and premotor cells in the paramedian pontine reticular formation has been shown to decrease with depth of sleep (Henn, Baloh, and Hepp 1984) and it was suggested that spindle activity might be gating the decrease in this nucleus (De Gennaro et al. 2000). In the case of REMs, PGO waves are suggested to lead to the rapid eye movements (Peigneux et al. 2001).

1.3. Novel methods for studying sleep

1.3.1. Importance of new technologies in sleep

Whether it is for sleep tracking in the research laboratories, clinics or at home, the demand for ease, applicability and precision is growing for the newly emerging technologies. Polysomnography (PSG) has been the gold standard for many laboratory and hospital settings, combining brain, heart, breathing and muscle movement monitoring. However PSG is a complex procedure that could almost only take place with the presence of experts in institutional settings. It needs a thorough setup procedure, requires the participant to be attached to multiple electrodes and wires, and the produced large datasets only become meaningful after detailed expert analysis. Therefore, due to costs of PSG, the participants are not tracked for longitudinal studies making the data not representative of long-term changes in sleep physiology. Home tracking customer-focused devices, on the other hand, are available at a very small financial cost to millions of people around the world, provided with easy-to-use software and hardware. The problem is that most of these devices have not been validated across enough participants and conditions to the degree that PSG studies have. Although being able to effortlessly acquire almost life long data sets, their precision, accuracy and analysis methodology are usually at question. These discrepancies beg for further research and development of new technologies that can provide researchers, clinicians and potentially end users a robust sleep tracking device. Here we will look into various existing technologies, their strengths and shortcomings.

The goal of sleep studies is usually to investigate sleep control mechanisms by monitoring or manipulating cerebral and other physiological processes in distinct sleep stages, measure sleep quality and diagnose sleep disorders, which are mostly neurological or psychiatric in nature. With these goals in mind, various techniques and approaches involving PSGs have dominated the field (Van Someren 2006; Sano, Picard, and Stickgold 2014; Willemen et al. 2014; Campbell 2009). In non-clinical fundamental research, where the aim might be to investigate particular processes that take place across different sleep stages, simplifying the PSG procedure and finding a straightforward method for classification has been a driving force (Fraiwan et al. 2012; Robert, Guilpin, and Limoge 1999). To this end, for both animal and human research simpler sleep classifiers have been proposed. These involved technical advancements in how to detect (absence of) movement patterns,simplified EEG recording and extraction, heart rate based sleep classifiers methods. While researchers came up with creative ways to track movement with touch sensors, EMG/EOG electrodes and accelerometers, sole usage of movement has been shown to represent fragmentation of sleep better than different depths of sleep (Morgenthaler et al. 2007; Ancoli-Israel et al. 2003; Watanabe and Watanabe 2004; Yaghouby et al. 2016). The progressive advancement of sleep stages are reported to be less reliably described only with movement activity. Novel EEG configurations through elimination of number of electrodes and algorithmic analysis have helped with speed and ease of distinguishing sleep stages (Koley and Dey 2012; Bagur et al. 2018). The complexity, invasiveness or just the presence of implanted electrodes remained a burden in many of these studies. A new method that is fast and simple enough is yet to be found.

Wavelet analysis of single heart beats have revealed a lot in terms of variability across sleep stages and participants through heart rate tracking (Karlen, Mattiussi, and Floreano 2009; Roche et al. 2003; M. H. Bonnet and Arand 1997). However, low time resolution of heart beats produced limited amounts of data, which made it hard to correlate with faster brain activity during sleep. Heart rate variability analysis, based on frequency domain components of the beat-to-beat intervals, provided additional information on the nonlinear dynamics of parasympathetic and sympathetic regulation (Pichot et al. 1999; Sztajzel 2004; Billman 2011). In light of these advances, usage of heart rate variability during sleep has potential to distinguish distinct sleep stages and sleep disturbances related to autonomic regulation (Chouchou and Desseilles 2014). Recent research indicates parasympathetic involvement in the regulation of stability and fragility periods in NREM sleep (Lecci et al. 2017). Nevertheless, considering the systemic interventions necessary to perturb cardiac activity, which may in turn impact sleep regulation, alternative non-invasive methods might help advance our understanding of sleep dynamics more simplistically.

Sleep tracking consumer products mostly aim for usage and design simplicity (Van de Water, Holmes, and Hurley 2011). Very few of these products have clinical precision or comparativeness (Kelly, Strecker, and Bianchi 2012). Although accelerometers as the primary component have been shown to acceptably detect overall activity, circadian rhythm and arousal patterns, their reliability for sleep stage classification has not been validated. While these devices provide an interesting tool for individuals to visualize their own patterns across days and correlate them with exercise, diet or sleep, they lack methodological clarification and precision (Baron et al. 2018).

Taken together, novel and robust sleep tracking methods hold promise for numerous health and wellness goals. From a clinical and research perspective, technological advances may allow researchers to collect longitudinal sleep-wake data, improving disease phenotyping, individualized treatment and optimization approaches. From a wellness standpoint, advanced and validated consumer products may allow individuals to reliably track their own sleep and eventually modify their behavior or seek professional help.

Importance and potential benefits of eye tracking in sleep

Complementing the missing points mentioned above, eye movement & pupil tracking during sleep through video imaging might provide the technological advancement that is needed in research, clinical and individual home settings. With the advancement of miniature video capture and processing technologies, eye tracking cameras can be negligibly small, providing hardware and software simplicity for both developers and users. Precise pixel information of eye movement and pupil size combined with infrared (IR) illumination as well as machine learning based tracking could give the answer to what the eyes are doing as opposed to the estimated triangulation approach of the EOGs and search coils. Apart from EOG electrodes and scleral search coils, there have been only a few attempts to track eye location with videography during sleep. These studies involve IR illumination, sleep masks or goggles that cover the eyes and video cameras that are recording the eyelids throughout sleep and trying to estimate eye movement information from the reflections on the skin (Kobashi et al. 2008; Wojewnik and Żmigrodzki 2014). Although less tedious and invasive than EOGs or scleral coils, these methods do not reveal the exact eye position and do not carry any information about pupil size. The sufficient level of detail is yet to be found in the existing technologies, we hope that the original research findings of this thesis may be able to fill the gap in the existing literature.

1.3.2. Goal of this thesis: predicting brain states from pupil size and eye movements in sleep

Sleep is composed of large scale brain wide activity, consisting of multiple stages with varying physiological processes at biochemical, cellular and network levels. Each of these stages are critical for the maintenance and improvement of various encounters during wakefulness in health and disease conditions. Given the prominence of sleep related disorders and other systemic, neurological and psychiatric disorders and their close ties with disturbances during sleep, it is of critical importance to reliably assess the central and autonomic nervous system dynamics throughout sleep. In this thesis, we propose a novel method to track eye movement and pupil size in both sleeping mice and humans, revealing their close relationship to cortical and autonomic dynamics that have so far been hidden behind closed eyes.

We first present a novel head-fixed sleep paradigm in combination with infrared back-illumination pupillometry (iBip) allowing robust tracking of pupil diameter in sleeping mice. We found that pupil size can be used as a reliable indicator of sleep states and that cortical activity becomes tightly coupled to pupil size fluctuations during NREM sleep. Pharmacological blocking experiments indicate that the observed pupil size changes during sleep are mediated via the parasympathetic system. We furthermore found that constrictions of the pupil during NREM episodes might play a protective role for stability of sleep depth.

Inspired by our findings in mice, we then present a novel pupil tracking method in sleeping humans combining polysomnographic recordings and infrared eye imaging. We found that pupil size is dynamic throughout sleep, with distinct size distributions across sleep states. Spectral analysis of the EEG signal and cross correlations showed that eye movement patterns and fluctuations in pupil size closely follow cortical activity. The fast changes in pupil size and its positive relationship to the heart rate makes it a promising candidate for tracking autonomic and cortical dynamics in sleeping humans. Similar to our findings in mice, we showed that constricted pupils act as a gating mechanism during deep consolidating parts of sleep, preserving the stability of critical processes. Our findings reveal a fundamental relationship between cortical activity, autonomic rhythms, pupil size and eye movements.

2. Materials and methods

2.1. Materials and methods of mouse experiments

All methods are in the following publication (see Appendix):

Yüzgeç Ö, Prsa M, Zimmermann R, Huber D. Pupil Size Coupling to Cortical States Protects the Stability of Deep Sleep via Parasympathetic Modulation. Curr Biol. 2018 Feb 5;28(3):392-400.e3. doi: 10.1016/j.cub.2017.12.049.

2.2. Materials and methods of human experiments

Open eye sleep procedure

In order to establish a simple pupil size tracking method during sleep, self experimentation tests were carried out. HTC Vive virtual reality headset ("VIVE Series Mainstream PC-VR for Gamers" 2011-2020) was combined with Pupil Labs add-on infrared eye tracking cameras (400x400 pixels, acquired at 30 Hz) ("Pupil Labs" 2020)(Fig. 13A). The volunteers were prepared for the polysomnography recordings by attaching the electrodes (details in closed eye naps). Several minutes before the start of the sleep session volunteers laid in the bed and their eyelids were taped open up to 10 mm by the experimenter. Different taping configurations and tapes were tested including Hypafix stretch medical tape, Nexcare Micropore paper tape, 3M Transpore polyethylene medical tape. Hypafix stretch medical tape showed the highest stretching capability, softest texture and ability to remain attached under high humidity conditions and was therefore used thereon.

Stripes of 15 mm wide and 40 mm long were cut along the stretching direction of the Hypafix stretch medical tape. Three pieces were utilized for the upper eyelid and one for the lower eyelid. Firstly, a single stripe was placed onto the center of the eyelid starting from 1 mm above the base of the eyelashes. Secure attachment was ensured with a soft finger rub on the tape. The tape was gently stretched towards above the eyebrow and securely attached to the orbital rim and the frontal bone. The initial eye opening was up to 15 mms, which was reduced to ~10mm by the end of procedure due to the tape's elasticity. Afterwards, the same procedure was repeated twice, taping the medial and lateral ends of the eyelids open, by attaching them towards the medial sinus or the temporal ridge. One single tape was used for the opening of the lower eyelid, attached to the center if the eyelid starting from 1 mm below the base of eyelashes, and was stretched several millimeters downwards. Same sequence was repeated for the other eye, completing the procedure under 2-3 minutes. Describing the procedure in detail to the participant (where and how the tapes will be attached, explaining the potential initial sensation of stretch, letting them test the flexibility of the tapes) and rapid application of the tapes were found helpful for the comfort of the participant and the quality of

eye recordings. Just before wearing the eye tracking headset, two pieces of single-use viscose make up sponges were soaked in boiled water and were placed to the inner sides of the headset. The sponges were fixed to the inner walls of the goggle with 3D printed elements. The volunteers were given earplugs for noise isolationand a radio-transmitting buzzer to report in case of an emergency. Finally they wore the eye tracking headset and the sleep session started in the light and sound isolated room.

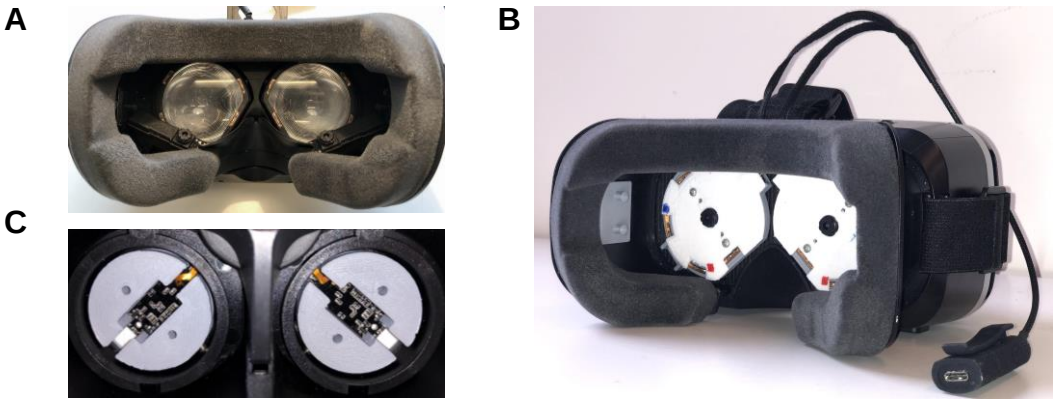

Figure 13: Pupil tracking goggles for sleep

A. Combined HTC Vive and Pupil Labs systems. In this configuration infrared cameras are located in the lower left and right sides of the lenses. B. Combination of Pupil Labs custom cameras and G-Gear virtual reality headset. Sponge holders are fixed on the sides with 3D printed parts (light gray). C. The cameras are fixed with 3D printed parts at 90° to the eyes.

Upon self-assessments of the possibility to sleep with eyelids taped open under described conditions, an ophthalmologically suitable, practical procedure to be used during naps was developed in collaboration with the University of Geneva Hospital Ophthalmology Department and Sleep & Cognition Laboratory at University of Geneva Basic Neurosciences Department. Following a complete ophthalmic examination, two healthy volunteers were admitted for the test procedure. Firstly, an eye stain test was conducted to observe the pattern of corneal dryness in the baseline condition with an orange dye (Fluorescein) under blue light. Then, the volunteers' eyelids were kept open up to 90 minutes while they were awake and wearing the humidified eye tracking goggles as described above. At the end of the session the eye staining test was repeated and the fluorescein marks did not reveal any significant dryness caused by the open-eye procedure.

The ophthalmologically tested procedure was then performed in the sleep rooms located at Brain and Behavior Laboratories at University of Geneva Medical Center. Alternative camera

resolutions and positioning angles were tested to optimize tracking and image quality. The original side location of the Pupil Labs cameras was changed to a central position, facing the eyeballs at a 90° angle when looking straight ahead. These cameras were then updated to a higher resolution (1080p) version, fixed at a side angle. The final eye tracking goggle was developed using high resolution infrared miniature cameras from Pupil Labs (up to 1080p, acquired at 30 Hz), scattered 880 nm infrared light source at 6 mW placed ~5cm from the eyes, 3D printed mounting elements to fix the cameras at 90° angle to the eyeballs and the cheaper GGear virtual reality goggles ("GGear Réalité Virtuelle" 2017)(Fig. 13B-C). This setup provided similar comfort to HTC vive setup at a lower weight, cost and higher image quality. The eye taping procedure was adapted to each participant, requiring longer or more tapes depending on the anatomy of the bones and tissue around their eyes. For example, participants with protruded orbital rims required longer tapes for their upper eyelids and additional tapes were used on the lower eyelids with participants with epicanthic folds.

This procedure was approved by the Geneva Cantonal Commision of Research Ethics (CCER) and was assigned the project ID 2019-01906.

Experimental design

Recruitment process: Healthy male and female individuals ranging from 18 to 40 years old, with no history of neurological, sleep or eye problems were recruited for the study. Individuals who scored lower than 5 in Pittsburgh Sleep Quality Inventory and complied with the demographic criteria (non-smoker, no regular medication, no regular consumption of alcohol and drugs) in the demographic questionnaire were invited for a 2-hour long assessment nap. Individuals who spent more than 70% of the session sleeping, showed no signs of physiological abnormalities in the polysomnography data continued with the ophthalmologic assessment. Individuals with no ophthalmological disorders were invited for a 2 hour long open-eye nap assessment. If they were able to sleep for a minimum of 70% of the session they were enrolled for the study (Table 1).

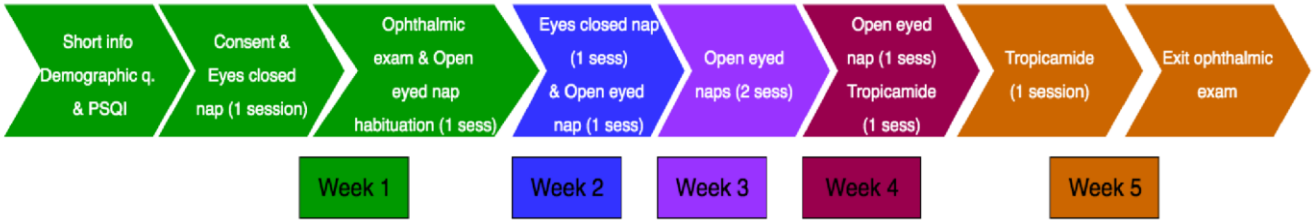

Table 1: Flow of experiments from recruitment to exit examination

Ophthalmic examinations: Before and after the open-eye naps, the participants went through the following exams to assess their eye health regarding: visual acuity, pupil size symmetry and reactivity, eye motility, bell's phenomenon, anterior segment inspection: eyelids, conjunctiva, cornea, anterior chamber, iris, phakic, corneal staining (Oxford grading scale), tear breakup time, Schirmer's test (in mm after 5 min), posterior segment inspection (dilated or with ultra-wide field retinal imaging): vitreous, optic disc, macula, retinal vessels, periphery. All procedures took place at University of Geneva Hospital Department of Ophthalmology.

Closed-eye naps: Polysomnographic data including EEG (electrodes F3,F4, Cz, Pz, O1, O2, A1, A2, F1 - Ground and between Fz & Cz – Reference according to the 10-20 system), EMG (3 electrodes on the chin), EOG (2 channels, left and right eye) and ECG (2 channels, diagonally placed between the right collar bone and below the left ribcage) was collected with a 16 channel recording system (V-amp 16, Brain Products). During the procedure, participants slept in a sound isolated, dark room and wore a humidified virtual reality headset. All sleeping sessions took place at the University of Geneva Brain and Behavior Laboratory sleep rooms. They started between 12:00 and 14:00 and lasted up to 2 hours.

Open-eye naps: Participants' eyelids were gently opened with medical tape up to ~1 cm as described in the open-eye procedure. Participants wore a humidified virtual reality headset (HTC Vive or V-Gear) with illuminated infrared LEDs (880 nm) and eye images were recorded with infrared cameras (Pupil Labs HTC Vive Add-on). Polysomnographic data was simultaneously collected, as described above. All sleeping sessions started between 12:00-14:00 and lasted up to 2 hours. The open-eye sleeping sessions were scheduled with 48 hours timing in between for each participant.

Flashlight (Tropicamide) experiment: Participants were prepared for an open-eye nap session as described above. One hour before the session, one of the eyes was instilled with Tropicamide while the other eye was instilled with artificial tears. A dark piece of sponge was placed over the nose to prevent the passage of light between the left and right side of the face. When the participant started the NREM1 phase of sleep, they were presented with flashlights (580 nm, 10 uW), randomly flickered to the left or the right eye at random intervals (5 to 10 s) for 500 ms. If the participants woke up, the flashes were stopped and were continued again with the start of NREM1 sleep.

Sleep scoring

Classification of sleep into Wake, NREM1, NREM2, NREM3 and REM phases and the scoring of arousals were done according to the AASM manual (Berry et al. 2015) by an experienced observer.

EEG analysis

Cortical electrodes were referenced to the contralateral mastoid electrode. For the cross correlation analysis, the reference signals were band pass filtered (2nd degree butterworth) for the desired frequencies (delta 0.1 to 4 Hz, theta 4 to 7 Hz, alpha 7 to 14 Hz, beta 15 to 30 Hz, gamma 30 to 49 Hz). The amplitude of filtered oscillations was obtained by computing the magnitude of its Hilbert transform. To find the best fit between the filtered EEG oscillations and the pupil diameter, the time constant of the low pass filter maximizing Pearson's correlation (R) between the two signals was evaluated for each sleep state separately (fminsearch function in MATLAB).

Heart rate analysis

Bipolar ECG electrodes were subtracted from each other and the subtracted signal was filtered between 1-150 Hz. To detect heartbeat based on the peak of R waves, *findpeaks* function in MATLAB was utilized. Heart rate was calculated as beats per minute by binning the pulse times into 60 s bins and calculating the mean of the inter-pulse-interval inverse values for each bin. Heart rate was up-sampled to 500 Hz by linear interpolation before calculating the cross-correlations.

