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The coordinating influence of thalamic nucleus reuniens on sleep oscillations in cortical and hippocampal structures – relevance to memory consolidation and sleep structure

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"Veritas consistit in adaequatione intellectus et rei"

- St. Thomas Aquinas

Contents

Abstract

Sleep is a fascinating and a bit mysterious behavior. Not only do so called “higher” animals like mammals sleep but also simpler organisms like jellyfish display rhythmic periods of quiescence which are interpreted as sleep. Despite it being almost ubiquitous across the animal kingdom, the function of sleep is still not fully understood. However, we do know that especially the brain is important for the initiation and maintenance of that state and that it is highly active during sleep. There has been a special focus on electric neuro-oscillations where research over the last 90 years has revealed that the brain displays quite distinct oscillatory patterns during sleep and its specific functions are slowly being brought to light, such as memory consolidation and communication between different brain regions. For example, it has been argued that newly formed memories are either stored in the hippocampus or at least dependent on it for reactivation and are later transferred to the neocortex or become independent of the hippocampus while being stabilized in the cortex, with a portion of the thalamus, the *nucleus reuniens thalami*, being possibly involved in this process as it is an anatomical relay between cortex and hippocampus.

The aim of my PhD project was to investigate the coupling of neuro-oscillations between prefrontal cortex, thalamus, and hippocampus in both a descriptive and manipulative way. Namely, we investigated the coupling between prelimbic cortex, nucleus reuniens of the thalamus and the CA1 portion of the hippocampus during unperturbed natural sleep, sleep after sleep deprivation and sleep with increased mnemonic demands after a learning task. Lastly, we optogenetically manipulated nucleus reuniens during sleep to assess its properties as a synchronizing link between prefrontal cortex and hippocampus.

We described the coupling of cortico-thalamic slow waves and spindles with ripples in the hippocampus by quantifying the amount of co-occurrence of the aforementioned events, describing the phase-locking of ripples to slow waves and spindles, and determining which oscillations drives the other.

Next we found that spiking behavior of nucleus reuniens is coupled to ripples and cortical slow waves.

Lastly, optogenetic manipulation showed that nucleus reuniens is involved in the precise phase-event coupling, in the co-occurrence of the mentioned events, and oscillatory drive between cortex and hippocampus. However, the effects we found on the neuro-oscillatory coupling were not accompanied by a change in memory performance after a learning task.

1. Introduction

1.1 Definition of sleep

Animals, including humans, show a wide range of different behaviors. When looking at human behavior, one sees that some behaviors are consciously planned and executed, while others are autonomously performed without willful execution and some even without full consciousness, like sleep. While it is a behavior which engages the whole body, it is mainly the brain which regulates the occurrence and execution of sleep.
Sleep is a behavioral state that to this day remains mysterious. It is not fully understood what goals it serves, even though we have come to understand at least some of its aspects, like electrophysiological features of the brain or adaptations of the immune system (see below), and it is not quite clear why it seems to be a necessary behavior that is displayed by all 'higher organisms'. Furthermore, sleep-like states or at least circadian modulations of behavior are even shown by simpler animals. It is a reversible state that can be characterized by perceptual disengagement and unresponsiveness to the environment, postural recumbency (i.e. lying down), quiescence of conscious and goal-directed behavior, and alterations of consciousness [1], only to mention the most striking features. Additionally, it is often referred to as one of two principal vigilance states besides wakefulness.

1.2 Electrophysiological observations in the brain during sleep

Since Hans Berger introduced the measurement of electric potentials of the brain with non-invasive electrodes in humans in 1929 [2], the electroencephalogram (EEG) has been a very important tool for observing the brain's activity during different tasks, behaviors and vigilance states (i.e. wakefulness and sleep). Thanks to this method, it was revealed that sleep in mammals and birds undergoes different stages and that it is subdivided [3]. Namely, it can be divided into a stage that shows rapid movements of the eyes as one of its characteristics besides EEG related characteristics, called *rapid-eye-movement sleep* (REM sleep) and a stage where this observation is absent, called *non-rapid-eye-movement sleep* (NREM sleep). Furthermore, NREM sleep can be further subdivided in certain species. In humans, we can identify three stages of NREM: NREM 1, NREM 2 and Slow-wave sleep (SWS). As for rodents, NREM has not been subdivided in stages until recently. However, a recent study could show that rodent NREM sleep does show three substages as well [4]. Studies in other animals suggest that fish and reptiles also have two sleep stages. However, it is not clear yet whether these two sleep states can be viewed as homologues or evolutionary predecessors of NREM and REM sleep. Electroencephalographic data for other classes of animals haven't been acquired yet and sleep in these animals can only be characterized through observation of external behavior [5].

Interestingly, wakefulness, NREM and REM sleep do not occur randomly. From one's own experience one can tell that humans preferably sleep during the night and are awake during the day, a pattern of sleep-wake regulation called *'diurnality'*. Mice on the other hand are

nocturnal as they sleep during the day and are active during nighttime. Furthermore, the occurrence of NREM and REM during sleep in general is quite well organized as well, in the sense that when an animal goes to sleep, it will first transition from wakefulness to NREM sleep in the case of mice and to the first stage of NREM in humans, or NREM 1. From there, humans will transition to NREM 2, followed by SWS [6]. Then following SWS in humans or NREM in mice, the organism will transition to REM sleep. After REM sleep, a brief period of wakefulness follows in rodents or NREM 1 in humans, respectively. From there on the sequence repeats. One sleep cycles lasts for about 1,5h in humans and is repeated 4-5 times over the night with increasing durations of SWS and REM sleep bouts with every cycle. In rodents, a complete cycle lasts only a few minutes and therefore many more occur during the day [7].
As alluded to above, wakefulness and the different sleep stages can be differentiated through their EEG characteristics: If the eyes are closed and the person is quietly resting, then the most dominant EEG rhythm are quite synchronous α-waves (alpha, 8-13Hz). When eyes are opened and the person engages in any mental activity, β-waves (beta, 13-13Hz) and γ-waves (gamma, >30Hz) become more dominant., which is also true for rodents. During NREM 1, the α-waves start to diminish and the person becomes drowsy, which is still an unstable state and is interrupted by brief moments of wakefulness. In NREM 2, K-complexes and sleep spindles emerge, which are described below. SWS is then characterized by high amplitude, low frequency slow waves (<1Hz), δ-waves (delta, 0.5-4Hz), θ-waves (theta, 4-7Hz) and a heightened threshold for awakening through external stimuli. As for mice, their NREM sleep is dominated by slow waves, delta waves and spindles, similar to SWS in humans. Finally, the sleeping brain transitions to REM sleep. The EEG patterns in humans resemble the ones during wakefulness with β-waves, γ-waves as well as small amplitude slow waves and the sleeping person experiences vivid dreams. Additionally, the brain induces muscle atonia in order to not live out the movements that occur in the dream [6]. Rodent REM sleep on the other hand is mainly dominated by highly synchronous θ-waves.

1.2.1 Electrophysiological characteristics of NREM sleep

The electrophysiological events described in the previous section underlie cellular and network mechanisms that are well described despite some open questions remaining to be answered. This section will present the most prominent oscillatory activities in the mammalian sleeping brain.

Slow oscillations
Occurring during NREM, slow oscillations (<1Hz) reflect the alternation between a depolarized UP state and a hyperpolarized DOWN state in cortical [8] and thalamic cells [9], [10] on the EEG level. This rhythmic activity not only emerges during sleep but also during deep anaesthesia, in isolated cortical slices, and in so called *'cortical islands'* after brain damage. Thus, slow oscillations are considered to be the default activity pattern of the cortex and parts of the thalamus which is then influenced by excitability levels, neuromodulators and inputs from connected areas [11]. An UP state is defined as a depolarization of both excitatory and inhibitory neurons of 7-10mV above the resting potential of -70mV, with varying spike rates between 5 and 30Hz during the UP state [8]. The cortical origin of slow oscillations are likely neurons of