Pupil tracking

DeepLabCut (Mathis et al. 2018) toolbox was used to detect pupil diameter and pupil centroid from the acquired eye images (680x400 pixels). To train the DeepLabCut network 20 frames from 5 subjects were chosen and 5 points (4 on the circumference, 1 in the center) were hand annotated. Pupil diameter was estimated as the longest line that connects between the two ends of the pupil from the detected dots on the circumference. Pupil centroid speed was calculated as the two second average displacement of the centroid in x and y coordinates. Before the start of each pupil recording session the participants were guided to make horizontal and vertical eye movements to help calculate the x-y axis of eye movements. They were instructed to look to the left, right, center, top and bottom, annotated by different colored markers (120° angle) for 4-5 repetitions in each direction.

3. Results

3.1. Results of mouse experiments

The results of the animal experiments related to this thesis have been reported in the following publication:

> **Yüzgeç Ö**, Prsa M, Zimmermann R, Huber D. Pupil Size Coupling to Cortical States Protects the Stability of Deep Sleep via Parasympathetic Modulation. Curr Biol. 2018 Feb 5;28(3):392-400.e3. doi: 10.1016/j.cub.2017.12.049.

3.1.1. Contributions of the publication

This publication provides the first continuous record of pupil size changes during sleep in relation to different brain states in mice. It also shows the close relationship of pupil size to ECoG, EMG and ECG activity throughout sleep. In this study, it is revealed that rhythmic pupil constrictions during sleep are mainly modulated by the parasympathetic autonomic pathway and might play a role in preserving the stability of deep, consolidating sleep. I took part in the conception, design and conduction of the experiments, as well as data analysis and the writing of the manuscript for this publication.

The published study aims to show that pupil size dynamics in sleep are indeed easily accessible in head-fixed mice with a novel infrared back illumination of the eyeball (Appendix, Fig. 1 A-D & F, Fig. S2 A-B). These dynamics reveal a relationship that has so far been hidden behind closed eyelids: a tight coupling to the ongoing brain and autonomic activity (Appendix, Fig. 2, Fig. S2 C-G, Fig. S3). For this study, I carried out the baseline head-fixed sleep experiments to describe the simultaneous dynamics of pupil size, ECoG and EMG activity across brain states. I then analyzed the acquired data extracting pupil size and classifying sleep into different stages, carrying out the spectral analysis on the ECoG data to help describe correlation between the pupil size and band-limited oscillations. In order to compare head-fixed sleep physiology to natural sleep, I carried out the free-sleep experiments, analyzed the data for sleep classification and comparative quantification to show similarities between free and head-fixed sleep in time and frequency domains. Additionally, I demonstrated the advantages of retinal back illumination to corneal infrared illumination by comparing light intensity information showing superior quality images of the former.

These baseline experiments showed that head-fixed sleep is comparable to natural free sleep in mice based on sleep architecture and ECoG spectral aspects (Appendix, Fig. 1, Fig. S1). Infrared back illumination substantially facilitates eye tracking in this position and reveals previously unknown dynamics. Our results indicate that pupil size is dynamic across

sleep stages and is strongly correlated to the ECoG activity, more so in sleep than in wakefulness (Appendix, Fig. 2). In NREM sleep, the alpha and more specifically the spindle band is tightly coupled to the pupil size (Appendix, Fig. S2 C-G). This relationship allowed us to predict different sleep states with more than 95% accuracy for NREM and wakefulness (Appendix, Fig. 2 B, D & E). Additionally, we showed that eye movements are also easily detectable with pupillography. With the addition of eye movement information, we suggested that REM sleep could also be identified reliably based on pupillography (Appendix, Fig. S2 B).

This study also sheds light on the mechanisms and potential functional role of pupil size control changes during sleep (Appendix, Fig. 3, Fig. S3). To quantify the involvement of the different autonomic pathways in the regulation of pupil size, I designed and carried out all of the pharmacological experiments. To describe the relationship between pupil size and other autonomic rhythms, I carried out heart beat data extraction from EMG data and correlated that to the pupil diameter in NREM sleep. By using common ophthalmic drops, noradrenergic (with Dapiprazole) or cholinergic inputs (with Tropicamide) to the pupil were pharmacologically blocked and the pupillary fluctuations across sleep simultaneously with ECoG and EMG were measured. These experiments showed that pupil size is primarily regulated by the parasympathetic pathway during sleep (Appendix, Fig. 3 B-G). The positive correlation between pupil size and heart rate also suggested the involvement of the parasympathetic pathway (Appendix, Fig. S3). Our results have shown that the pupil is smallest when the spindle power is at its largest. This may suggest that pupil constrictions provide an additional protection mechanism at the level of the periphery for consolidating sleep. I therefore designed light stimulation experiments combined with pharmacological manipulations to test if the pupil constrictions during NREM sleep have a protective role in preserving the stability of sleep (Appendix, Fig. 4 A, B). By instilling antimuscarinic Tropicamide into one eye and leaving the other eye intact, I was able to determine the deep phases of NREM sleep based on small pupil size. Upon detecting these phases light stimulation was triggered randomly to the pharmacologically treated or the control eye. These experiments revealed that, indeed, mice were pushed towards an arousal-like state shown by the changes in EMGs, delta, alpha and gamma power in the ECoG signal when the pharmacologically dilated pupil was stimulated (Appendix, Fig. 4 C-F). I wrote the introduction, methods, results and discussion sections of the original manuscript corresponding to the experiments and analyses that I conducted.

3.2. Results of human experiments

3.2.1. Pupil tracking is possible in sleeping humans through IR pupillography

Based on our findings in mice, we implemented pupil tracking during sleep in humans. 16 young (18-40 years) and healthy, well sleeping individuals, as determined by the Pittsburgh Sleep Quality Index, and without any eye problems were recruited. After ophthalmologic and polysomnography assessments, 12 participants were confirmed to have healthy eyes and good sleep and were consequently admitted for the open-eye habituation nap. Participants attended a two-hour long daytime nap while simultaneous EEG, EMG, ECG, EOG recordings and pupil tracking were carried out (Fig. 14 A-C). In order to image the pupils, we gently opened the eyelids of the participants with soft medical tape up to ~ 1cm (Fig. 14E). 11 participants managed to sleep with their eyes open without any disturbance and for longer than one hour, and thus were admitted to the study. The positioning of the frontal bone of one participant was not optimal for eyelid opening hence was excluded from the study.

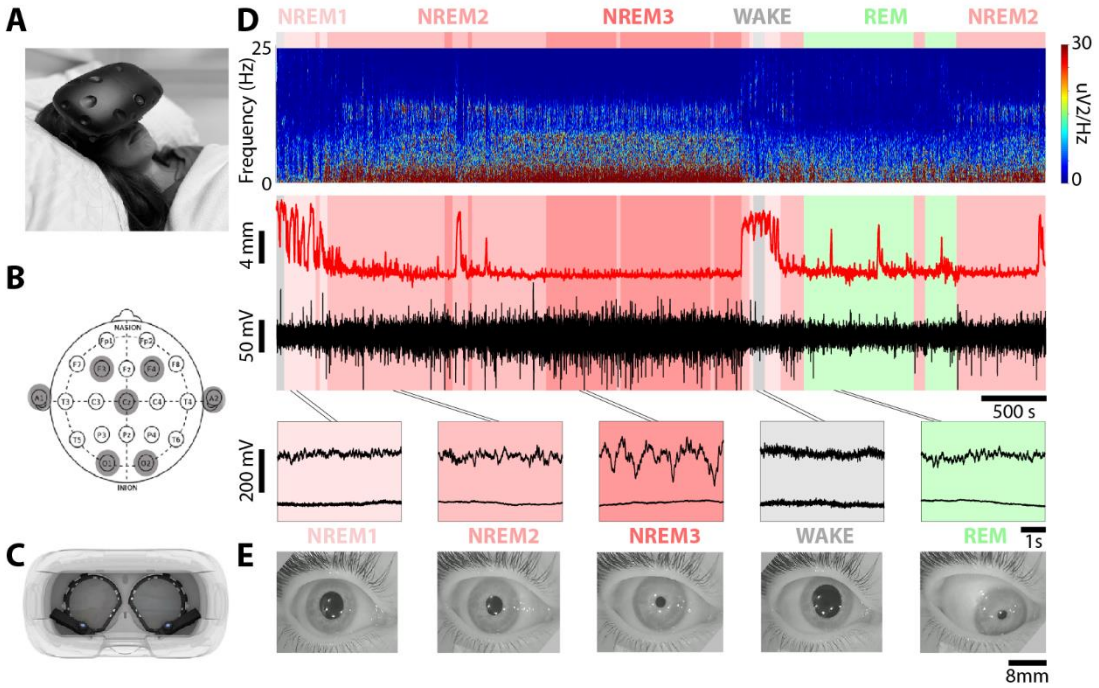

Figure 14: Pupil tracking and simultaneous polysomnography during sleep

A. Participant with modified virtual reality headset during sleep. B. Electrode locations of the EEG recordings (from ersnet.org). C. Internal view of the headset, implanted with miniature cameras and infrared LEDs (from pupil-labs.com). D. Top row: Power spectrogram of F3 EEG signal during wake, NREM and REM sleep states. Second row: F3 EEG signal (in black) and pupil diameter (in red) during different sleep states. Third row: a close-up of the EEG (top) and EMG (bottom) signals during WAKE, NREM, and REM states. E. Images of pupils under infrared illumination in WAKE, NREM and REM states.

Under humidified, sound and light isolated conditions all remaining ten participants fell asleep within the first 2 to 10 minutes and slept up to 120 minutes. Similar to closed-eye sleep, WAKE state was characterized by low amplitude and high frequency EEG activity and high muscle tone (Fig. 14 D). As the participants transitioned into sleep and from NREM1 to NREM3, their muscle tone decreased and high frequency activity reduced. In NREM2 K-complexes, spindle activity was observed and slow waves started to emerge. NREM3 was characterized by large amplitude slow oscillations and spindles. In REM state rhythmic theta activity was observed; the muscle tone was at its lowest and twitches were observed in the EMG electrodes. There were no significant differences observed in the polysomnographic activity between open and closed eye naps based on the sleep architecture, time and frequency characteristics of the naps (Fig. 15). These findings show that open eye sleep tracking procedure allows us to access the pupil diameter without significantly changing sleep dynamics.

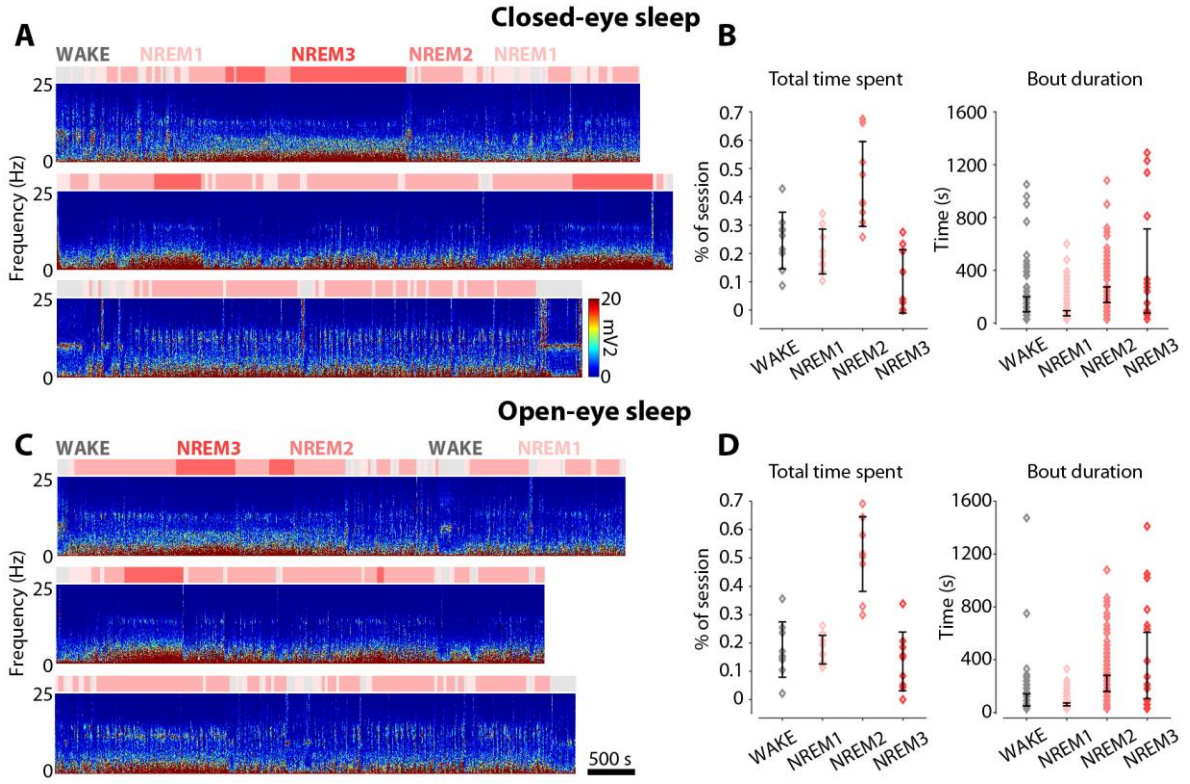

Figure 15: Comparison of time and frequency characteristics of open and closed eye naps

A. Power spectrum and hypnograms from closed-eye naps from 3 different participants. B. Left: mean (± s.e.m.) percentage of time spent in different stages of sleep. Right: mean (± s.e.m.) duration spent in each stage of sleep during closed eye naps (n=8 sessions from 4 participants). C. Power spectrum and hypnograms from open-eye naps from the same 3 participants as in A. D. Left: mean (± s.e.m.) percentage of time spent in different stages of sleep. Right: mean (± s.e.m.) duration spent in each stage of sleep during open-eye naps (n=9 sessions from 4 participants).

3.2.2. Pupil size and eye movements are dynamic across sleep stages

Firstly, we asked whether the pupil size is dynamic during sleep and distinguishable across different sleep stages. Our recordings revealed that pupils remain dilated during the wake state (Fig. 15A). As NREM1 emerges, pupil size starts oscillating between large and medium size, with slow rolling eye movements. In the NREM2 state, pupils are mainly constricted and slow rolling and drifting eye movements can be observed. Anecdotally, in this state the eyes were observed to move independently from each other (non-conjugate) and exhibited vergence movements. Additionally, in NREM2 sleep we observed that slow eye movements come to a halt upon the emergence of spindle bursts (Fig. 16C). In NREM3 state, pupils are maximally constricted and eyes tend to fixate (Fig. 13 A-B, Fig. 16A). In REM state pupils are constricted, horizontal, saccade-like as well as smooth eye movements can be observed (Fig. 16A-B).

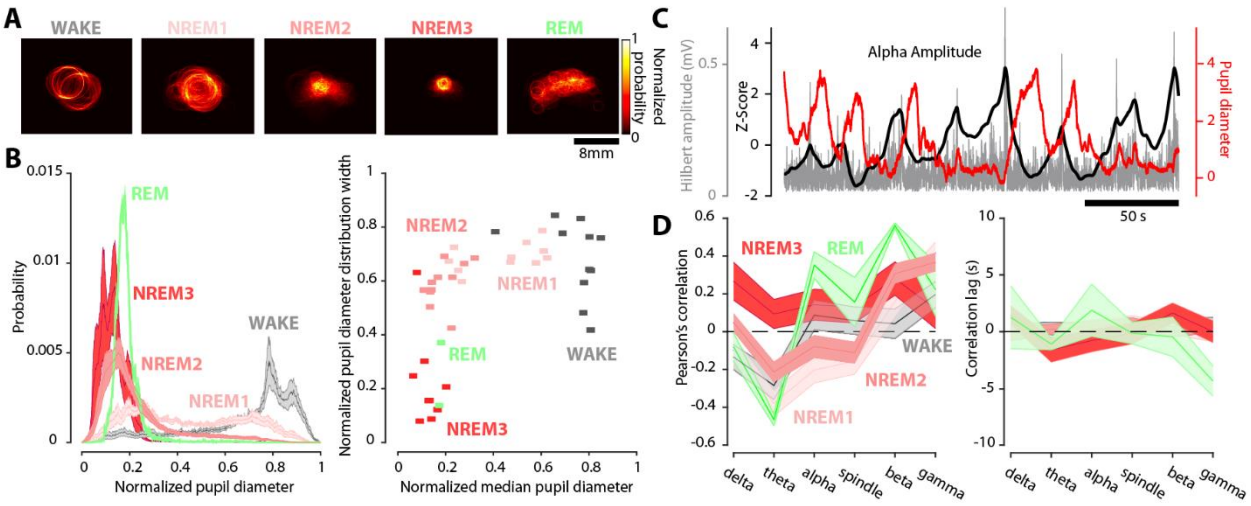

Figure 16: Pupil size fluctuations in different sleep states and its coupling to brain oscillations

A. Probability of the pupil's contour indicating size and movement in one representative session across sleep stages. The pupil diameter has been normalized to its maximum recorded value in each session. B. Left: The distribution of pupil diameter during REM, NREM, and WAKE states (n = 11 sessions from 8 participants, Mean ± SEM). Right: Median pupil diameter versus its distribution width (the middle range containing 96% of the data points) of the 11 sessions. Note that the two REM bouts are from one participant. C. Example session illustrating co-fluctuations of pupil size (red) and the Hilbert amplitude of frontal EEG oscillations in the 7-15 Hz frequency band depicted in gray and its low-passed trace in black. D. Mean (±SEM) Pearson's correlation between EEG oscillatory magnitude of each frequency band and pupil diameter (n = 11 sessions) and the corresponding correlation lags, where negative values indicate that changes in oscillation size lead changes in pupil size (delta: 0.1-4 Hz, theta: 4-7 Hz, alpha: 7-15 Hz, spindles:12-15 Hz, beta:15-30, gamma: 30-48 Hz).

Plotting of the pupil centroid over the eye images revealed the difference in the range of eye movements across different stages (Fig. 17A). Pupil centroid data was then down sampled to 1 Hz and the square root of the difference between x and y locations between each data point was calculated as the eye movement speed. On average, eye movements were quantified to be fastest in REM sleep, slower in NREM1, NREM2 and WAKE consecutively, and slowest in NREM3 (Fig. 17B). Additionally, aligning the pupil centroid movements to the frontal EEG activity revealed the emergence of spindle activity time to the pausing of eye movements in the x and y axes (Fig. 17C). In all NREM states, upon the occurrence of arousals, eyes were observed to roll upwards and in some cases downwards, similar to the Bell's phenomenon (Francis and Loughhead 1984). Plotting the median pupil diameter versus pupil distribution width for each sleep episode (Fig. 16B) revealed five separable clusters, each corresponding to one of the five brain states. This separation could be improved with the integration of additional eye movement parameters and further data collection. This may imply that pupil size can be used as a handle for brain state identification during sleep.

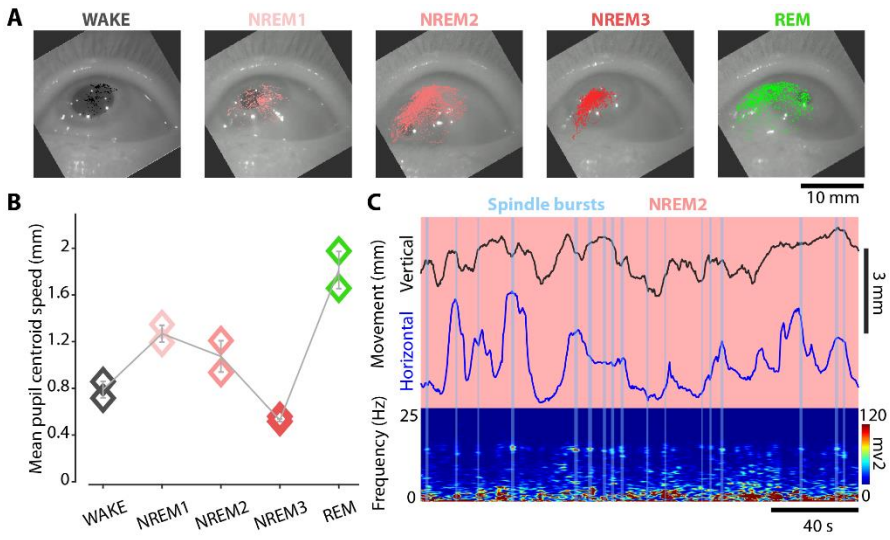

Figure 17: Eye movements across sleep detected by pupil tracking

A. Pupil centroid is plotted on representative images from each sleep state. B. Pupil centroid speed in 2 sessions from the same participant. C. Bursting EEG activity corresponds to slow or absence of moment of the centroid in NREM2 sleep.