the deep layers, particularly layer 5, as has been demonstrated by numerous studies *in vitro* and *in vivo* [12]–[15]. The cellular mechanism for UP state initiation are not fully clear and there are studies pointing either towards larger intrinsic excitability that leads them to begin firing during Down states, stochastic release of synaptic vesicles or specific pacemaker cells (see [11] for a review). The UP state is mainly driven by NMDA and non-NMDA glutamatergic synaptic input and Na^+-influx [11], [16].
DOWN states are periods of hyperpolarized (7-10mV) membrane potentials below the resting potential, marked by absence of spiking, overall absence of synaptic input to the neurons, some mediation by Cl^--influx through inhibitory synapses and increased K^+-conductance [8], [17]. The termination of an UP state, which then leads to a DOWN state, is not fully understood but it seems to involve an increase of excitatory conductance, synaptic depression, thalamic dys-facilitation, fast inhibition, or extracellular K^+-dynamics. These mechanisms could potentially result in changes in K^+-conductance, potentially via ATP-dependent K^+-current, $GABA_B$ receptor engagement, or Ca^{2+} and Na^+-dependent K^+-currents (see [11] for a review).

Importantly, both excitatory and inhibitory neurons participate parallelly in the alternation between UP and DOWN state and their interaction is even at the basis of slow oscillation generation [15], [18]. The conductances of excitatory and inhibitory synapses are at similar levels during UP states as they are high at the beginning of it and then decrease progressively [19], [20].
For DOWN and UP states to be visible on the EEG, there needs to be a certain number of neurons that are synchronous and thus generate a big enough electrical signal. Indeed, the spiking during UP states and quiescence during DOWN states cause a synchronization in neighbouring neurons through synapses between nearby neurons, so that a slow wave can span up to 7 mm of cortical tissue in cats [21] and even travel across the cortex. In the case of humans, slow waves often originate in frontal regions and then travel to posterior regions [22].
Since neurons in the thalamus are excitatory except for the ones from the reticular nucleus [23], generation of thalamic slow oscillations are based on intracellular mechanisms. They involve interplays between the hyperpolarization activated I_H current, which consists of both a Na^+-influx and K^+-efflux, a voltage-sensitive Ca^{2+}-current I_T and the Ca^{2+}-activated non-selective cation current I_{CAN} [16]. Importantly, I_H activation results in net depolarization as Na^+-influx predominates.

Delta waves
Like slow oscillations, delta waves occur during NREM and are mainly of thalamic origin. In the course of sleep, excitatory inputs from wakefulness promoting areas in the brainstem and basal forebrain [24] start to diminish, causing thalamic neurons to become progressively hyperpolarized. In this hyperpolarized state, an interplay between I_H current, I_T-current, and hyperpolarizing K^+- and Cl^-currents takes place. In a state of hyperpolarization, I_H is activated and causes a net depolarization due to Na^+-influx. It is also permeable for K^+ but the Na^+-influx outweighs the K^+-efflux. At about -65mV, I_T gets activated and further depolarizes the cell to the point where action potential threshold is reached, resulting in an action potential burst. Then, K^+-efflux and Cl^-influx are activated because of the depolarization and they hyperpolarize the cell again, starting the cycle anew [25]. As with slow oscillations, if the oscillations are visible on the EEG level, it means that a sufficiently big number of neurons participate. In this case, synchronization is established through the hyperpolarization affecting all thalamic neurons. These neurons are within the thalamic nuclei quite monotonous in their cell properties,

thus behaving similarly to inputs. Additionally, the delta wave propagates to cortex via thalamic neurons projecting to layer IV pyramidal neurons (called thalamocortical projections) which send projections reciprocally back to thalamus (i.e., corticothalamic projections) [26]. Synchrony of neurons participating in the delta oscillation is then further established through this interplay between thalamus and cortex.
As implied in the beginning of the paragraph, there is data indicating that layer 5 pyramidal neurons in the cortex display oscillations in the 1-4Hz range under conditions that mimic the neuromodulatory state during deep NREM sleep [8], [27].