3.2.3. Pupil size correlates with band-limited EEG activity in sleep

Pupil size changes have previously been shown to be tightly coupled to oscillations in EEG activity in awake animals (McGinley, David, and McCormick 2015; Reimer et al. 2014; Vinck et al. 2015). Additionally, our findings in mice demonstrated the tight correlation between band-limited EEG oscillations and pupil size changes in sleep (Yuzgec et al. 2018). We therefore asked whether the pupil-EEG coupling observed in sleeping mice is also present in sleeping humans and how it differs across different brain states. To test this, we looked at the

correlation between pupil diameter and the power in band-limited EEG oscillations. Similar to the dynamics in mice, we found co-fluctuations of pupil size and amplitudes of bandlimited EEG oscillations in sleeping humans. Our analysis revealed that the coupling between the EEG activity and the pupil size differs across sleep stages (Fig. 16 C-D), being the strongest relationship observed in the NREM1 and REM sleep. In NREM1 sleep pupil diameter correlated negatively with low frequencies (delta, theta and alpha bands) and positively with higher frequencies (beta and gamma bands). In WAKE and REM states, pupil size correlated negatively with alpha and theta bands, and positively with the rest of the mentioned bands. While in NREM2 sleep pupil diameter correlated positively with delta, beta and gamma power and negatively with theta and alpha power, in NREM sleep the coupling of the pupil-EEG was observed to be always positive. This relationship was comparable across different EEG electrodes. The lag in time of the changes in pupil size and EEG oscillations differed across sleep stages and bands, negative lags indicating the EEG leading pupil. While our results indicate that the coupling of pupil-EEG activity is overall stronger in NREM1, NREM2 and REM sleep than in WAKE, this relationship is weaker in NREM3 sleep than WAKE. Compared to our findings in mice, pupil-EEG coupling appears weaker in human sleep. However, it should be noted that sleep state classification was done automated on 4-second windows in mice, while sleep was classified based on 30s windows in humans by experienced observers according to the AASM guidelines. The 30s-window classification of sleep usually is prone to including different states in one window, leading into mixed frequency characteristics. It should also be noted that the REM sessions in this analysis was taken from a single subject. Therefore, an automated classification based on a larger cohort and smaller window size might lead to clearer description of the pupil-band limited EEG oscillation coupling.

3.2.4. Autonomic markers correlate with pupil size during sleep

Our previous experiments in mice had revealed that the positive correlation between the pupil diameter and heart rate, indicating a predominantly parasympathetic drive for the control of pupil size. Based on these findings we asked whether pupil size and heart rate follow similar trends in humans. Cross correlation analysis revealed that pupil size and heart rate are negatively correlated across all sleep stages, with NREM1 sleep the strongest and NREM3 the weakest coupling (Fig. 18). Therefore, the heart-pupil relationship is observed to be different than in mice.

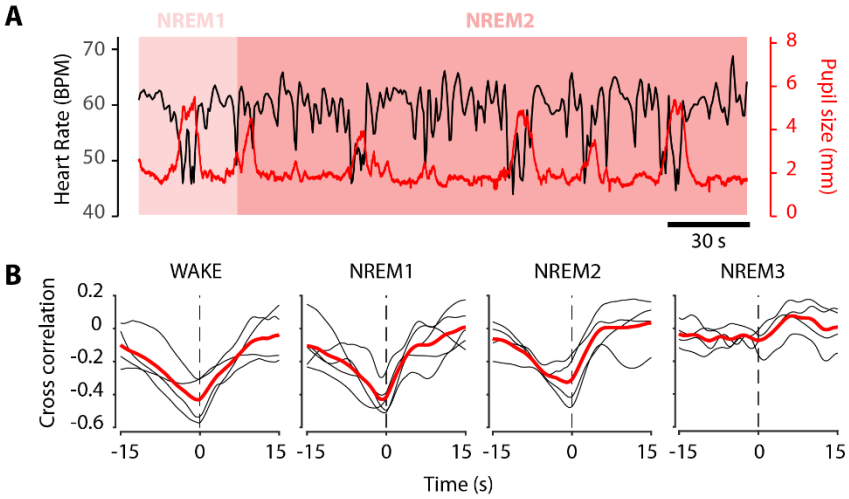

Figure 18: Heart rate and pupil coupling in human sleep

A. Example trace of pupil size and heart rate in NREM1 and NREM2 sleep. B. Cross-correlation of pupil size and heart rate across different brain states. Time=0 indicates zero lag between the two signals (N=4 participants).

We then asked, whether pupil size correlates with other autonomic indicators, namely arousals. Previous studies indicate that sympathetic nervous system activation is involved in the occurrences of arousals during sleep (Somers et al. 1993; Halász et al. 2004) and pupil dilations have been shown to indicate increased sympathetic activity through projections from the locus coeruleus (Bradley et al. 2008; Joshi et al. 2016). Frequent arousals and high sympathetic activity have been implied in autonomic imbalances and sleep disorders (Halász et al. 2004; Miglis 2016; Michael H. Bonnet and Arand 2010; Somers et al. 1995). Therefore we took a closer look into the arousal periods during sleep. Throughout sleep classification, arousals have been marked by experienced observers, as the periods of brief (<10 s) decrease in sleep activity in EEG and increased muscular activity in EMG, which are often followed by sleep rather than wake state and are mostly accompanied by K-complexes. In our experiments, we observed that participants have between 30 to 80 arousals per 2 hour nap (Fig. 19A), mostly in NREM1 and NREM2 stages. In all instances, pupil size increases rapidly with the start of the arousal (Fig. 19B) ($p<10^{-10}$, Student's t-test). The average traces revealed that the arousals are mostly preceded by the K-complexes (180 of 230 arousals).

Our results indicate that pupils dilate rapidly time-locked to arousals during sleep. Given the involvement of sympathetic pathway in sleep arousals and pupil dilations during wakefulness, these results may suggest the potential involvement of the sympathetic nervous system in regulation of pupil size during NREM sleep stages. As the pupil size changes and EEG activity precede the observer-marked arousal onset by a few seconds, upon further validation of our results on larger cohorts, pupil size could potentially be used to predict arousals during sleep.

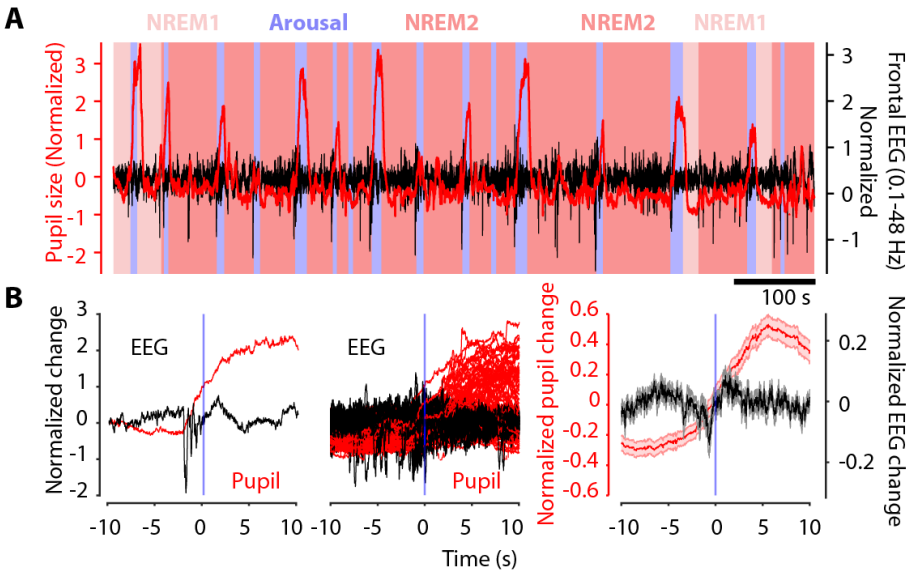

Figure 19: Pupil size and arousals

A. Increase in pupil size can be observed at the time of arousal, mostly coinciding with the K-complexes. B. Example of a single arousal trace (Left). All 80 arousal traces from a single sleep session (Middle). Average of pupil size and EEG changes across 4 participants and 5 sessions, a total of 230 arousals (Right). Time = 0 indicates the beginning of the arousal period marked during sleep scoring.

3.2.5. Pupil constrictions preserves stability of NREM sleep

Our previous experiments in mice revealed that pupil constrictions may act as a protector from visual stimuli, preserving the stability of deep sleep. We therefore asked whether pupil fluctuations may play a functional role in human sleep as well. In order to test this, similar to the mouse experiments we temporarily dilated one pupil by instilling antimuscarinic eye drop, tropicamide, while we left the other eye intact as control (Fig. 20A). We blocked the passage of light between the two sides of the pupil tracking goggles by placing a dark sponge above the nose area. Then, we presented brief, yellow light flashes randomly to the left or the right eye every 5 to 10s, each lasting 500ms (Fig. 20B). Light flashes continued across all stages of sleep unless the participant went into WAKE state. If the participant was determined to be in WAKE state, the light stimulation stopped until the participant was in NREM1 sleep again. During the experiments we simultaneously recorded EEG, EMG, ECG, EOG and pupil activity.

The light stimulation experiment showed that when the light flashes are presented to the pharmacologically treated eye, participants displayed a larger event related potential measured by the difference between peak to trough amplitude, compared to the control eye along with increased EMG activity (Fig. 20C-J)($p<10^{-10}$, Student's t-test) and a larger EOG response ($p<10^{-7}$, Student's t-test). Additionally, EEG activity in the frontal and central regions and the EOG activity were 10% larger in amplitude (Fig. 20G,H)($p<10^{-4}$, Student's t-test). In the occipital regions, the evoked potential amplitudes were comparable for both trial types, however there was 10% larger rebound activity in the drugged trials compared to the control

(Fig. 20I)(p=0.04, Student's t-test). Intriguingly, in the parietal cortex electrode the event related potential amplitude was larger for the control condition than the drugged-eye trials, although this was not statistically significant (Fig 20J). These results may suggest that, at the sensory processing level in the occipital and parietal regions the control and drugged conditions are similar. What propagates to the central and frontal regions, which are associated with conscious perception, may be determined by the function of the pupil. In the drugged-eye stimulations, the visually evoked potentials lead to a larger activation in the conscious processing areas, suggesting that pupil size might be gating the external disturbances from reaching the consolidating brain during sleep.

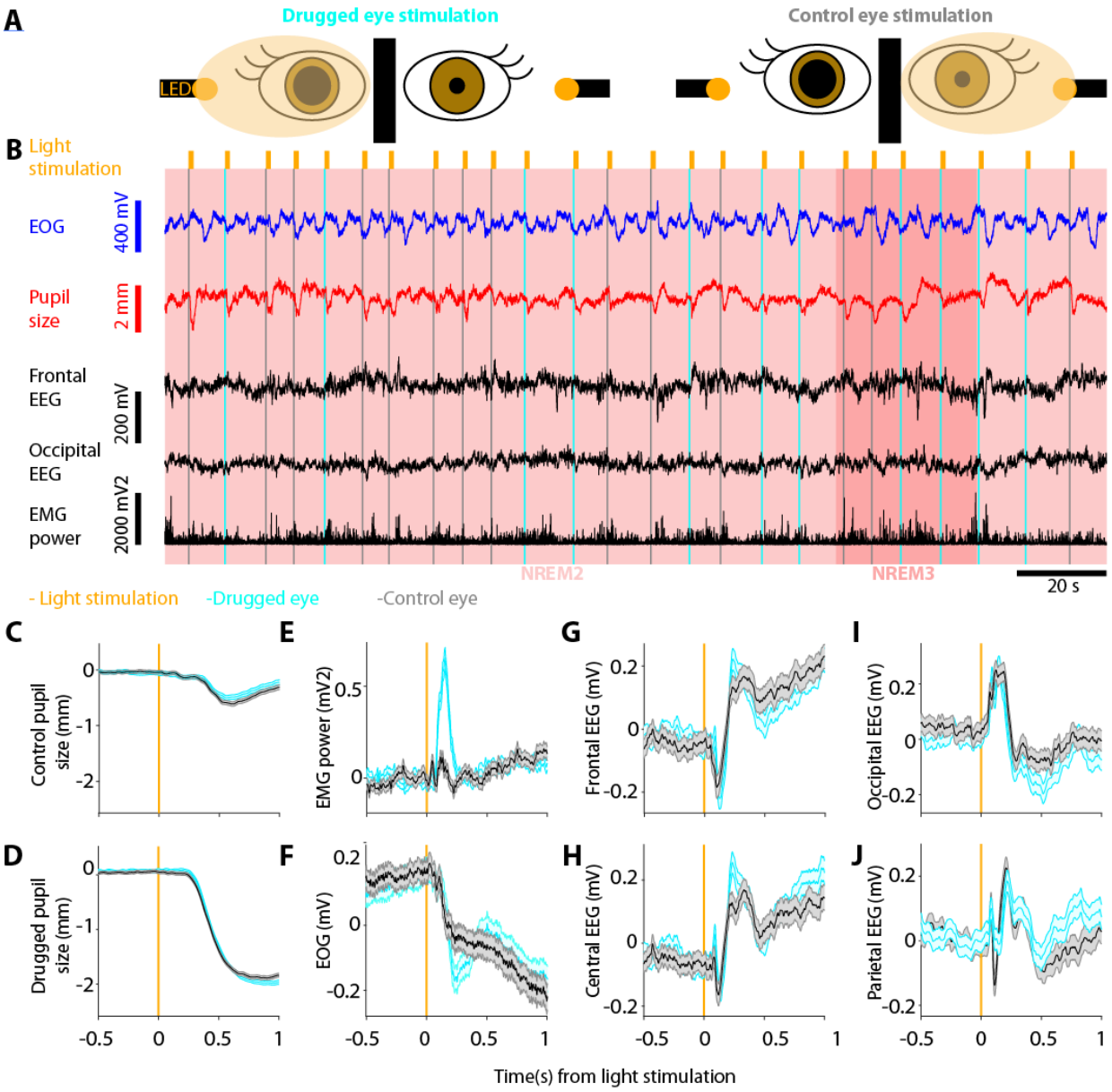

Figure 20: Potential Role of Pupillary Constrictions during NREM Sleep

A. The light-stimulation experiment schematics. B. The timing of the light flashes marked in yellow, color coded by cyan for drugged eye stimulation and gray for control eye stimulation trials. EOG, pupil, EEG (0.1-48 Hz) and EMG activity at the time of stimulation are shown during a typical experiment. Control eye pupil size, EOG and contralateral EEG activity displayed in this figure. C-D. Pupil size at the time of light stimulation (three participants, three sessions, 930 control and 919 drugged-eye stimulation trials, mean [±SEM] in C–J). EMG power in 1-150 Hz (E), EOG (F), frontal EEG (G)(F3 or F4, contralateral to stimulation eye), central EEG (H)(Cz electrode), Occipital EEG (I)(O1 or O2) and parietal EEG (J)(Pz electrode) change in 0.1-48 Hz at the time of light stimulation.

4. Discussion

The development of novel eye imaging methods enabled us to track pupil size throughout sleep in both mice and humans. Our observations of pupil and eye movements led to new findings: pupil size is dynamic across sleep and these dynamics are distinct across different brain states. Band-limited oscillations are coupled to pupil size in mice and this relationship enabled us to reliably predict different sleep states. For humans this relationship persists in a similar fashion, although is weaker. While in mice, pupil size could be mainly regulated by the parasympathetic pathway during sleep, in humans the parasympathetic and sympathetic pathways may both be involved in the modulation of pupillary dynamics. For both species, pupillary constrictions in NREM sleep may play a functional role in preserving the stability of sleep when faced with sensory stimulation. These findings might have several future implications. Pupil tracking could be used as an alternative method for brain state and sleep tracking for mice and humans. Additionally, pupil size can provide real-time autonomic state information with unprecedented speed. Closed loop brain state tracking systems could be developed based on real-time pupil tracking to interact and intervene with experimental subjects. Using pupil tracking in healthy individuals and those with sleep or neurological disorders may lead to new findings in sleep and disease mechanisms as well as new diagnostics and potential treatments.

4.1. Evaluation of the results of mice experiments

4.1.1. The first continuous record of pupil dynamics in sleep: advantages of open-eye and head fixed sleep

Our published results in mice have provided the first and detailed record of pupil size changes in relation to brain and autonomic states. We have shown that mice are able to sleep under head-fixation in a comparable way to their natural sleep. In this position mice sleep with their eyes open, which made it possible to track their pupil size throughout sleep. Based on these observations, the 30° angled, head-fixed sleeping position we developed allowed us to track the changes in pupil size with high accuracy and enabled us to record ECoGs with as little movement noise as possible (Appendix, Fig. S1A). The animals were habituated to sleep in this position within a week's time on daily sessions of up to 4 hours. Their ability to habituate to sleep in a head-fixed position raises questions regarding studies that are done in "quiet wakefulness" (Poulet and Crochet 2018; Poulet and Petersen 2008). The time and frequency characteristics of open-eye head-fixed sleep were similar to the natural sleep of the animals (Appendix, Fig. S1B-C). While additional two-photon calcium imaging experiments (not included in the scope of this thesis) have indicated that the infrared illumination from the

imaging laser is sufficient to track pupils, the iBip system has proven to be a cheap and simple method that provides high contrast images.

Previous studies that reported the pupillary changes in non-wake conditions include video imaging of the eyes in anesthetized rodents and (Pais-Roldán et al. 2020; Szkudlarek, Herdzina, and Lewandowski 2008; Blasiak, Zawadzki, and Lewandowski 2013), anesthetized and sleeping deafferented cats (Borgdorff 1975; Berlucchi et al. 1964). These studies provided first-hand information on how pupil size relates to different brain states in altered states of consciousness. However, a continuous record of pupil size changes across all sleep stages was missing from the picture. Our findings have bridged this gap and are in line with the previous studies. The infraslow oscillations that we observed in the pupil dynamics of sleeping mice were at a similar time scale (~50-100 s) as the ones reported in anesthesia and drowsy states. Additionally, confirming previous findings, our experiments showed the presence of coordinated infraslow oscillations in the cortical activity. These findings may point to a common regulatory mechanism for infraslow oscillations in the pupil size and cortical activity that persists across different consciousness states in mammals.

The mentioned experiments of pupil tracking in anesthesia and sleep involved peculiar methods including suturing or gluing of the eyelids open or spontaneous opening of the eyelids to access pupil size. In our experiments we did not need to provide any additional reinforcement for eye opening: the head-fixed position combined with infrared back illumination of the retina proved to be enough for the mice to sleep naturally with their eyes open. The infrared illumination did not heat up the skull and the iBip method was effectively usable on the same animal for recordings over a month. Perturbation experiments to investigate the neural basis of sleep regulatory substrates usually involve systemic or cerebral injections, which may impact the whole body physiology and potentially, sleep architecture. Conversely, intervening with pupillary muscles to study their sleep regulatory roles was more simplistic: topical eye drops that locally impact pupil muscle receptors without any effect on the central nervous system. Taken together, our results showed the first record of pupil size in sleep through a novel, non-invasive, and natural-like method, revealing its relationship to the cortical and autonomic rhythms that had been so far hidden behind closed eyelids.

4.1.2. Pupil size during sleep predicts brain states

In our studies, spectral analysis of the ECoG signal across sleep stages showed a band-limited coupling of the cortical activity to the pupil size: with alpha and spindle bands showing the tightest relationship to pupil constrictions (Appendix Fig2C-D). Periods of high spindle activity have previously been shown to include consolidation periods, perturbation of which can severely impact memory and performance during wakefulness (Astori, Wimmer,

and Lüthi 2013; Antony et al. 2018). The rhythmic fluctuations in the spindle band has also been previously reported to modulate fragility and continuity periods in sleep (Lecci et al. 2017). Given the tight correlation of the pupil to the spindle oscillations, sleep pupil tracking could, therefore, potentially provide a precise measurement to detect these consolidation periods and perturb them in real time.

Previous studies involving pupil tracking in wake and other states have supported that pupil is a reliable marker of autonomic and cortical changes and could be used to determine different brain states in research experiments as well as practical applications (Hogervorst, Brouwer, and van Erp 2014; Bradley et al. 2008; Onorati et al. 2013). Wake recordings showed the possibility to detect emotional, cognitive, arousal states based on pupil, with potential applications to warn of dangers and predict user performance (Torsvall and Akerstedt 1987; DiNuzzo et al. 2019; Wainstein et al. 2017; Nishiyama et al. 2007). In anesthetized conditions, complementing EEG, blood pressure and breathing recordings, pupil size was shown to indicate anesthesia depth and periods of cortical activation (Blasiak, Zawadzki, and Lewandowski 2013; Wildemeersch et al. 2018). Given the close coupling to autonomic and cortical activity changes shown in our results, pupil size tracking promises to be complementary and potentially better than other auxiliary measures to detect fast and slow changes in brain states.

In order to detect brain states in practical ways, various techniques have been described as alternative to multi-channel EEG and EMG recordings. These included simplified EEG recordings, movement based detectors, heart rate based estimations and multivariate approaches (Bagur et al. 2018; Yaghouby et al. 2016; Virkkala et al. 2007; Harper, Schechtman, and Kluge 1987; Watanabe and Watanabe 2004). Despite remarkable engineering efforts to simplify brain state detection through analytical ways, these methods failed to provide an innovative alternative that is less invasive and computationally simpler than the existing methods. Many of these proposed procedures still involved implanted or attached electrodes and high level analytics of acquired physiological signals. Video based solutions also did not produce the accuracy of physiological recordings (Fisher et al. 2012; Gilestro 2012). The anatomical and functional proximity of the pupil and eye movement control centers to brain state regulatory substrates make them a physiologically compelling candidate for sleep applications. Through verifying on a larger cohort, pupil size and eye movement information is promising readout to predict sleep stages and brain states in an accurate, physiologically relevant, and non-invasive way.

4.1.3. Infraslow oscillations in pupil size during sleep

Infraslow oscillations have been observed across in vivo and in vitro studies in multiple species and brain regions (Llinas and Yarom 1981; Deschênes et al. 1984; Steriade, Nuñez, and Amzica 1993; Fellous and Sejnowski 2000). They have been reported in the thalamus of cats and rodents, cortical activity in mammals and reptiles and their presence was observed across anesthesia, sleep and wakefulness (Libourel et al. 2018; Shein-Idelson et al. 2016). Memory consolidation periods and the behavioral manifestation of sleep disorders have also been shown to follow these rhythms (Vanhatalo et al. 2004; Watson 2018; Ferri et al. 2017; Hughes et al. 2011). Besides electrophysiological recordings, these changes were also detected with functional magnetic resonance imaging (Pais-Roldán et al. 2020).

Our frequency analysis of the pupil size and the ECoG signal have revealed rhythmic commonalities in the two signals: the infraslow fluctuations during NREM sleep (Appendix, Fig. S2E-G). While the pupil size oscillates between its smallest and largest size within 50-100 s, the power in the spindle band (or sigma, 12-15 Hz) also fluctuates between high and low power at a similar time scale. The infraslow changes we detected are similar to those frequently reported in animal physiology (P. Achermann and Borbély 1997; Steriade et al. 1993; Lewandowski and Błasiak 2004; Blasiak, Zawadzki, and Lewandowski 2013; Lecci et al. 2017) as a common denominator of body-wide rhythms. The changes at the infraslow range are often implied in brain activity, indicating periods of responsiveness or alertness. Fluctuations in the spindle power in the sigma band has been shown to correlate with arousability from sleep (Lecci et al. 2017). Given the presence of simultaneous infraslow oscillations in the pupil size and the power of the spindle activity in our recordings, pupil size may be used as a noninvasive measure for detecting fragility and continuity periods in sleep.

Various regulatory mechanisms have been suggested regarding the regulation of infraslow oscillations in the brain: from intrinsic cellular mechanisms in the vasoactive intestinal polypeptide (VIP)-expressing neurons in the suprachiasmatic nucleus (Miller and Fuller 1992; Aton et al. 2005), to zeitgeber properties of multiple interacting substrates and retinal modulation (Szkudlarek, Herdzina, and Lewandowski 2008; Steriade et al. 1993; Ibata et al. 1989). The widespread infraslow activity has also been suggested to be vascular modulated, but this hypothesis is not confirmed through stimulation or perturbation experiments (Drew et al. 2020). Despite unclear regulatory mechanisms of infraslow rhythms, given their importance in the context of sleep regulation and disorders, their simplistic detection through our novel pupillography remains as a beneficial advancement for sleep physiology research. This could be done through real-time closed loop feedback applications to record neural activity specific to fragility or continuity periods in sleep or intervene through sensory or neural stimulations.

4.1.4. Potential origins of pupil-brain coupling during sleep

Pupil size has been shown to be a closely coupled physiological marker to brain and behavioral states. In wakefulness, sensory processing, cortical gain, attention and cognitive availability, stress and emotional responses have been shown to tightly correlate with rapid pupillary changes (Bradley et al. 2008; Partala and Surakka 2003; Pedrotti et al. 2014; Reimer et al. 2014). Additionally, slower co-fluctuations at the range of tens of seconds were also observed in pupil size, brain activity and behavioral states (Palva and Palva 2012). During wakefulness, the locus coeruleus was shown to regulate most of the co-modulation of pupil and brain activity via the sympathetic pathway (Y. Liu et al. 2017; Murphy et al. 2014).

During altered state of consciousness such as drowsiness and anesthesia, in addition to the brainstem substrates, the thalamus, suprachiasmatic nucleus and olivary pretectal nuclei (Blasiak, Zawadzki, and Lewandowski 2013) have been shown to contribute to regulating pupil size changes. Much less is known for the co-modulation of brain and peripheral processes, however there are two main views: firstly, pupil/eye muscle control centers might have a role in regulation of sleep and secondly, there might be a higher order structure that imposes common rhythms to eye muscles and the cortex.

Recent studies showed that the brainstem regions that control pupil size and eye movements also promote NREM sleep (Zhang et al. 2019). Adjacent to the third cranial nerve in the Edinger Westphal, the periocculomotor region was one of the first candidates to be involved in sleep regulation in the early 20th century (von Economo 1930). Similarly, pontine regions have been frequently implied in the regulation of REM sleep and rapid eye movements (Henn, Baloh, and Hepp 1984; Nelson, McCarley, and Hobson 1983). Pontine activity has been shown to be at a comparable time scale as slow eye movements during NREM sleep in humans as well. In the light of these data, it is plausible that pupil and eye muscle control centers might be involved in sleep regulation. Future recordings in these regions further in natural and disrupted sleep conditions would be needed to investigate the relationship.

A compelling line of research shows that several substrates that are involved in circadian and ultradian regulation may be the common driver of the cortical and pupillary fluctuations during sleep. Suprachiasmatic nucleus, olivary pretectal nuclei and intergeniculate leaflet work in coordination in the regulation of circadian rhythms, with feedback provided from the retina and Edinger Westphal (Morin and Allen 2006; Smeraski et al. 2004). These substrates also interface with Locus Coeruleus and have wide range projections to almost all areas in the brain, including cortex and others that modulate vital signs. In this regard, thalamus has also been implicated in the generation and transmission of slow oscillations across brain regions (Neske 2015; Steriade et al. 1993). Additional

experiments with pupil tracking and subcortical recordings during sleep could reveal more about common denominators of the co-fluctuations in these substrates.

4.1.5. Pupil as an indicator of autonomic activity during sleep

Pharmacological manipulations of the pupil size with eye drops revealed that pupillary oscillations are only abolished when the parasympathetic input was blocked (Appendix, Fig. 3A). Blocking the parasympathetic pathway with anti-cholinergic tropicamide instillation reduced pupillary oscillations and uncoupled the pupil-ECoG relationship. These findings suggested that pupillary changes in sleep are primarily modulated by the parasympathetic pathway, through rhythmic constrictions of the sphincter muscle. Whether the cholinergic modulation of the pupillary changes during sleep is locally or or globally regulated would be an interesting investigation in the future. The cholinergic tone is considered to be highest in REM sleep, which is in line with our observation of maximally constricted pupils during REM (M. G. Lee et al. 2005; Fraigne et al. 2015). Two-photon calcium imaging of the noradrenergic and cholinergic axons in the layer I of cortex during wakefulness showed the coupling of pupil dilations to the activity of both types of axons, but more tightly to noradrenaline (Reimer et al. 2016). Similar experiments could be carried out to understand the cortex-wide cholinergic modulation timed to pupil during NREM and REM sleep. If a close relationship is established, measuring pupil size could be an indicator of neuromodulatory input into the cortex.

Our experiments also showed that the pupil size and heart rate are positively correlated in NREM sleep. Heart rate is considered to be parasympathetically modulated during sleep through the vagus nerve in the mice. In line with the existing literature, our findings confirm pupil's ability to track parasympathetic activity. Additional heart rate variability analysis could provide a more detailed description of the pupil-heart rate coupling through high and low frequency oscillations that might be common for both substrates. There are indications that depending on the size or species, perhaps also based on being a predator/prey, the autonomic balances might change. Therefore, whether the positive pupil-heart coupling during sleep persists across different species is another topic for investigation.

4.1.6. Open eyed sleep and sleep protective mechanisms

Our findings in mice have been possible due to the fact that mice can naturally sleep with their eyes open. Other species such as avians, dolphins and humans have also shown this kind of sleep behavior, with one or both of their eyes open (Gole 1999; Mascetti and Vallortigara 2001; Correia Pereira and Firmato Glória 2010), a phenomenon called lagophthalmos, meaning rabbit eyes. In humans open eyelid sleep might be due to nerve damages, other times this is for self protection from predators during vulnerable sleep

(Rattenborg, Lima, and Amlaner 1999). Additionally, some animals display hemispheric sleep for energy conservation purposes and prevention from potential threats during sleep. Given these examples, it is possible that mice might be sleeping with their eyelids partially open due to unfamiliarity and stress from being in the experimental setup. Our anecdotal observation that mice start closing their eyes after a month of being familiarized to head-fixation supports this view. In addition to infraslow changes of fragility and continuity in sleep, this kind of "readiness" might be allowing a window of opportunity to assess the safety of their environments. As the time and frequency characteristics of sleep does not change significantly, head-fixed sleep is a useful paradigm for pupil tracking and other physiological recordings.

Our experiments revealed that pupillary constrictions also act as a periphery based sleep protection mechanism, determining the amount of light that reaches the retina and cortical areas. With constricted pupils, mice are less likely to be pushed towards wakefulness upon light stimulation. Previous research also showed that there are numerous physiological mechanisms that prevent animals from waking up from sleep easily, protecting the continuity of their sleep. Central nervous system based mechanisms including thalamic gating, the occurrence of K-complexes upon sensory stimulation and spindle power modulation allow distinct levels of responsiveness to stimulation (Halász 2016; Fernandez and Lüthi 2020). Peripheral mechanisms include various modulations at the receptor level leading to changes in efficiency of sensory processing, closure of multiple eyelids in some species, and posture adapted to cover sensory organs (Kato et al. 2003; Velluti 1997). Taken together, it would be interesting to investigate the role of pupil size in sleep disorder models such as insomnia and aging to understand its contribution to maintain or shift into deep sleep.

4.2. Evaluation of the results of human experiments

4.2.1. Open-eyed sleep in humans: comparing to natural sleep

Our human experiments have shown that pupil tracking in sleeping humans is surprisingly easy and comfortable. Upon the ophthalmologic confirmation of participants' eye health, they were habituated to sleep with their eyes open within one single session. Gentle opening of the eyelids with medical tape combined with well cushioned, humidified goggles provided the safety and comfort for the participants while enabling us to track pupil size and eye movements with high resolution. Thanks to the light and sound isolation in the sleeping rooms at the end of many sessions the participants needed to be woken up from the naps by the experimenter. There was a larger proportion of participants who were not able to sleep at all during a daytime nap (25% participants) rather than sleeping with their eyes open. Out of the good sleepers and ophthalmologically suitable participants only 2 were excluded due to

lack of sleep or suitability to the setup (20%. In that regard, finding participants who could nap for 2 hours remained the bigger challenge. Unlike many other studies where participants need to sleep in unconventional conditions such as MRI scanners, our participants did not need to be sleep deprived to fall asleep easily (Moehlman et al. 2019). Participants reported the open-eyed sleep to be similar to a meditative state at the beginning of the session and later similar to a "normal" sleep. They expressed not being able to tell if their eyes were open or closed after a few minutes in the dark and at the end of the session. In all, pupil tracking during sleep with open-eyed procedure has shown to be easy, cheap and comfortable.

Time and frequency characteristics of different sleep stages were shown to be similar to closed-eyed sleep (Fig. 14D), including the decrease of high frequency activity and muscle tone in NREM1, emergence of K-complexes and spindles in NREM2, slow waves and spindles in NREM3, minimal muscle tone and high theta activity in REM sleep. The sleep architecture did not reveal any unusual state changes or unusually frequent arousals (Fig. 15). Despite these similarities, it should be kept in mind that the recordings were done in the form of short naps. While recording naps facilitated easier data acquisition and gave us the first impression of the pupil at sleep, whole night sleeps would be necessary in order to assess the full extent of the relationship between pupil size, cortical and autonomic activity.

Another consideration for the human experiments is the nature of day-time naps and how that could potentially impact the results. Although all our sessions were recorded in the afternoon between ~12:00-16:00, they did not strictly start and end at the same time. Considering that metabolic and circadian factors build up towards the night and regulate sleep dynamics, our results might be confounded due to the lack of these factors in the afternoon. While most participants were good sleepers, very few of them reported to be frequent nappers in their day to day lives. Additionally, each participant had different day time activities with varying stress or cognitive loads. It is unclear how this diversity would impact autonomic regulation during a daytime nap. It is possible that during overnight sleep, the modulation of the autonomic activity would be more standardized and pupil-cortical coupling would be stronger. Therefore in the follow up experiments in this research project it would be essential to test varying circadian and metabolic factors in relation to pupil size changes during sleep.

4.2.2. The coupling of pupil size and eye movements to cortical activity in sleeping humans

Simultaneous pupil tracking with polysomnography recordings revealed that the pupil size and eye movements across sleep are dynamic (Fig. 14-16). In contrast to previous records of snapshots of pupil size in sleeping infants and adult humans (Oswald 1960; Krastel, Alexandridis, and Rating 1996) where pupils were reported to be constricted only, our findings

describe variability across sleep stages. During wakefulness pupils remain mostly dilated, with the emergence of NREM1 sleep pupils change between constriction and dilation. Similar pupillary oscillations were previously reported in video recordings of drowsy humans' eyes, suggesting those studies might have included bouts of NREM1 sleep (Yoss, Moyer, and N. 1970). In NREM2 sleep pupil size continues to oscillate but at a much smaller scale. In NREM3 and REM sleep pupils remain mostly constricted, with the difference of near-absence of movement in the former and rapid eye movements in the latter case. The speed of eye movements are estimated to be fastest in REM sleep, then in NREM1, WAKE, NREM2 and NREM3 stages, respectively. These findings differ from the mice, where eye movements were observed to be at a much slower scale in NREM sleep, although not quantified. Therefore, in sleep there seems to be a larger diversity of pupil size and eye movement "repertoire" in humans, as also observed in the cortical activity and in awake behaviors. Taken together, pupil size and eye movements remain to be distinctive across different brain states in sleeping humans but with more diverse parameters than mice.

Spectral analysis of the EEG signal across different brain regions has revealed a similar coupling of the pupil size to the cortical activity that was observed in mice, although weaker. In the lower frequencies including delta, theta and alpha bands, pupil appears to correlate negatively with the EEG activity in NREM1 and NREM2 sleep. In the higher frequencies, this relationship is inverted to a positive correlation. In NREM3 sleep a consistent positive correlation is observed between pupil size and EEG across all frequencies. In REM sleep a similar relationship as in NREM1 is observed, though with a stronger coupling in the theta band and an earlier shift to the positive correlation above alpha band. These phenomena were similar across different EEG electrodes (frontal, central, parietal and occipital). Additionally, we repeatedly observed moments of eye fixation corresponding to the high amplitude spindle activity in NREM2 sleep and rapid eye movements with rhythmic cortical activity in REM sleep. Though a weaker relationship than mice is observed, these first time ever reports of pupil-cortex coupling in humans is promising and open for technical and analytical improvements.

The correlation between the pupil and EEG signal at the infraslow range in the human pupil data was not investigated due to filtering properties of the EEG recording system. An initial analysis of the pupil size fluctuations did not reveal any conclusive results in the 50+ s range but showed a peak of activity at around 10 s. The 10 s range fluctuations may be related to the changes in the pupil to respiratory activity (Borgdorff 1975; Ohtsuka et al. 1988), although breathing rate was not recorded in our sessions. Additionally, how the nap procedure vs. regular sleep that was recorded in the mice (recordings were done during the light cycle) impacts the infraslow pupil dynamics based on circadian factors needs to be investigated.

Further experiments including longer, overnight recording sessions and respiratory tracking are necessary to establish the infraslow changes in the pupil size during sleep.

4.2.3. Predicting brain states with pupils: a comparison

Previous studies comparing the capacity of different physiological measures to predict sleep and arousal states indicated that out of all the measures ranging from breathing rate, skin conductance and eye parameters, pupil is the best predictor after EEG (Hogervorst, Brouwer, and van Erp 2014; Aktaruzzaman et al. 2017). Given the anatomical and functional proximity of the pupil and eye muscle control centers to sleep regulatory circuits, the well established correlations in mice data and the promising human prospects, it would be of large interest for the sleep community to have a pupil-based sleep tracking system to predict arousal and brain states.

Our human data showed the close relationship between the pupil size and band-limited oscillations in the EEG signal across different sleep stages. In addition, spontaneous dilations of the pupil size during NREM sleep were shown to be tightly aligned to arousals during sleep, mostly coupled with K-complexes. A careful examination of the eye movements during sleep revealed that the pupil centroid not only moved at different average speeds in distinct sleep stages, but EEG markers corresponded to specific eye movement patterns such as fixation-timed spindle bursts. Additionally, a variety of eye movement patterns were observed such as convergence, saccades, rolling and drifting movements. Taken together, both pupil size and movement parameters could be used to develop a brain state prediction method during sleep.

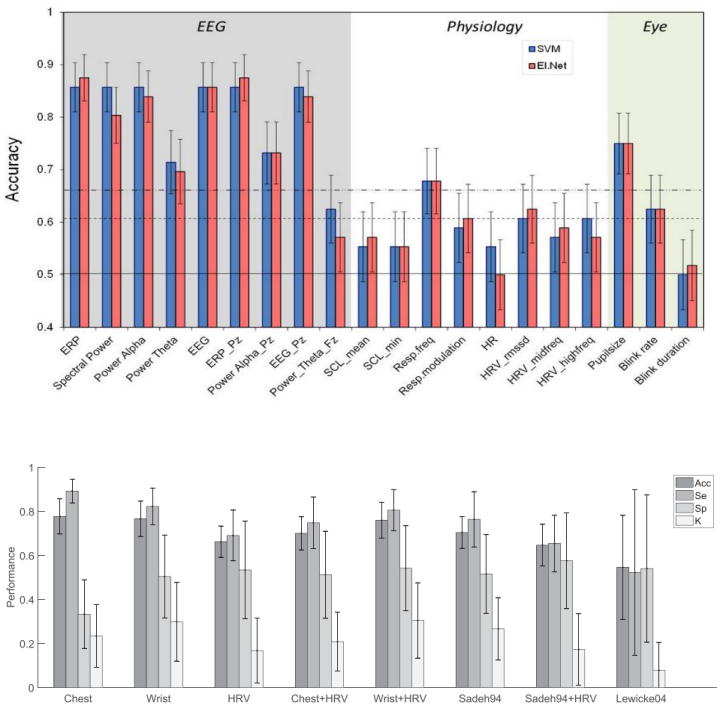

Figure 20: Comparison of pupil size prediction performance in relation to other measurements

Pupil data provides accuracy comparable with EGG data (top, (Hogervorst, Brouwer, and van Erp 2014)) Prediction accuracy of different sleep stages based on combined heart rate and actigraphy is less than 80%, (bottom, (Aktaruzzaman et al. 2017). Our published results using pupil shows accuracy above 95% (Appendix, Fig. 2B). REM prediction accuracy is expected to increase to above 95% by integrating pupil movement information to the prediction algorithm.