Sleep Slow Waves
When analysing EEG data or local field potential data[1] ('LFP'), it is difficult to differentiate slow oscillations and delta waves (K-complexes are easier since they occur during NREM 2) and thus they are often grouped together in the field as *'sleep slow waves'* [16] when analysing EEG and LFP data, usually from 0.5-4Hz. It is important to keep in mind that slow oscillations and delta waves as two distinct events were established using single cell recordings with patch methods, where data show both fluctuations of the membrane potential and action potentials. The fine resolution of the data gets lost as the scales of the signal increase up to hundreds of neurons in the case of LFP and thousands of neurons in EEG.
Sleep slow wave activities (SWA) have been associated with homeostatic functions of sleep, as Vyazovskiy et al. and Aeschbach et al. [28], [29] found that during the night both the amplitude and the slope of slow waves decrease, which can be interpreted as a global weakening of cortical synapses whose strength has increased during the day due to repeated usage and is brought down during sleep. A proposed function is the reestablishment of an energetically favourable state of the brain, where important information has been successfully stored, unnecessary has been discarded and plasticity of the brain is maintained, to then gather new information again (see section on memory consolidation) [30], [31].

K-complex
K-complexes appear spontaneously during NREM 2 of humans, while they are not clearly described in rodents [32]. On a mechanistic level, they are thought to be a single cycle slow oscillation [16]. They are often followed by a spindle (see below) and an alpha burst and can be evoked by sensory stimulation but only in NREM sleep, not REM [33]. They seem to be multimodal, meaning that different types of sensory stimuli can evoke them and there is evidence that the differing qualities within the type of stimulus and behavioral relevance of the stimulus have an influence on the amplitude of the K-complex. However, this relationship seems to depend on the brain state as well and the evidence is often contradicting. As for 'spontaneous' K-complexes, it is not quite clear whether they are truly spontaneous or if they are elicited by interoceptive processes, since they have been associated with blood pressure changes and heartbeat increases.
Similar to slow oscillations, they preferably occur in the frontal cortex, which is indicative that they are not the result of direct cortical arousal by sensory information reaching the cortex. It is assumed that K-complexes are either elicited via sensory signals that are relayed through nonspecific thalamic nuclei or via the ARAS in the brainstem.

[1] same type of measurement of electric potentials, but on a smaller scale, i.e., the signal of a couple of hundreds of neurons is measured as compared to tens of thousands on the EEG level.

Lastly, the function of K-complexes seems to be a sleep-preserving response to potentially arousing stimuli and an emergent event of deepening sleep [34], [35].

Sleep Spindles

Spindles are oscillatory events in the range from 11-16Hz and show a striking waxing and waning pattern in the course of their ~6-15 cycles in the time span of 0.4-2 seconds [32]. The main impulse generator of spindles are GABAergic cells of the reticular nucleus thalami (TRN) [36]. Similar to delta waves, I_T plays an important role in rhythm generation. However, TRN cells express a subtype of the Ca^{2+}-channel with a threshold that lies at more positive potentials. So, when a TRN cell depolarizes, Ca^{2+} flows into the cell and causes further depolarization with a spike burst. Next, a Ca^{2+}-activated K^+-channel called I_{KCa} is activated which hyperpolarizes the TRN cell again. This hyperpolarization is already sufficient for the I_T to switch from inactive to closed and therefore another Ca^{2+}-influx can occur. After a few of these cycles, a CA^{2+}-activated cation channel called I_{CAN} is activated, causing a depolarization of the neuron positive to -55mV, which leads to tonic spiking of the cell and thus the spindle cycle is terminated [25].

Synchrony is achieved through local axonal collaterals within TRN. Gradual increase and decrease of cell recruitment within TRN results in the waxing and waning visible in the EEG. The resulting spindle is transferred to thalamocortical cells in the other thalamic nuclei, which in turn project to the cortex. TRN cells receive in turn excitatory input form thalamocortical and corticothalamic cells, resulting in synchrony and initiation of another spindle cycle.

Moreover, a few studies indicate that there might be a second spindle generator located in cortical regions. One based that claim on hemodynamic changes during spindles recorded in humans with MRI [37]. Another found different responses of fast and slow spindles after administration of a sedative drug [38]. However, this hypothesis requires further experimental confirmation.

The function of spindles seems to be associated with memory consolidation and learning. They show a positive correlation with intellectual abilities in humans [39]. Several studies have found an increase in spindle activity after procedural [40]–[42] and declarative [43], [44] learning tasks in humans and after reward learning in rats [45]. Their exact role in consolidation of memories is not fully understood but they seem to mediate 'replay' of cortical firing patterns during sleep that encode bits of information acquired during wakefulness [46] and are potentially enabling long-term potentiation (LTP) in cortical neurons [47], [48].

Sharp-wave ripples

Sharp-wave ripples (SPW-R) are events that occur only in the hippocampus and are the result of the most synchronous population event in the mammalian brain. They occur mainly during NREM sleep but also during prolonged periods of immobility (quiet wakefulness) and transient halts during locomotion [49]. On an LFP level it is composed of two parts: the sharp-wave and the ripple. The sharp wave is a large deflection with negative polarity in the stratum radiatum of CA1 that lasts 40-100ms and is the result of depolarization of apical dendrites of pyramidal CA1 neurons induced by the synchronous bursting of CA3 pyramidal cells. Ripples are short-lived fast oscillatory events in the CA1 pyramidal layer which are caused both by firing of pyramidal and inhibitory neurons following a sharp wave, thus creating a compound event called sharp-wave ripple.

On the anatomical side, CA3 pyramidal cells have extensive axonal collaterals that target other pyramidal cells in both CA3 and CA1. The collaterals to other CA3 neurons create a recurrent

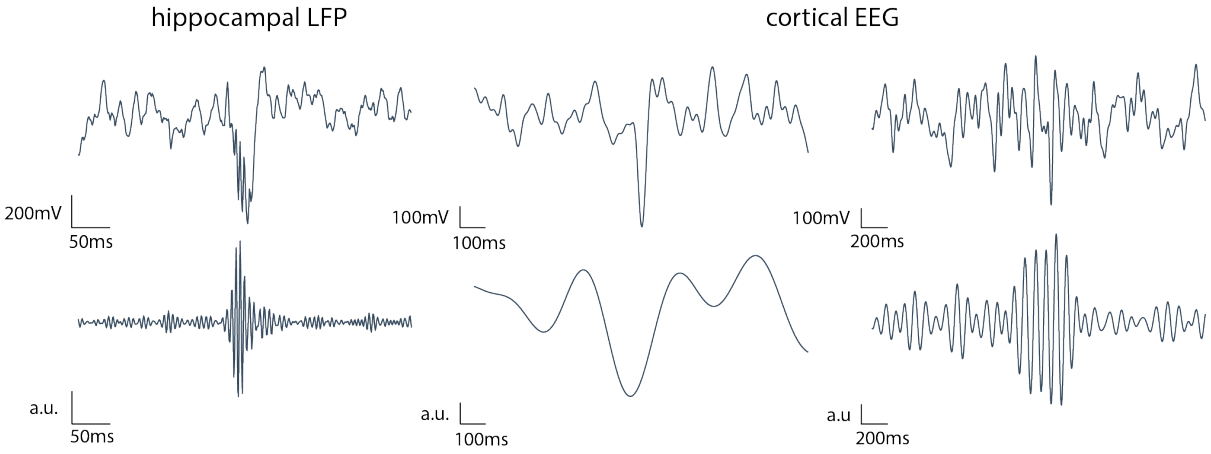

Figure 1.1. Typical oscillatory events during NREM sleep

Exemplary traces of sharp-wave ripples (left), slow waves (middle), and spindles (right). The top traces represent the raw signals while the bottom traces depict the same signal but filtered in the respective frequency ranges. Note, that the ripple is nested in a sharp down deflection, representing the sharp wave part that is transmitted from the stratum radiatum to the stratum pyramidale where the signal was obtained. The slow wave is marked by a distinctive negative deflection representing the DOWN state, followed and preceded by a positive deflection reflecting the UP state. Sleep spindles are often not well visible in raw EEG signals of mice, but filtering reveals the strong waxing and waning characteristic of that event.

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system that can give rise to large numbers of neurons firing in synchronized bursts, which happens spontaneously [50], and which can be suppressed by cholinergic-muscarinic input [51] and cannabinoid receptors [52]. The burst firing of CA3 pyramidal cells induces then a ripple in the CA1 region via Schaffer collaterals [53].

Ripples consist of highly synchronous firing of CA1 pyramidal cells as well as inhibitory interneurons, mainly Parvalbumin (PV) expressing basket cells [49]. Clamp recordings from single pyramidal neurons show that the negative peaks during the ripples represent spike activity while the positive peaks come from somatic IPSCs, indicating a strong input of interneurons during this phase of the ripples [54], [55]. However, the generation of the fast rhythm of ripples (varying ranges from 100-250Hz in rodents [50], [56], 100-120Hz in monkeys [57] and 80-160Hz in humans [58]) is still quite uncertain.