Unlike mouse sleep, where rhythmic and low variability fluctuations were observed in the pupil size, EEG and sleep architecture, human sleep is a more complex and diverse phenomenon. The range of defining characteristics within each sleep stage and diversity among participants is wider than it has been observed in mice. Therefore to predict distinct brain states reliably based on pupil parameters, it would be necessary to have a more elaborate computational approach, such as machine learning. This may include multilayer neural networks that would be trained across many participants (from ten participants) across several nights of data each. Given the qualitative characteristics of the human pupil data in sleep, in addition to simple frequency-based approaches, pattern detection and multivariate systems could reveal more conclusive results.

4.2.4. Pupil as an indicator of autonomic activity in human sleep

Our findings in mice had shown that the fluctuations in pupil size were completely abolished with the instillation of tropicamide, showing the dominance of parasympathetic input into pupil muscles during sleep. While the instillation of tropicamide into the participant's eyes almost maximally dilated the pupils (up to 8 mm), with the light flashes, we were still able to observe small changes in the pupil up to 0.8 mm. As it was previously shown in human infants (Krastel, Alexandridis, and Rating 1996), tropicamide alone might not be enough to create a consistent dilation of the pupil and therefore there may be other mechanisms controlling pupil size in humans during sleep than cholinergic input.

Additionally, pupil size in mice was shown to be positively correlated with parasympathetically modulated heart rate during NREM sleep. Contradicting with these findings, we found that in humans pupil size and heart rate are negatively correlated across all NREM stages (Fig. 17). Other studies have also shown a similar relationship between human EEG and heart rate in NREM sleep, which displayed contrasting dynamics to the ones found in mice (Lecci et al. 2017; Abdullah et al. 2010). The exact mechanism that would lead to this negative relationship is currently unclear. In previous studies including ours, with a of ~10 s, the pupil-heart or EEG-heart relationship could be inverted to a positive one. As the heart rate is largely modulated by the baroreflex (Di Rienzo et al. 2009), that takes effectively about 10 s to kick in through each breathing cycle, the delay could be explained by the time constant of breathing cycles. It must be kept in mind that there could also be additional hormonal or chemical mechanisms that are involved in this relationship, which remain to be explored.

4.2.5. Human open-eyed sleep and sleep protective mechanisms

The light stimulation experiments during sleep revealed similar dynamics to the ones we observed in mice (Fig. 19, Appendix Fig. S1). Upon stimulating the pharmacologically dilated or the intact pupil, the pupils showed a time locked, consensual light reflex, however the reverberating effects of this stimulation in the frontal cortex were different for two trial types. While the occipital cortex responses were comparable in both cases, indicating a similar visually evoked response, there was a larger event related potential in the central and frontal regions upon the stimulation of pharmacologically dilated pupil. Functionally, this might imply that pupil size functionally modulates the amount of sensory information that reaches to the conscious-processing regions in the brain, gating the relevant information that might lead the individuals to wake up.

Our findings are comparable to other sleep experiments where processing of auditory or olfactory stimuli were tested, and their relevance determined the modulation of conscious processing during sleep (Portas et al. 2000; Hennevin, Huetz, and Edeline 2007; Bar et al. 2020). For example, while during sleep random words played back to sleeping participants were only detectable in the primary auditory cortex, calling their names resulted in activations in frontal regions and led to a memory of being called their name during sleep. Regarding the visual system, it is possible that pupil size may help determine how far the effects of stimulation reach in cortical processing, allowing windows of opportunity for continuous sleep or waking. In addition to gating mechanisms based on cortical processing, peripheral changes have been described in sensory physiology that impact stimulus processing during sleep (D. A. McCormick and Bal 1994; Murakami et al. 2005). Further experiments could be carried out comparing pupil modulated sensory gating with other sensory modalities and in the closed eyelid condition to determine how well it performs to prevent participants from waking up.

4.3. Differences between mice and human results

On a final note to compare our findings in mice and humans we will focus on the biological, technical and analytical differences between our participants, methods and results. Firstly, from an experimental perspective the animal and human experiments were carried out in different time points of their daily cycles. While the mice in our sleep experiments had their sessions at their usual sleep hours in the day-night cycle, humans slept during their usual wake times. The autonomic rhythms and metabolic processing were known to be different in circadian sleep and wake times, which might impact experimental results (Huang et al. 2011; Burgess et al. 1997; Aston-Jones et al. 2001). Additionally, the human sleep sessions were in the form of two hour naps instead of overnight sleep. Moreover, while the cortical activity

was recorded with ECoG electrodes in mice, in humans we utilized scalp electrodes, all of which may impact the observed dynamics and the relationships.

Secondly, there were various differences in the analysis of human and mice sleep data that might contribute to the diverging results. For sleep classification for mice, we utilized an automated approach, setting threshold limits for band-limited oscillations in the ECoG and EMG amplitude to decide on different sleep stages with 4 s windows. In the human data analysis, sleep stages were detected by experienced observers who visually inspected EOG, EMG and EEG channels simultaneously and decided on the sleep stages on a 30 s window basis, as advised by the AASM manual. Although that is the gold standard for human research, increased window size leads to the mix of frequency characteristics and may therefore weaken the correlation of pupil size per sleep stage based on band-limited oscillations. Alternatively, to determine the relationship between pupil size and cortical activity in human data more detailed machine learning approaches could be carried out rather than simple correlations. These might include multi layer neural networks and decision trees that utilize multivariate approximations to establish which pupil size and eye movement patterns correspond to which EEG activity patterns.

Lastly, inherent biological differences between mice and humans need to be considered when evaluating their compatibility. Mice and humans show differences in sleep architecture, brain size and neuromodulatory regulation (Brown et al. 2012; S.-H. Lee and Dan 2012). In humans individual differences in physiology based on genetics and environmental factors are larger than inbred lab mice. The varying parameters in sleep habits, brain physiology as well as daily routines may contribute to the different results in the experiments. Further analysis, methodological changes and larger cohort size would be helpful to explain the phenomena in more detailed and paralleled ways.

4.4. Practical implications of pupil tracking in sleep

Importance of determining brain states in non-wake conditions through alternative methods

Decades of research show that brain states determine performance, learning outcomes, development phases, disease condition and progression and many more (Alhola and Polo-Kantola 2007; Diekelmann and Born 2010; Anderson and Bradley 2013; Dahl 1996). During wakefulness humans can not only report their subjective experience regarding the change in their internal states but mostly, these states also present through behavioral manifestation. On the other hand, during sleep, anesthesia and other altered states of consciousness, there are more subtle changes in the behavior, and reduced reactivity to stimulation while there are still substantial changes happening in the brain states. Therefore

it remains a crucial endeavor to detect and potentially intervene with alternating brain states in these situations.

Currently the gold standard measure for detecting brain states is through EEG recordings, for both rodent and human research. While this is a thorough and accurate procedure, it is also a costly and complex method that is highly prone to electrical noise. In addition to EEG, other auxiliary measures are usually taken to monitor muscular or peripheral changes including heart rate, skin conductance and breathing rate. These methods are informative yet have slower time constants than rapidly changing brain states. With these in mind, usually periods of arousal, epileptic seizure, autonomic collapse during sleep are not optimally detected. Taken together, pupil tracking in sleep has a high potential to facilitate simpler experimentation and implementation and guide into new directions in physiology and sleep research.

4.4.1. Simplification of sleep tracking and cost reduction

In sleep research the most common method used for physiological tracking of brain and autonomic states is through polysomnography, with the main element being EEGs. These multi-channels systems, even with the most simplified versions (Khushaba et al. 2013) require expertise in implementation, analysis and interpretation of results. The procedure usually involves multiple experts and expensive hardware costing thousands of dollars only for equipment. The experiments have a notoriously long setup time, making it tiresome for both the experimenter and participants, in many cases leading to dropout.

Pupil tracking in sleep is a simple procedure that requires very little expertise from the experimenter. The hardware involves inexpensive equipment costing less than CHF 1’000 and implementation only takes a few minutes, which reduces setup time as well as cost related to hardware and operation. This procedure is also confirmed as surprisingly comfortable by the participants. Unlike EEG signals, video-based eye tracking is less prone to electronic or movement noise that might contaminate data in critical moments of eg. sleep seizure detection.

Moreover, image processing is a faster and simpler procedure than EEG analysis. Most of the EEG acquisition hardware comes with specific and proprietary software, operation of which often requires paid-authorization and knowledge in signal processing. Pupil tracking based on images can be done for free utilizing cutting edge open source tool boxes with well documented user interfaces (Mathis et al. 2018). With the advancement of machine learning techniques the data from only a couple of sessions can be used to extract necessary parameters from all acquired experimental sessions. Extracting parameters from eye images is simple: linear measures (pupil diameter, pupil centroid location), extracted rapidly across

participants and sessions that are shown to be coupled to brain and autonomic activity. Given the negligible size of the extracted parameters, pupil data has also the potential to be acquired in very large scales and analyzed over big datasets. Taken together, pupil tracking carries a high potential to be a cheap, comfortable and practical alternative to be used in sleep experiments.

4.4.2. Technological advantages for closed-loop interventions

Given the advancements in high-performance miniaturized electronics and availability of real-time processing capabilities of image processing toolboxes, pupil tracking during sleep has a high potential for real-time applications for research and interventions. Currently, a bundle of high performance graphics, central processing units and microprocessor-level microcontrollers can fit inside the palm of the hand ("Teensy 4.1 Development Board" n.d., "Cortex M7," n.d., "Camera Module V2" n.d.). Together with high resolution miniature infrared cameras, designing compact and comfortable pupil tracking goggles for sleep is no longer beyond imagination (Fig. 21).

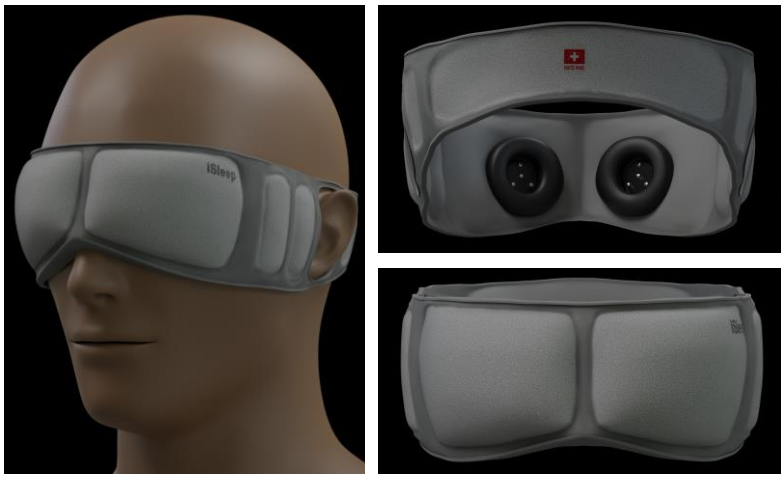

Figure 21: New 'iSleep' goggles Graphical rendering of the next generation, comfortable and compact iSleep goggles prototype. Combining soft fabric, 3D printed parts and a rechargeable battery for the camera and LEDs, iSleep will provide enhanced comfort for the users. Design by Javier Gesto and Ozge Yuzgec with Blender software.

Real-time pupil tracking sleep goggles could be used for instant detection of brain states, interacting with the participants and potentially interfere with them in critical moments. Implementation of a real-time pupil tracking and brain/autonomic state detecting sleep goggles could potentially be used in basic and applied sleep research to determine the memory consolidation periods and instances of high/low autonomic pressure in relation to ongoing cortical processes. Pupil tracking could also be implemented in combination with other visual, auditory or olfactory stimulations for interacting purposes and potentially be used as a guide for interventions. For example, in the case of obstructive sleep apnea, it is of utmost interest

to intervene with the patient through nerve stimulation at or if possible before the moment of blockage and loss of breath. Pupil tracking could help guide treatments or diagnostics developments that involve such in real-time interventions. Development of a compact and clinically proven eye tracking system for end users could potentially enable simplistic and accessible home treatments including light stimulation therapy for circadian disturbances with optimal performance.

4.4.3. Enabling the discoveries in autonomic dynamics in sleep

More than a century long research and inventions have explained the role of sleep regulation in relation to learning, development, disease progression and survival of animals (Rasch and Born 2013; Anderson and Bradley 2013; Diekelmann and Born 2010; Imeri and Opp 2009). While the discovery of EEG was crucial for describing physiological sleep, magnetic resonance imaging, optogenetics, peripheral recordings, and video tracking helped put the pieces of the sleep puzzle together. Development of a real-time, reliable and simplistic pupil tracking system is bound to open doors for many more regarding autonomic regulation.

Current research indicates that the autonomic nervous system contributes to the regulation of brain activity changes that result in the long-term strengthening or weakening of memory traces (Whitehurst et al. 2020, 2016). Additionally, in relation to autonomic rhythms, neuromodulatory influences have been shown to shape memory processing during sleep (Hasselmo 1999; G. R. Poe et al. 2020), which can be monitored by pupil tracking (Bradley et al. 2008; Sharon, Fahoum, and Nir 2020). It would be of interest to the community of sleep researchers to use pupil tracking not only as a handle for consolidation periods but also as a measure of autonomic activity related to memory formation. This has so far not been achievable with current measures such as heart rate or skin conductance, that are less reliable or slower. The combination of pupil tracking with existing methods in animal research will enable detailed descriptions of autonomic changes and their relationship to neural circuit dynamics through two-photon calcium imaging and electrophysiological recordings.

Describing autonomic rhythms during sleep at high resolutions can also help researchers explain not only sleep disorder mechanisms but potentially other neurological or psychiatric disorders where autonomic disturbances are implied (Bremner 2009; Moseley et al. 2013; Barbanti et al. 2002; Benarroch 1993). The range of applications of pupil tracking in experimental clinical research can include insomnias, apneas, parasomnias, nightmare disorders, sleep epilepsy, migraines and glaucoma (Larson and Behrends 2015). Pupil tracking in sleep could be experimented in novel approaches towards understanding disease predisposition, progression and early diagnosis, prediction of disease episodes and treatment outcomes.

4.5. Outlook and conclusion

The necessary steps to be taken after this thesis include experimental, analytical and technical improvements. Additional experiments with genetically modified and potentially blind mice would be helpful for a circuit-based interrogation of the role of pupil size in autonomic modulation and visual stimulus gating. Then, an automated approach to sleep state classification and a machine learning approach to determining pupil and cortical dynamics would be necessary. Night time sleep recordings in humans with full polysomnographic assets including high density EEG could help describe various interacting mechanisms involved in pupil size regulation in relation to other physiological measures in sleep. To facilitate future human applications, pupil tracking with closed eyelids potentially involving infrared illumination from the temples and contact lenses with integrated infrared sensors would be highly beneficial.

Our technical efforts to track pupils during sleep have led to development of a novel method in sleep monitoring. Using this method, our experiments with mice and humans have revealed the tight relationship between pupil size and eye movements to the cortical and autonomic dynamics, which was so far hidden behind closed eyelids. Utilizing this relationship has the potential to change the way to analyze and investigate cortical and autonomic nervous system dynamics in sleep physiology in a completely novel way with unprecedented speed and precision. Pupil tracking in sleep could help substantially reduce research materials and operational costs while enabling researchers to explain sleep regulation and disorder mechanisms. Implementation of sleep pupil tracking in clinical research could open doors to new diagnostic methods, treatment options and disease state assessment tools. New engineering approaches towards pupil tracking in sleep may enable user applications, providing the next-generation home-care system in the wearables market to be used for self-monitoring and lifestyle improvement.

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APPENDIX

Article

Current Biology

Pupil Size Coupling to Cortical States Protects the Stability of Deep Sleep via Parasympathetic Modulation

Highlights

- Infrared back-illumination allows accurate pupillometry in sleeping mice
- Brain activity and pupil diameter are tightly coupled during sleep
- The parasympathetic system is the main driver of pupillary changes during NREM sleep
- Pupillary constrictions might have a protective function to stabilize deep sleep

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In Brief

Using infrared back-illumination pupillometry in head-fixed sleeping mice, Yüzgeç et al. show that pupil diameter is tightly coupled to cortical states during sleep. Pharmacological and light-stimulation experiments reveal that the pupillary constrictions are parasympathetically driven and might have a protective function to stabilize deep sleep.

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Current Biology
Article

Pupil Size Coupling to Cortical States Protects the Stability of Deep Sleep via Parasympathetic Modulation

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SUMMARY

During wakefulness, pupil diameter can reflect changes in attention, vigilance, and cortical states. How pupil size relates to cortical activity during sleep, however, remains unknown. Pupillometry during natural sleep is inherently challenging since the eyelids are usually closed. Here, we present a novel head-fixed sleep paradigm in combination with infrared back-illumination pupillometry (iBip) allowing robust tracking of pupil diameter in sleeping mice. We found that pupil size can be used as a reliable indicator of sleep states and that cortical activity becomes tightly coupled to pupil size fluctuations during non-rapid eye movement (NREM) sleep. Pharmacological blocking experiments indicate that the observed pupil size changes during sleep are mediated via the parasympathetic system. We furthermore found that constrictions of the pupil during NREM episodes might play a protective role for stability of sleep depth. These findings reveal a fundamental relationship between cortical activity and pupil size, which has so far been hidden behind closed eyelids.

INTRODUCTION

Fluctuations in cortical states may determine learning efficiency, impact performance, and predict decisions [1–3]. Electrophysiological measurements, such as electrocorticograms (ECoGs), are reliable indicators of different cortical states and have as such been used to determine optimal conditions for sensory processing or cognitive performance [4]. Other physiological measurements, such as pupilometry, can be used as a non-invasive proxy for tracking vigilance states during behavior. Variations in pupil size can predict arousal [5], vigilance levels [6], and emotional responses [7] and reveal choice inclinations [8]. Due to its inexpensive and simple methodology, pupilometry has attracted not only the interest of neuroscientists, but also that of market researchers, athletes, and engineers. It became as such the focus for the development of practical applications, such as drowsiness detection while driving [9], fatigue [10], and mental health assessments [11] or affective state classification [12].

More recently, studies in rodents and non-human primates have investigated interactions between cortical states and pupil diameter and found a close coupling during quiet wakefulness [13–15]. Follow-up studies combining optical imaging, electrophysiology, and modeling revealed that the link between brain activity and pupil diameter might be modulated by noradrenergic and cholinergic systems [16] and confirmed the occurrence of transient periods for optimal sensory processing.

Given the existing link between pupil size and cortical states during wakefulness, we asked whether a similar relationship persists during sleep. Changes in cortical states are very prominent during different sleep phases, while cognition- and sensory-related processes are strongly reduced. Would the pupil thus still fluctuate and what kind of changes would these fluctuations be related to during sleep? Might the pupil play a specific functional role, disturbance of which might interfere with sleep? In this study, we combined a novel approach allowing pupillometry in head-fixed naturally sleeping mice together with electrophysiology and pharmacology to find the answers to these questions.

RESULTS

Head-Fixed Sleeping Paradigm

The main challenge for pupil tracking during natural sleep is that mice often close their eyes, incline their head, or curl up (Figures 1A and 1B). Since pupil tracking is greatly facilitated during head-fixation as compared to freely moving conditions [17], we developed a setting allowing mice to fall asleep under head-fixed conditions. Using X-ray-based posture analysis, we adjusted the head angle and body position to mimic the body's natural position observed during quiet wakefulness and sleep (Figures 1B, 1C, and S1A; STAR Methods). Mice were gradually habituated to sleep in this position within 7 days in sound- and light-isolated boxes. ECoGs and electromyography (EMG; STAR Methods) of the neck muscle were used to monitor and quantify sleep states (Figure 1E). Recordings started while mice were awake and lasted up to 4 hr, most of which was spent in non-rapid eye movement (NREM) sleep with intermittent periods of rapid-eye movement (REM) sleep and wakefulness (awake) (Figure 1E). Similarly to sleeping mice in freely moving conditions [18, 19] (Figures S1B and S1C), awake states were characterized by high-amplitude, continuous EMG activity and high-frequency,

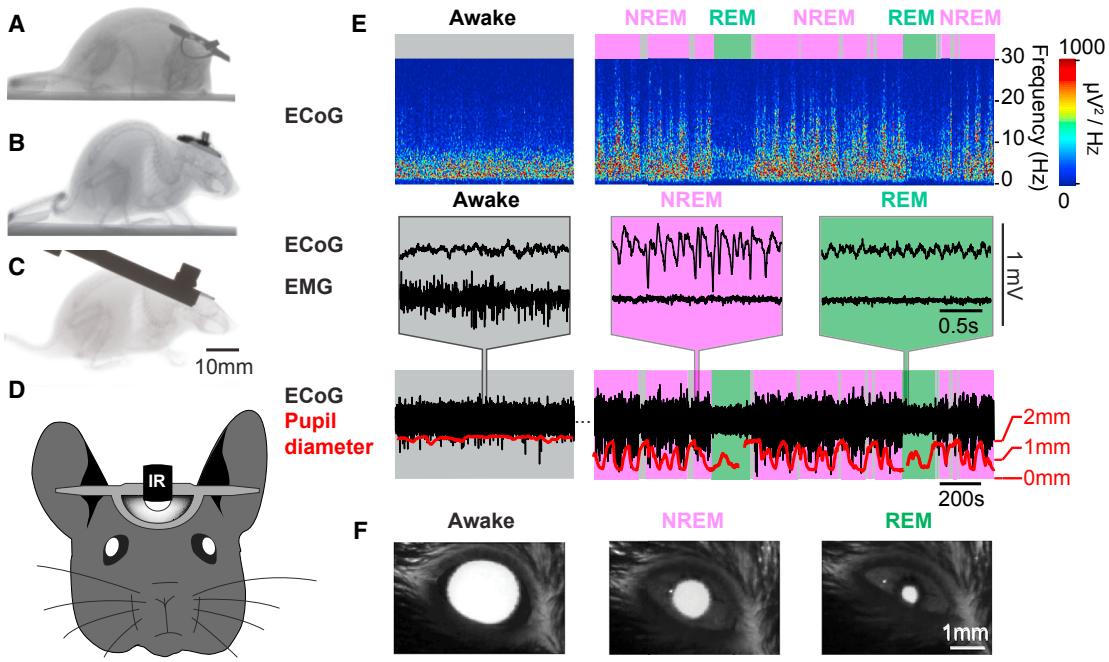

Figure 1. Head Fixed Sleep and iBip Pupil Tracking

(A) X-ray images of a naturally sleeping mouse in a curled-up position.

(B) Position of a sitting mouse during natural sleep.

(C) Head-fixed mouse with a head angle of 30 degrees.

(D) Front-view schematics of the infrared back-illumination pupillometry (iBip) pupil-tracking system. The 940 nm infrared light-emitting diode (LED) is placed above the skull, which allows the bright LED light to penetrate the head and back-illuminate the pupils.

(E) Top row: power spectrogram of M1 ECoG signal during awake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep states. Second row: a close-up of the ECoG and electromyography (EMG) signals during awake, NREM, and REM states. Third row: ECoG signal and pupil diameter during different cortical states. Data are from a habituated head-fixed mouse. The pupil diameter data are missing due to eye blinks at the beginning or end of REM periods.

(F) Images of pupil with infrared back illumination in awake, NREM and REM states.

See also Figures S1 and S2 and Movie S1.

low-amplitude oscillations in the ECoG signal. NREM sleep was defined by high-amplitude ECoG and low or absent EMG activity. REM sleep was characterized by prominent ~7 Hz oscillations in the ECoG signal and complete disappearance of EMG activity (Figure 1E). Surprisingly, we found that head-fixed mice consistently sleep with their eyelids partially or fully open (Figure 1F), which allowed us to have access to the pupil diameter during continuous, natural-like sleep. Thus, we found that mice are able to sleep under head-fixed conditions, showing sleep patterns comparable to natural sleep under freely moving conditions (Figures S1B and S1C), yet with open eyelids [19, 20].

High-Contrast Infrared Back-Illumination Pupillometry

To enhance the contrast of the pupil for reliable tracking during head-fixed sleep, we developed infrared back-illumination pupillometry (iBip; see STAR Methods). For iBip, a 940 nm LED light source was placed on the skull of the mouse above frontal cortex, thereby illuminating the structures inside the head including the brain and the back of the eyes. When we imaged the eyes with infrared video cameras, the pupils appeared brightly illuminated (Figure 1F; Movie S1) and allowed reliable high-contrast tracking of their diameter and movement dynamics during natural sleep (Figures 1E, S2A, and S2B).

Pupil Size as an Identifier of Awake and Sleep Brain States

We first asked whether pupil diameter is qualitatively different during different sleep states. The average distribution of pupil size in darkness revealed that during the awake state, the pupil remains dilated most of the time (Figure 2A). During REM sleep (Figure S2B; Movie S1), it remains mostly constricted, whereas during NREM sleep, the pupil's diameter continually oscillates between small and large (Figure 2A). Plotting the median diameter versus its distribution width for each session yields three clearly separable clusters of points each corresponding to one of the three brain states (Figure 2A). Indeed, a K-means clustering analysis correctly partitioned 46 out of the 48 points into three sleep state categories.

The latter result implies that pupil size can be used as a reliable signal for brain state identification during sleep. To explicitly test this, we trained a neural network machine learning algorithm by providing the pupil diameter signal to its input layer and the sleep state label, identified based on the characteristics of corresponding ECoG and EMG signals, to the output layer. Each training trial consisted of a 100 s chunk of data, and we trained the neural network separately for each session by including datasets from all sessions but that one. The data of the remaining

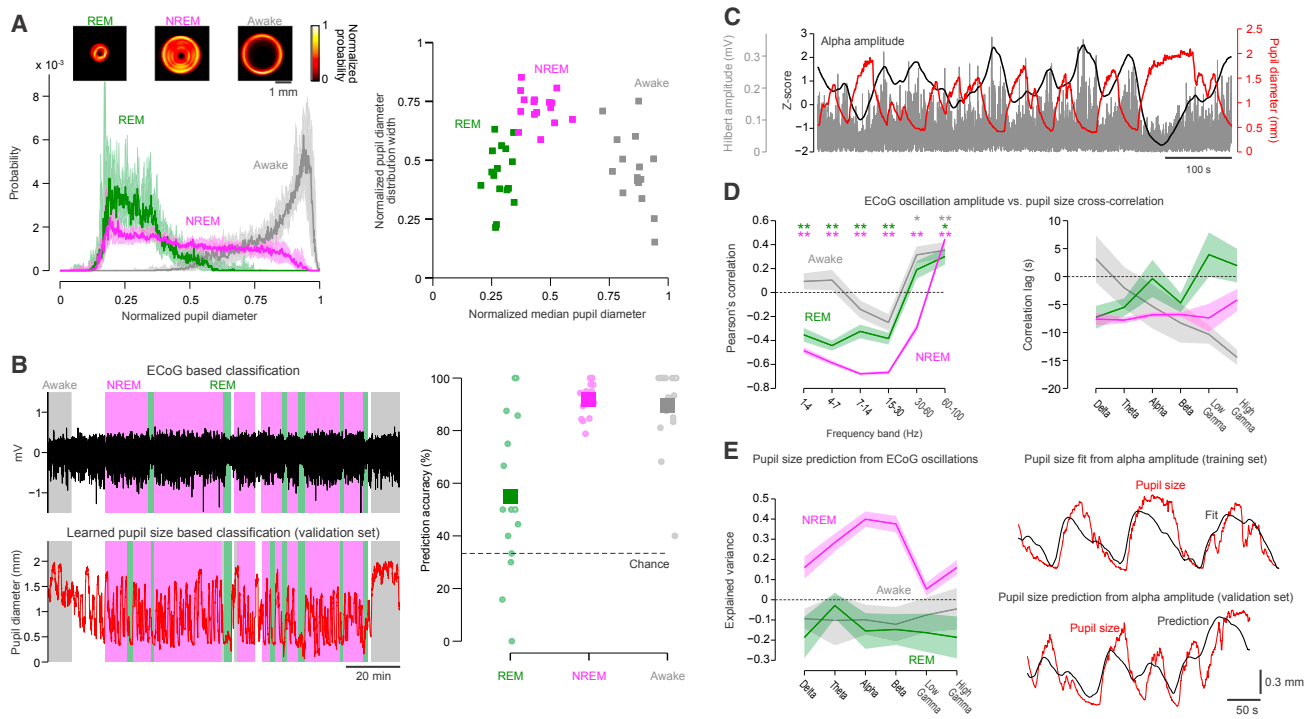

Figure 2. Pupil Size Fluctuations in Different Sleep States and Its Coupling to Brain Oscillations

(A) Left: median distribution (shaded regions are quartiles) of pupil diameter during REM, NREM, and awake states (n = 16 sessions). The pupil diameter has been normalized to its maximum recorded value in each experimental session. Insets show the location probability of the pupil's contour in one representative session for each state separately. Right: median pupil diameter versus its distribution width (the middle range containing 95% of the data points) of the 16 sessions.

(B) Top: ECoG recording of an example session with the identified sleep states based on delta, theta, and EMG power classification criteria (see STAR Methods for details). Bottom: predicted sleep states based on the pupil diameter recording (red trace) of the same session using a trained neural network algorithm (see STAR Methods for details). Note that the episodes marked in white include mixed NREM and awake bouts shorter than 100 s and were therefore excluded.

(C) Example session illustrating co-fluctuations of pupil size (red) and the Hilbert amplitude of ECoG oscillations in the alpha frequency band depicted in gray and its low-passed trace (denoted as alpha amplitude) in black.

(D) Mean (±SEM) Pearson's correlation between ECoG oscillatory magnitude of each frequency band and pupil diameter (n = 16 sessions) and the corresponding correlation lags, where negative values indicate that changes in oscillation size lead changes in pupil size. *p < 0.01, **p < 0.001 (Student's t test, Bonferroni corrected).

(E) Left: mean (±SEM) variance accounted for between measured and predicted pupil size based on ECoG oscillations of each frequency band. Right: example session comparing the measured pupil size (red) to the fitted (top) and predicted (bottom) pupil size with a general linear model using alpha amplitude as a regressor.

See also Figure S2 and STAR Methods for details.

session was used to evaluate prediction accuracy. In this manner, the learned classification based on pupil diameter correctly predicted all three sleep states (Figure 2B) more often than expected by chance (REM: 58%, NREM: 96%, awake: 95%; REM: $p = 0.016$, NREM: $p < 0.001$, awake: $p < 0.001$, Wilcoxon signed-rank test). These results reveal that pupil size during natural sleep is a reliable identifier of different sleep states.

Co-variation of Pupil Size and Brain Oscillations

Temporal changes in pupil size were previously found to be tightly coupled to oscillations in bandlimited EEG power in awake animals [14–16]. We therefore asked whether such coupling is also present during sleep and how it differs between the different brain states. In order to test this, we looked at the correlation between pupillary activity and traditional brain rhythms [21–24]. Striking co-fluctuations of pupil size and amplitudes of bandlimited ECoG oscillations were indeed observed in sleeping mice (Figure 2C). This analysis revealed that the strongest coupling is observed for the alpha (7 to 14 Hz) and beta (15 to 30 Hz) frequency bands (Figure 2D) in the NREM sleep state, where brain oscillations and pupil diameter are inversely correlated. Analysis at finer frequency resolution showed that peak negative correlation occurs in the spindle band (12 to 14 Hz; Figure S2C). Positive correlations were only found in the high-gamma band (60 to 100 Hz) and were similar in all three states. Brain oscillation changes led in time the changes in pupil size as indicated by the negative cross-correlation lags (Figure 2D).

To assess how steady these correlations are throughout a session in the different sleep states, we tested whether the oscillation amplitude can actually be used to predict pupil size (n = 5 mice). For each session, low-pass-filtered ECoG oscillation amplitudes were scaled to fit the pupil signal, and the fitted parameters were cross-validated on separate data of the same session (see STAR Methods). Surprisingly, only magnitude fluctuations of low-frequency oscillations (alpha and beta bands) during NREM sleep could reliably predict changes in pupil diameter

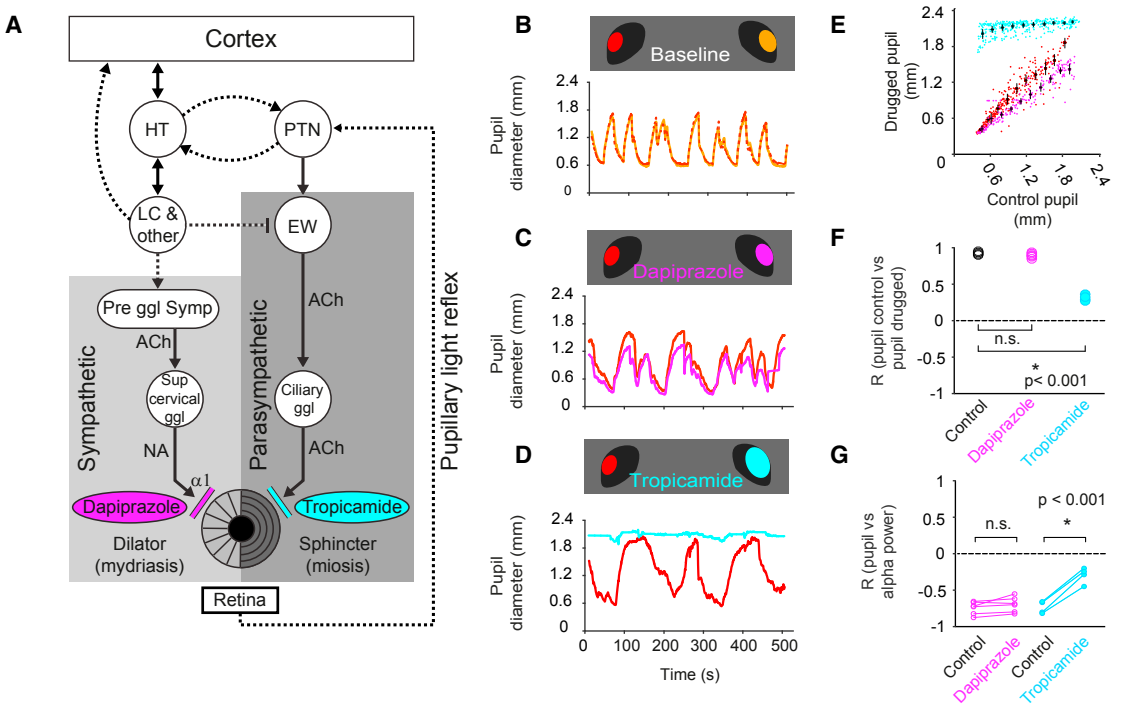

Figure 3. Mechanisms Underlying Coupling of Pupil Diameter and Brain Activity

(A) Schematics of the regulation of pupil size in relation to cortex through sympathetic and parasympathetic pathways (adapted from [28]).

(B) Pupillary oscillations in both eyes in baseline condition in NREM sleep.

(C) Pupillary oscillations in intact and dapiprazole-instilled eyes in NREM sleep.

(D) Pupillary oscillations in intact and tropicamide-instilled eyes in NREM sleep.

(E) Pupil size comparison of the eyes in baseline, dapiprazole-instilled, and tropicamide-instilled conditions in NREM sleep. Colored dots correspond to individual NREM bouts, and black dots are means (±SEM) of binned data (ten equally sized bins between the minimum and maximum size of the control pupil).

(F) Cross-correlation of the pupil diameters in opposing eyes in NREM sleep in baseline, dapiprazole-instilled, and tropicamide-instilled conditions.

(G) Cross-correlation of pupil diameter and the alpha power in the contralateral M1 in control, dapiprazole-instilled, and tropicamide-instilled conditions.

See also Figure S3 and Movie S1.

(Figure 2E). Peak prediction accuracy was again observed to occur in the spindle band when the analysis was performed at a finer frequency resolution (Figure S2D). Given the inverse correlation, it follows that during NREM sleep, increases in the size of low-frequency brain oscillations predict pupil constrictions, and decreases predict pupil dilations. These two phenomena seem to reflect the fragile and deep sub-states of NREM sleep [25], respectively. These results also reveal that the covariation of cortical oscillations and pupil diameter is stronger during sleep as compared to the awake condition (Figure 2D) [14–16]. The fluctuations of pupil size and amplitude of ECoG oscillations were found to be periodic, with an infra-slow modulation frequency ranging from 0.01 to 0.02 Hz (Figures S2E–S2G). These infra-slow modulations have been shown to affect not only cortical activity, but also heartbeat [25, 26]. We indeed found that the changes in pupil diameter were positively correlated with changes in heart rate during NREM periods (Figure S3).

Mechanisms of Pupil Size Control in Sleep

During wakefulness, the pupil diameter is driven by an equilibrium of the sympathetic and parasympathetic systems [27]. Whereas the parasympathetic pathway mediates the constriction of the pupil during relaxation and upon light illumination, pupil dilations during arousal or locomotion are mediated by the sympathetic system. In wakefulness, the sympathetic changes are closely coupled to cortical oscillations and are correlated with fluctuations in noradrenergic and cholinergic afferents [16]. The question therefore arises, is it the parasympathetic or the sympathetic system that mediates the tight coupling between pupil size and cortical states that we found to occur during sleep?

To assess their respective roles, we pharmacologically blocked the action of each of these two pathways at the level of the pupil. The sympathetic pathway was blocked by using an adrenergic alpha-1 receptor antagonist (dapiprazole), and the parasympathetic input to the pupil was inhibited by a cholinergic antagonist (tropicamide; Figure 3A). The drugs were applied to a single eye before the beginning of the sleep session, and the other eye was used as simultaneous control. To assess the effect of the drugs, we compared the correlation between ECoG in the alpha power band and pupil size in the NREM sleep state, which was found to be the most prominent co-fluctuation that exists between brain oscillations and pupil dynamics (Figure 2).

Under natural conditions, the pupils of the two eyes co-varied and were both correlated with the ECoG alpha power (Figures 3B, 3E, and 3G). Blocking the sympathetic afferents by

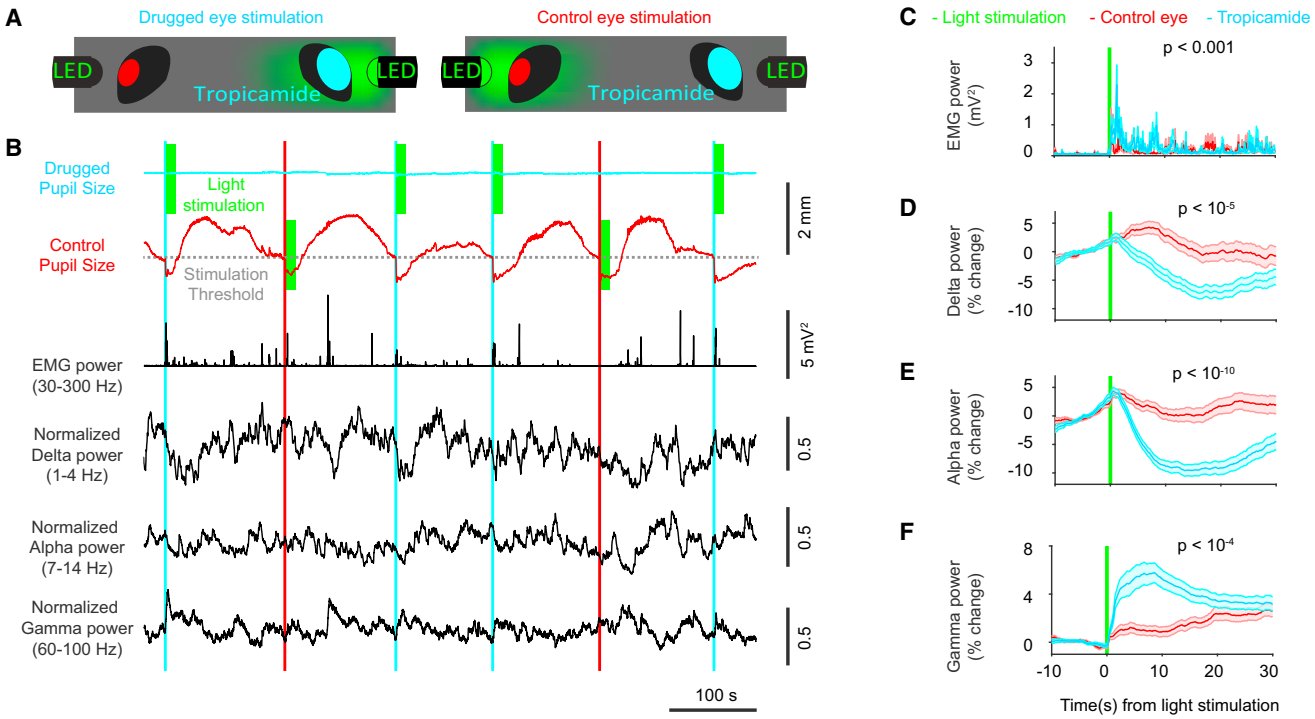

Figure 4. Potential Role of Pupillary Constrictions during NREM Sleep

(A) Schematics of the light-stimulation experiment.

(B) Pupil size traces of the drugged and control eyes with EMG and bandlimited ECoG power signals in a typical stimulation experiment. 1-s-long light stimulations are marked with green vertical bars on the pupil diameter traces.

(C) EMG activity at the time of light stimulation (two animals, 11 sessions, 196 control and 207 drugged-eye stimulation trials, mean [±SEM] in C–F).

(D–F) Delta (D), alpha (E), and gamma (F) power change at the time of light stimulation.

application of dapiprazole led to a reduction of the maximum pupil diameter (Figures 3C–3F; $p < 10^{-10}$, Student's t test) but did not significantly change the correlation with the control eye pupil size (Figure 3F; $p = 0.61$, Student's t test) or with the power of alpha oscillations (Figure 3G; $p = 0.41$). In contrast, blocking the parasympathetic drive by tropicamide application dilated the pupil and removed its prominent fluctuations during NREM sleep (Figures 3D and 3F; $p < 10^{-6}$). More importantly, tropicamide treatment abolished the coupling that existed between pupil size and cortical activity (Figure 3G; $p < 0.001$). These results suggest that fluctuations in pupil diameter during sleep are mainly driven by the parasympathetic pathway.

As an independent measurement of the parasympathetic drive, we also monitored the heart rate (extracted from the EMG signal; see STAR Methods). We found that pupil diameter and heart rate are positively correlated during NREM sleep (Figure S3). These findings complement previous reports showing that heart rate is correlated with parasympathetic modulation and also with cortical activity during NREM sleep [25, 26, 29].

Potential Function of Pupil Size Changes during Sleep

Might the observed pupil size fluctuations be playing a functional role during sleep? For instance, could pupil constriction be acting as a protector from visual stimuli in order to preserve the stability of deep sleep? In order to investigate the potential protective function of pupil constrictions, we artificially dilated one pupil with tropicamide, while leaving the other pupil intact as a control. The control pupil also allowed us to monitor the depth of NREM sleep (Figure 2). We then stimulated either the eye with the dilated pupil or the control eye with 1-s-long flashes of light (510 nm, 90 μW) at the moment of putative deepest NREM sleep (i.e., at local minima of control pupil diameter and high alpha and delta power; see STAR Methods) [25] (Figure 4A). We simultaneously monitored the pupil diameters of both eyes, as well as EMG and ECoG activity (Figure 4B).

As expected, we found that briefly illuminating the control pupil caused a pupillary light reflex (Figure 4B), yet it only had a minor effect on the sleep state (Figures 4B–4F, red trace). In contrast, when the dilated pupil was illuminated with equal amounts of light, the animals showed robust signs of change in sleep states (Figures 4B–4F, blue trace). Besides the expected light-reflex-related constriction of the contra-lateral control pupil, the light stimulus triggered a sharp decrease in ECoG delta (Figure 4D; $p < 10^{-5}$, Student's t test) and alpha (Figure 4E, $p < 10^{-10}$) power, whereas it provoked significant increases in power in the high-gamma range (Figure 4F; $p < 10^{-4}$) and EMG activity (Figure 4C; $p < 0.001$, Wilcoxon rank-sum test). Given that high power in alpha and delta oscillations are associated with NREM sleep whereas high gamma power is associated with arousal [21], we conclude that light stimulation resulted in an arousal-like state change. These changes are reminiscent of state transitions toward arousal induced by brief optogenetic

manipulations [20, 30, 31]. Taken together, these results indicate that sleep states are altered differently upon sudden illumination depending on pupil size. We thus suggest that sustained pupil constrictions during deep sleep might have a protective role preventing light-induced wake-up.

DISCUSSION

Our study reveals that pupil size is dynamic during sleep and tightly coupled to the different sleep states. The deeper the sleep, the more the pupil constricts. This coupling is primarily mediated via the parasympathetic system and might provide a protective function by blocking visual input during deep sleep.

Pupil diameter and cortical states have been shown to be coupled to various degrees during wakefulness [13–15]. In our study, we show that cortico-pupillary coupling is enhanced during NREM compared to awake or REM states (Figure 2). We find that this correlation is strongest and negative in the alpha band (7 to 14 Hz, with a peak in the spindle band) and positive at higher frequencies (60 to 100 Hz) of cortical activity. This is consistent with previous reports during awake states in which the low alpha band (2 to 10 Hz) best predicted the decrease in sensory discrimination performance [15]. It is also reminiscent of recent reports in which slow fluctuations in the sigma range (10 to 15 Hz) were identified to best predict the depth and stability of NREM sleep [25, 32]. In the context of sleep versus wakefulness, this might also explain a higher correlation in our study compared to previous findings. We speculate that this tight coupling during sleep is not only due to the absence of external stimuli or locomotion, but is also related to the strong cyclical fluctuations of various neuronal and physiological parameters during NREM sleep, imposing a broad synchronicity on many processes.

The slow fluctuations in the ~0.01 to 0.02 Hz range found to pattern the alpha rhythm and pupil diameter during NREM sleep have previously been described across several species and brain regions. These infra-slow oscillations have been termed "cyclic alternating patterns" in humans [33], but they have also been found modulating the 10 to 15 Hz power in mice [25], the hippocampal EEG rhythms [34, 35], and activity in locus coeruleus neurons during sleep in mice [36]. Similar fluctuations and coupling with the pupil diameter have been reported during urethane anesthesia in rats [37]. Due to its ease of implementation and cost efficiency, monitoring pupil diameter with iBip thus provides a non-invasive and reliable handle (Figures 2A, 2B, and S2A) to this ultra-slow rhythm, facilitating the identification of sleep stages or fragility periods during natural sleep.

One of the potential origins of such infra-slow oscillations might be the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN shows prominent ~0.01 to 0.02 Hz oscillations [38, 39] and is connected to the main modulatory systems [40], which might alter cortical activity [16]. The SCN also has a direct and reciprocal connection with the pretectal nucleus (PTN) [41, 42], where the infra-slow oscillations are also observed [41–44] (Figure 3A). This pathway would provide a direct link to the parasympathetic modulation of the pupil via the Edinger-Westphal and the ciliary ganglion (Figure 3A). Alternatively, the thalamus has been suggested to modulate not only slow oscillations [45], but also alpha- and spindle-range activity in the cortex [46, 47]. Thalamic nuclei have been shown to oscillate at an infra-slow rate [48] and to take part in maintaining the oscillations in SCN-PTN loop that impact pupillary constrictions [43]. Moreover, cholinergic activation of thalamic nuclei is reported to induce alpha rhythms [49]. Further experiments including subcortical recordings during sleep could reveal more about the role of different structures in generating infra-slow rhythms in the cortex and pupil size.

Blocking the sympathetic pathway with dapiprazole (an alpha adrenergic receptor antagonist) did not abolish the fluctuations or the correlation with ECoG oscillations (Figures 3C, 3F, and 3G). In contrast, tropicamide, a cholinergic blocker inhibiting the parasympathetic pathway to the pupil, significantly reduced the pupillary fluctuations and uncoupled them from the ECoG activity (Figures 3C, 3F, and 3G). Furthermore, we show that changes in the parasympathetically modulated heart rate [29] correlate positively to the pupillary oscillations in NREM sleep. These findings suggest that the observed dynamics of pupil diameter are not mediated by a decrease in sympathetic tone in the dilator muscle, but rather are mediated by an increase in the parasympathetic drive, causing an active pupil constriction during periods of deep sleep. Whether the cholinergic modulation of the pupil during NREM sleep is local or whether similar afferents can also affect other circuits, including the cortex, will have to be addressed in future experiments. The cholinergic tone in the brain is believed to be increase mostly during awake and REM states, yet there are reports of basal forebrain activity also in NREM sleep [50, 51].

How well these findings are applicable to other species, including humans, is not yet clear. Coupling of cortical activity and heart rhythm during sleep has been shown to be different in mice and humans [25, 26]. Also, blocking the parasympathetic input to the pupil was not sufficient to maintain complete dilations during sleep in children [52]. We can speculate that these inter-species differences might be due to differences in the balance of parasympathetic versus sympathetic drive during sleep or to different levels of baseline activity in autonomously regulated effector organs. The exact mechanism of how opposing autonomous systems act during sleep in both species remains an intriguing question for future sleep research.

This study was made possible by the fact that head-fixed mice can show an incomplete closure of their eyelids during natural sleep (nocturnal lagophthalmos). In humans, lagophthalmos can be caused by various conditions, including facial palsy (damage of the seventh nerve [53]). The reasons and mechanisms why this occurs in head-fixed sleeping mice are currently unclear. Anecdotally, we observed that the lagophthalmos decreased over weeks of repeated sleep sessions in some mice and might therefore be related to the habituation of the imposed body position. To reliably measure the pupil diameter during partial eyelid closure, we developed iBip. This technique was inspired by pupil tracking during *in vivo* two-photon calcium imaging, where the infrared light from the Ti Sapphire laser used for fluorophore excitation illuminates the back of the eyes [54]. The simplicity and low cost of iBip will most likely facilitate reliable pupil tracking in future studies during sleep and awake conditions. If used with higher frame rate (>60 Hz), iBip is ideally suited to track the eye movement dynamics during REM sleep (Figure S2B; Movie S1). Eye movements during tonic and phasic REM periods have been shown to have neural and muscular

correlates in adult and developing mammals [55–57]. Currently, the state-of-the-art rodent eye-tracking systems include implanted coils or electrodes around the eye area [57, 58]. Our technique would therefore provide a simple and noninvasive alternative for REM sleep studies.

Finally, we provide the first evidence for the protective role of pupil constrictions during sleep. Although eyelids have primarily a protective function, they still transmit light sufficiently [59] to cause changes in cortical states [60]. Light stimulus into the pharmacologically dilated eye during NREM sleep resulted in a change in ECoG and EMG signals that were similar to state transitions toward arousal induced by brief optogenetic manipulations [20, 30, 31] (Figures 4D–4F). In contrast, light stimulation to the control eye had only a minor effect on sleep states. Until recently, one of the primary mechanisms for regulating cortex and sensory stimuli interaction during sleep was thought to be the thalamic gating hypothesis [61] (but see [60, 62]). Our study provides an additional, periphery-dependent gating mechanism for protecting the brain from waking up during phases of deep sleep. We hypothesize that pupillary constriction might ensure the continuity of NREM sleep periods [25], which is considered to be critical for memory consolidation [20, 63].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- CONTACT FOR REAGENT AND RESOURCE SHARING
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- METHOD DETAILS
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- QUANTIFICATION AND STATISTICAL ANALYSIS
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  - Relating ECoG oscillations to pupil size fluctuations
  - Changes in ECoG signals with light stimulation

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one movie and can be found with this article online at https://doi.org/10.1016/j.cub.2017.12.049.

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AUTHOR CONTRIBUTIONS

O.Y., R.Z., and D.H. conceived the study. R.Z. developed the head-fixed sleep paradigm, conducted pilot head-fixed sleep experiments including the X-ray analysis, and discovered the coupling between pupil diameter and cortical activity. O.Y. conducted all experiments described in this paper including the design of pharmacology and stimulation experiments. O.Y. and R.Z. manually scored the data. O.Y. and M.P. analyzed the data. M.P. developed the video-acquisition and pupil-size-tracking systems. O.Y., M.P., and D.H. wrote the paper.

DECLARATION OF INTEREST

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Tropicamidum, % 0.5	Thea Pharma	Tropicamide 0.5% SDU Faure
Dapiprazole, % 0.5	Angelini	Glamidolo
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	Charles River Laboratories	C57BL/6J
Software and Algorithms		
Electrophysiology acquisition interface	Open-ephys.org	Open Ephys GUI
Other		
USB camera, 0.3MP Mono Firefly MV USB	Point Grey Research	FMVU-03MTM
Micro-video lens, 25.0 mm FL, No IR-Cut Filter, f/2.5	Edmund Optics	#56-776
LED, 940 nm 5mm T-1 3/4	Everlight Electronics	IR323
Electrophysiology acquisition board	Open-ephys.org	Acquisition board
Headstage, 32 channels	Intan Technologies	RHD2132/RHD2216
ECoG wires, 75 μm	Science Products	AU-3T
EMG wires, 75 μm	Science Products	SS-2T/HH

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daniel Huber (daniel.huber@unige.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Seven wild-type (C57BL/6, 10 to 11 week old) male mice were used for this study. Animals were caged individually, kept at 12 hr dark/light cycle and were placed under a water restriction regime (1 mL/day). The habituation sessions and experiments were performed in the second tierce of the light period. All procedures were reviewed and approved by the local ethics committee and the authorities of the Canton Geneva.

METHOD DETAILS

Surgeries

To implant the titanium frame for head-fixation, mice were anesthetized with 2% isoflurane-oxygen mix and kept warm on a heating pad (T/PUMP, TP500C). Injections of carprofen (Rimadyl, 50 mg/mL, 1:20 dilution in NaCL, 50 μL sub-cutaneous), buprenorphine (Temgesic, 0.3 mg/mL 1:2 dilution in NaCl 25 μL intramuscular) and dexamethasone (Mephamesone-4, 4 mg/mL, 20 μL intramuscular) were given. The eyes were covered with Vaseline. Local anesthetic lidocaine (Rapidocain 10 mg/mL, 50 μL subcutaneous) was applied to the scalp before removal. A titanium head bar was glued to the dried skull with cyanoacrylic glue (ergo 5300 elastomer). Small craniotomies for the ECoG electrodes were drilled over primary motor cortex (in right hemisphere 1.5 mm lateral (L), 1.5 mm rostral (R) of bregma) and cerebellum (all animals, midline 6 mm R). Custom electrodes for ECoG recordings were made from Teflon coated gold wires (75 μm, AU-3T, Science Products). The electrodes were soldered to a miniature connector (Millmax). For EMG recordings, custom electrodes were fabricated with twisted Teflon coated half hard stainless steel wires (75 μm, SS-2T/HH, Science Products). The coating of the EMG electrodes was stripped off with sharp tweezers and used as bipolar electrodes. These wires were guided into the neck muscles with a 24 G needle and immobilized in the muscle by bending the ends to a hook. Finally, the electrodes and the titanium frame were covered with transparent dental acrylic (Lang Dental Ortho-Jet Powder B1320). The animals were taken off the anesthesia, left to recover in a clean cage placed on a heating pad for 2 hr. Recordings started after an additional recovery period of 7 to 10 days.

Handling and sleep training

Animals were handled for 10 min per day for 6 consecutive days. To habituate the mice to sleep under head-fixed condition, their body was placed in a small plastic box (80x80x50 mm) which was filled with cotton bedding to comfortably accommodate the

animal's body. The head of the animals was kept at a 30° angle to imitate the natural sleep position in during head fixation (Figure 1C). During habituation and recordings the mice were held in a sound and light isolated Faraday cage. Through the first three sessions the mice were kept head-fixed for 10, 20 and 30 min and were delivered random water rewards through a lick port. In later sessions water delivery period remained 30 min while the duration of the head fixation increased by 20 to 30 min reaching to ~4 h in 10 to 12 sessions. The state of the mouse was continuously monitored with an IR USB camera (Point Grey Firefly MV USB FMVU-03MTM).

X-Ray imaging

X-Ray imaging to determine the body position during different head-fixed conditions was conducted using a custom lead shielded BV-25 (Philips) X-Ray machine equipped with a 120 × 70 mm CMOS detector (1207, Dexela). The X-Ray source was run at 52 kV. The effective dose in the path was determined with a RadeEye B20 Geiger counter (Thermo Scientific) and the cumulative dose during the lifetime was kept below 10mSv per animal. Images were acquired and analyzed using custom MATLAB code. During the X-Ray imaging the mice were continuously monitored with an IR USB camera (Point Grey Firefly MV).

Electrophysiology

ECoG and EMG data were acquired at 1 kHz using a 16 (for natural sleep recordings in the home cage) or 32 channel head stage (for head-fixed recordings) (RHD2132/RHD2216 Amplifier Board, OpenEphys). The signals from the ECoG electrode were referenced to the cerebellar electrode and the bipolar EMG channels were subtracted from each other. Signals were high-pass filtered at 0.1 Hz and a Fast Fourier Transform (FFT) was used to calculate the power spectrum of the ECoG and EMG signals with a 2 s sliding window sequentially shifted in 0.1 s increments. Each 2 s interval of data was first multiplied by an equal length Hamming window before applying the FFT. For the analysis of natural sleep recordings cortical electrodes were referenced to their contralateral counterparts to eliminate movement artifacts on the ECoG signal.

Heart beat detection

Heart rate was extracted from the EMG signal by detecting the prominent biphasic pulses (≈10 ms duration) occurring rhythmically. For this purpose, the EMG signal was filtered between 30 and 300 Hz and heart pulses were detected with the *findpeaks* MATLAB function applied to the squared absolute values of the filtered signal. Heart rate was computed by binning the pulse times into 1 s bins and calculating the mean of the inter-pulse-interval inverse values for each bin. Heart rate was up-sampled to the time base of pupil size by linear interpolation before calculating the cross-correlation between their Z-scored values.

Pupil tracking with iBip

Pupils of both eyes were monitored with two separate digital USB cameras (Point Grey Research 0.3MP Mono Firefly MV USB FMVU-03MTM) and acquired and saved at 10 frames/s (240x376 pixels 8-bit greyscale images) with a custom video acquisition system written in MATLAB. The timestamp of each frame relative to a digital trigger were saved in the image headers and used for post hoc alignment of pupil and electrophysiology data. To obtain high contrast images of the pupil we used infrared back-illumination pupillometry (iBip). iBip consists of a 940 nm LED (Everlight Electronics, 3 mW, 40 mA, 1.2 V) positioned on the skull above frontal cortex. Light reached the head through the polished dental acrylic of the head-cap. Light emission to the side of the LED was blocked with black tape shielding. The LED at this intensity level did not produce any additional heating of the illuminated area (measured by Peaktech 5140 digital thermometer).

Pharmacology and light stimulation experiments

Before the start of the recording session, one eye of the mouse was instilled with a drop of cholinergic receptor antagonist Tropicamide (Tropicamide, Thea Pharma, ~100 μL, 0.5%) or the alpha receptor antagonist Dapiprazole (Glamidolo, Angelini, ~100 μL, 0.5%), while the other eye was left intact (n = 7 mice). Animals were given 1 to 2 days of break in between pharmacological experiments. The side of the drugged eye was switched every session. For the light stimulation experiments a LED light source (510 nm, 90 μW) was positioned 20 mm in front of each eye (n = 2 mice). 510 nm wavelength was chosen as the center of the mouse green cone spectrum [64] and the 90 μW were found to be sufficient to induce changes in the ECoG bands (as determined in pilot experiments). The LEDs were mounted in 5 mm wide black tubes to restrict illumination to the targeted eye only. Light pulses (1 s) were triggered manually during NREM sleep when the control pupil diameter was in a decreasing phase and reached ~0.5 mm.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sleep state classification

Sleep classification was carried out semi-automatically in several consecutive steps. First, delta and theta power signals were calculated by summing the ECoG power in the 1 to 4 Hz, and 6 to 10 Hz frequency ranges, respectively. The EMG power was summed in the ranges between 30 and 300 Hz. NREM sleep was identified by 50 s uninterrupted periods where delta power was two standard deviations larger than its mean and EMG power smaller than its mean (similar to [65]). A state was marked as REM sleep when the theta/delta ratio was two standard deviations larger than its mean and EMG power was two standard deviations smaller than its mean. Everything else was marked as awake. All sleep stages were proof read and corrected by two experienced observers. Micro

awakenings during NREM were identified manually and marked as awake. NREM bouts that were interrupted by micro awakenings were considered continuous. All analysis was carried out with custom MATLAB scripts.

Pupil diameter detection

To extract the pupil diameter, single frame images were first centered on and cropped around the pupil (90x90 pixels). The greyscale pixel values of the cropped image were classified into 2 clusters (dark and bright pixels) using K-means clustering. A binary image, obtained by setting the dark pixels to 0 and the bright pixels to 1, was used to find connected components (bwconncomp function in MATLAB) and the largest component was deemed to correspond to the pupil. An ellipse was fit to the component's contour (linear least-squares fitting) and its major axis was defined to be the pupil diameter. Eye blinks and periods with closed eyelids were manually detected and excluded from analysis. The pupil diameter traces were upsampled to 1 kHz by linear interpolation to match the ECoG signal sampling and low pass filtered at 2 Hz (except for light stimulation experiments). Converting pupil size to mm was done by calibrating the images to a video recording of 1 mm square graph paper.

Pupil-based sleep state classification

A neural network with one hidden layer was used to classify sleep states based on pupil diameter. For each sleep session classification, the network was first trained on data from all other sessions (n = 5 mice). Training trials consisted of 100 s pupil diameter traces (sampled at 10 Hz) at the input layer and the corresponding sleep state label at the output layer (awake, REM or NREM). Sleep bouts that were shorter than 100 s were omitted in this analysis. The hidden layer size was set to 30 nodes. The trained network coefficients were then used to predict sleep states of the session not used for training. Prediction accuracy was calculated as the % of the 100 s trials correctly classified in each session and separately for each of the three sleep states.

Relating ECoG oscillations to pupil size fluctuations

The recorded ECoG signals of each session were bandpass filtered into the traditional delta (1 to 4 Hz), theta (4 to 7 Hz), alpha (7 to 14 Hz), beta (15 to 30 Hz), low (30 to 60 Hz) and high gamma (60 to 100 Hz) frequency bands using a bidirectional second order Butterworth filter, thus eliminating possible phase shifts introduced by filtering. The frequency definitions correspond to cut-off values at -3 dB attenuation. The instantaneous amplitude of each bandlimited oscillation was obtained by computing the magnitude of its Hilbert transform. To assess how the magnitude of cortical oscillations changes relative to fluctuations in pupil size, Pearson's correlation (R) was calculated between the low-pass filtered (bidirectional single pole IIR filter, i.e., exponential decay impulse response) Hilbert amplitude signals and pupil diameter. For each frequency band, the time constant of the low pass filter that maximized $|R|$ was evaluated for each sleep state separately (fminsearch function in MATLAB, n = 5 mice).

We used general linear modeling (GLM) to assess the extent to which ECoG oscillations of each band can be used to model changes in pupil diameter. The GLM consisted of two regressors: the filtered ECoG oscillatory amplitude and a constant term. The former trace was first standardized by taking its z-score. The low pass filter time constant and the two GLM coefficients that minimized the sum of squared errors between the fitted and actual pupil diameter were evaluated on half of the data in each session. The other half of the data, not used for parameter fitting, was used to validate the prediction by computing the explained variance as $1 - var(p_m - p_{pr})/var(p_m)$ where p_m is the measured and p_{pr} the predicted pupil diameter and $var()$ symbolizes the signal's variance.

The spectral content, in the infra-slow frequency range, of pupil fluctuations and bandlimited ECoG oscillation amplitude changes during NREM sleep (Figures S2E–S2G) was obtained using the Fast Fourier Transform (FFT). Hilbert transforms of the bandpass filtered ECoG signals or the pupil diameter trace were first normalized to their respective mean values for each NREM bout separately and then multiplied by an equal length Hamming window. The traces were padded with zeros for the number of FFT points to be equal to a higher power of 2. The obtained absolute FFT values were first interpolated at a 0.001 Hz frequency resolution and then averaged across all NREM bouts of an experimental session.

Changes in ECoG signals with light stimulation

For light stimulation experiments, % change was calculated with respect to a 10 s baseline preceding stimulus onset. Drug and control conditions were compared (red and blue traces in Figures 4C–4F) based on mean values of a 20 s post stimulation period.

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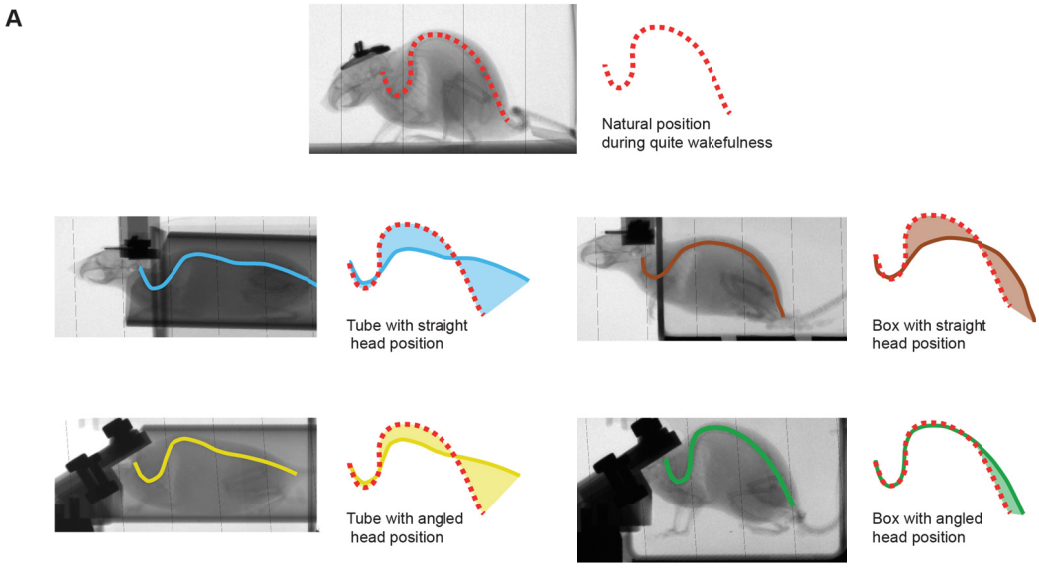
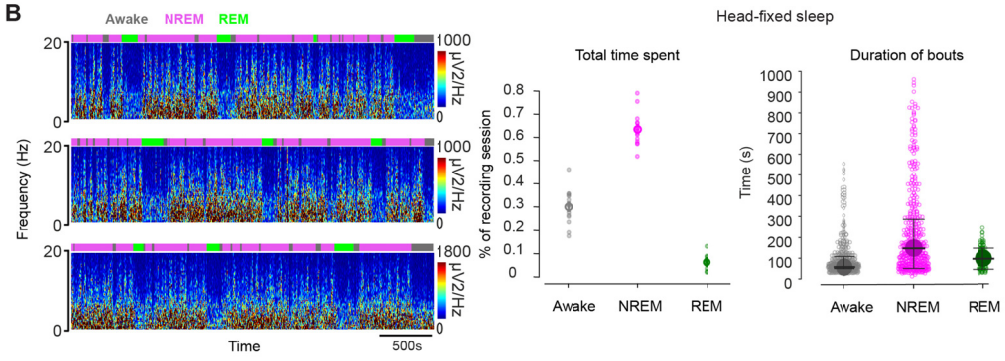
Supplemental Information

Pupil Size Coupling to Cortical States Protects the Stability of Deep Sleep via Parasympathetic Modulation

Özge Yüzgeç, Mario Prsa, Robert Zimmermann, and Daniel Huber


A
Natural position
during quite wakefulness
Tube with straight
head position
Box with straight
head position
Tube with angled
head position
Box with angled
head position


B
Awake
NREM
REM
Head-fixed sleep
Total time spent
Duration of bouts
Frequency (Hz)
Time
500s
% of recording session
Time (s)
Awake
NREM
REM

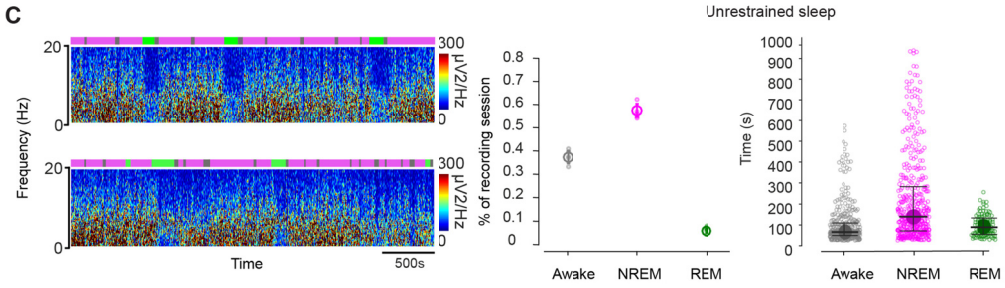

C
Unrestrained sleep
Frequency (Hz)
Time
500s
% of recording session
Time (s)
Awake
NREM
REM

Figure S1. Comparison of posture, time and frequency characteristics of head-fixed and unrestrained sleep (Related to Figure 1).

A. Top row: natural position of a mouse during quiet wakefulness. The shape of the spine is marked with a dotted red line. Second row, left: body position of a mouse and the related spine shape (compared to the freely moving condition in red). In figures on the left column, the mouse is held in a tube. In figures on the right column, the mouse is in a cotton-filled box. In the figures on the second row, the mouse's head is held straight and in the figures on the third row, the mouse's head is held at a 30° angle. Note the close similarity between the natural body position and the mouse sitting in a box with an angled head (green trace). The configuration illustrated in the lower right figure (green trace) was used for this study. **B.** Left column: power spectrum and hypnograms from head-fixed sleep recording sessions with 3 different animals. Middle column: mean (± s.e.m.) percentage of time spent in different stages of sleep. Right column: median (± quartiles) duration spent in each stage of sleep during head-fixed recordings (N= 16 sessions from 4 animals in middle and right columns). **C.** Left column: power spectrum and hypnogram examples in unrestrained sleep recording sessions from two mice. Middle column: mean (± s.e.m.) percentage of time spent in different stages of sleep. Right column: median (± quartiles) duration spent in each stage of sleep during unrestrained conditions (N = 5 sessions from 2 animals in middle and right columns).

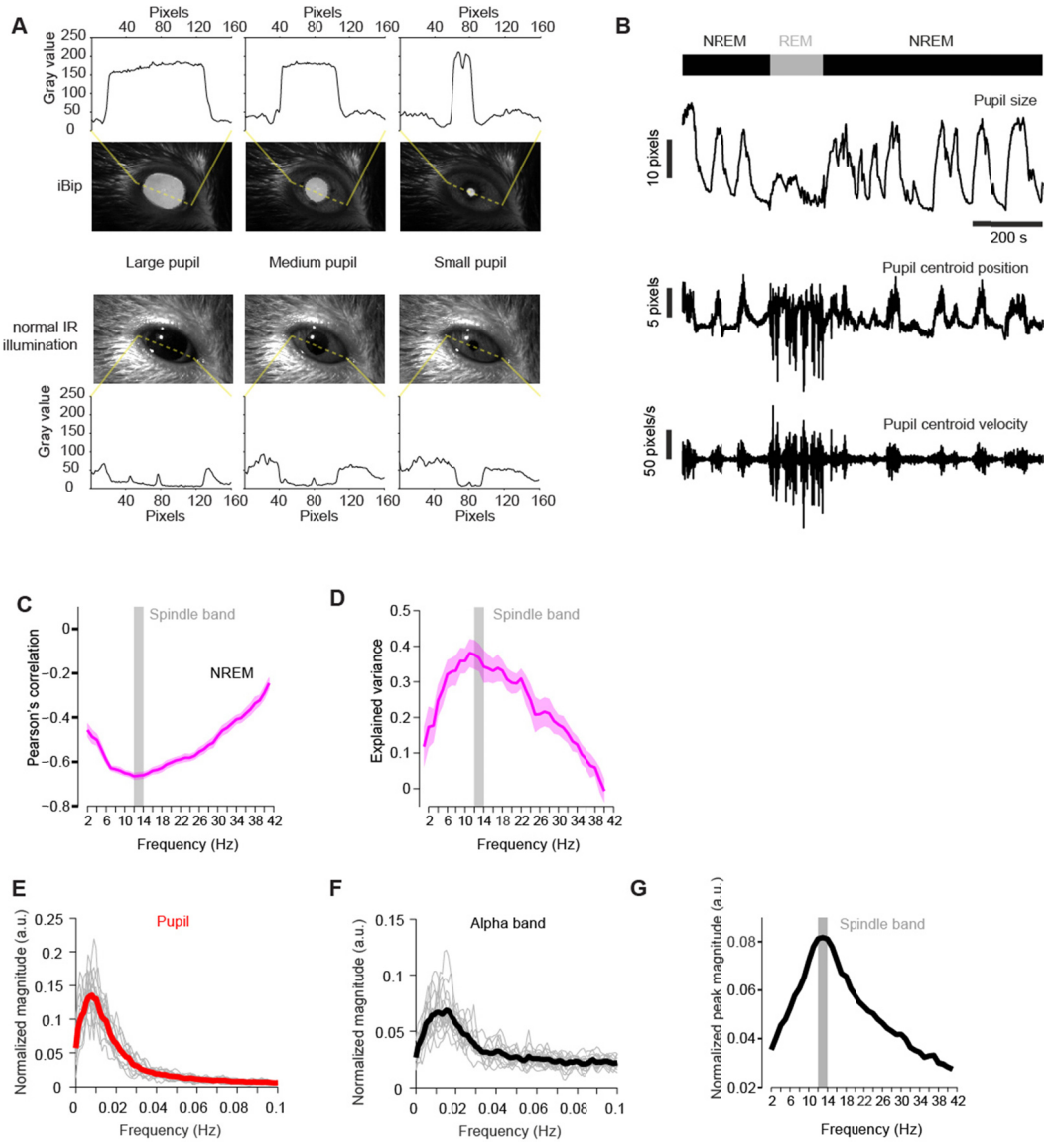


Figure S2. Tracking of pupil diameter and rapid eye movements with iBip, fine scale description of pupil – brain co-fluctuations in NREM sleep (Related to Figure 1 and Figure 2).

A. Comparison of the eye contrast in a sleeping mouse acquired with the iBip system (top) and with a conventional infrared illumination approach (bottom), for large, medium and small sized pupils (left to right). The greyscale values correspond to image pixels marked with the dashed yellow line. **B**. Pupil size, pupil centroid position and velocity in a representative sequence of NREM and REM sleep episodes. Position and velocity traces show apparent rapid eye movements during REM sleep. **C.** Mean (± s.e.m.) Pearson's correlation between pupil size and infra-slow changes in ECoG oscillatory magnitude within 2 Hz wide frequency bands (abscissa values indicate the band's center frequency) during NREM sleep (N=16 sessions). **D.** Mean (± s.e.m.) variance accounted for between measured and predicted pupil size based on infra-slow changes in ECoG oscillations of the same frequency bands as in C, during NREM sleep. **E.** Spectral composition of pupil size reveals the

existence of infra-slow oscillations (0.01 to 0.02 Hz). Grey traces correspond to NREM bouts of individual experimental sessions (N=16 sessions) and colored traces to their means. **F**. Spectral composition of ECoG oscillatory magnitude in the alpha frequency band (7 to 14 Hz) during NREM sleep shows an infra-slow peak (0.01 to 0.02 Hz) (grey: 16 individual sessions, black: mean). **G.** Absolute peak values of infra-slow slow changes in ECoG oscillation magnitude for the same narrow frequency bands as in C, during NREM sleep.

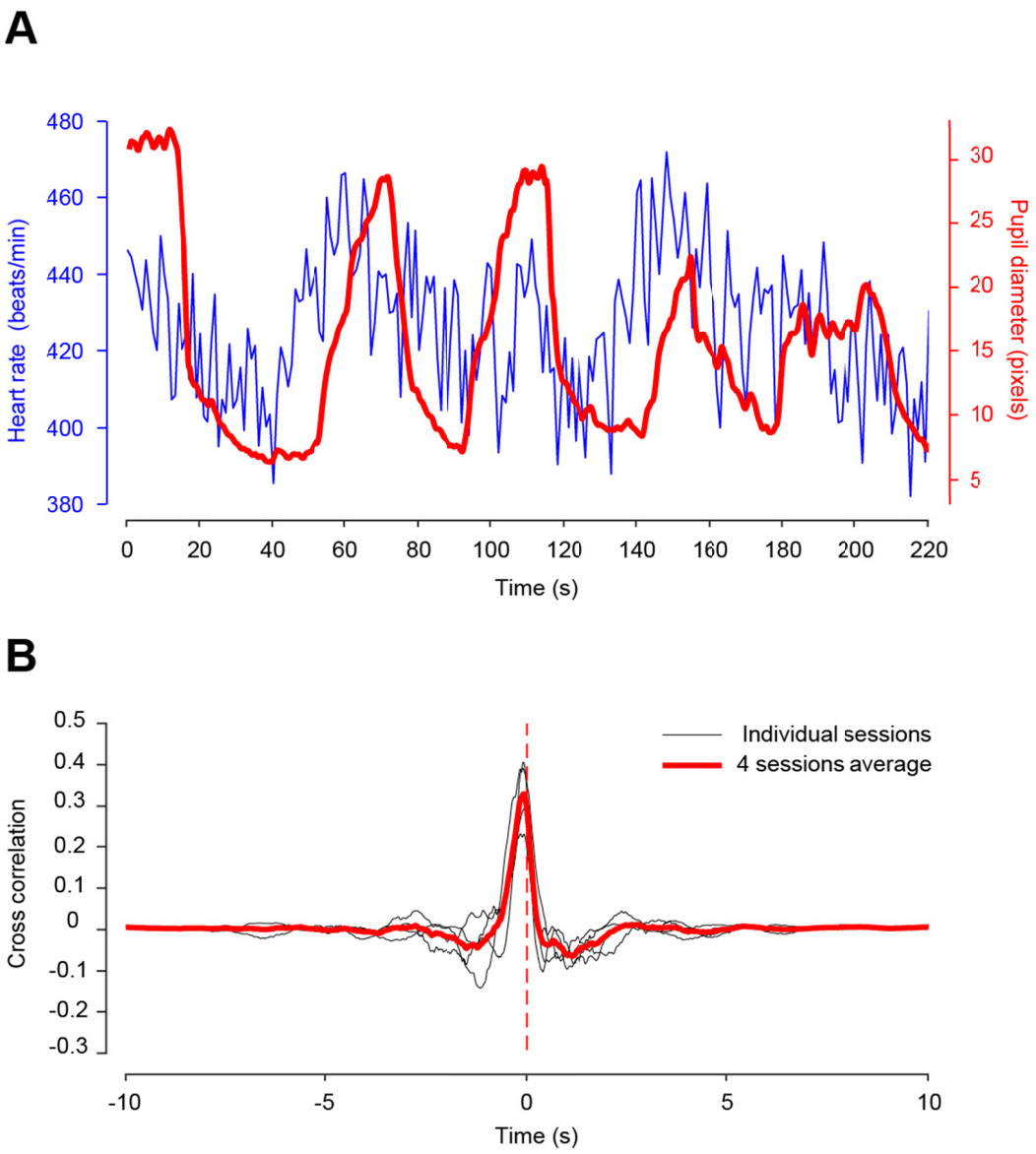


Figure S3. The relationship between pupil diameter and heart rate during NREM sleep (Related to Figure 3).

A. Example of concomitant fluctuations of pupil diameter and heart rate extracted from the EMG signal in a NREM sleep bout (see STAR Methods). **B.** Cross correlation between heart rate and pupil diameter Z scores in N = 4 individual sessions from 3 animals (black traces) and their average (red trace). Negative lags indicate that heart rate changes lag those of pupil size.