One rhythm generator could be based on interactions between pyramidal and interneuron interactions [49], whereby pyramidal neurons excite inhibitory neurons which in turn inhibit pyramidal neurons with no interactions between interneurons among themselves. So far, artificial ripple-like oscillations could be induced by optogenetically stimulating either pyramidal cells alone or both pyramidal and interneurons with a protocol that mimics the SPW-R envelope to induce spiking [59]. This model is also often associated with gamma oscillations [60], [61], which raises questions about whether small adjustments in the circuitry would be sufficient to go from gamma to ripple frequency [49].

A model that is more strongly supported by the current evidence, is based on interactions among interneurons and the entrainment of pyramidal cells in a second step [49]. Indeed,

there is data suggesting that PV basket cells are necessary [62] for ripple generation but $GABA_A$-receptor mediated inhibition is too slow to give pace to >200Hz rhythms. Thus, PV interneurons do not act all synchronously on pyramidal cells during a ripple, but rather through interactions with each other, they form assemblies which act time-shifted on pyramidal cells and on the respective other interneuron-assembly [49]. However, to this day the formation of PV basket cell assemblies remains to be demonstrated.
Lastly, Traub and his colleagues proposed that the rhythms are generated via gap juctions between axons of pyramidal cells [63]–[66]. However, a lot of the results in the mentioned studies as well as some others leave room open for different explanations and to this day the existence of gap junctions between pyramidal cells in CA1 has not been shown.
Sharp-wave ripples have been implicated in memory consolidation during sleep in a now extensive number of studies. First, it has been demonstrated that certain CA1 pyramidal neurons increase their firing in a particular place in the environment the animal is currently located in. Thus, they are called place cells [67] and their function is to encode the environment. Then, Wilson, McNaughton and Skaggs [57], [68] showed that a sequence of firing cells which represents the trajectory on a linear track that was explored by the animal previously is preserved or *"replayed"* during sleep. In two following studies, it was then shown that this replay happens in a time-compressed manner during SPW-R's [69], [70]. Thus SPW-R's may reflect a reactivation of a part of a *"cognitive map"* [71] and therefore information that is replayed during a SPW-R's in the hippocampus is transferred to the neocortex for long-term storage or the hippocampus binds and provides contextual information for other elements of the learned experience which are encoded in the neocortex (see section on memory consolidation). Indeed, data show that novel experiences, new environments and learning all increase SPW-R-occurrence [56], [72]–[75]. Furthermore, there are several studies showing learning impairments when SPW-R's were perturbed during sleep after a learning task [76]–[78]. Additionally, SPW-R -frequency can induce synaptic modifications. Thus, CA1 pyramidal neurons might induce LTP or spike time dependent plasticity (STDP) in downstream neurons [79]–[82].
Lastly, as mentioned above, SPW-R's occur during quiet wakefulness as well. During these ripples, a *"reverse replay"* has been reported, where the firing sequence of place cells is reversed [83]. Studies suggest that SPW-R's during wakefulness serve the maintenance of a cognitive map and thus help in decision making [84], recapitulation of the immediate past trajectory [85], and planning of future behavioral routes (*"preplay"*) [86]–[88]. The role of the reversed SPW-R's seems to be involved in recapitulation and planning to move backwards along a known trajectory.

Summary

What is important to keep in mind when considering sleep oscillations is the fact that they do not occur totally randomly at any time and independently of each other. But rather they co-occur and are sequenced within as well as across brain regions, which has been demonstrated in numerous studies [77], [89]–[98]. Namely, cortical sleep spindles tend to appear after the DOWN state of a cortical slow wave at the transition to the UP state [91], [92], [94], ripples tend to be nested in troughs of cortical spindles [98] and during the UP state of cortical slow waves [95]. One possible function of this orchestration of sleep oscillations is thought to be memory consolidation during NREM sleep: cell assemblies firing during sleep in a sequence that was formed during wakefulness have also been observed in the cortex [46], [99] in association with UP states. These assemblies have been shown to fire during or shortly after (40ms) a

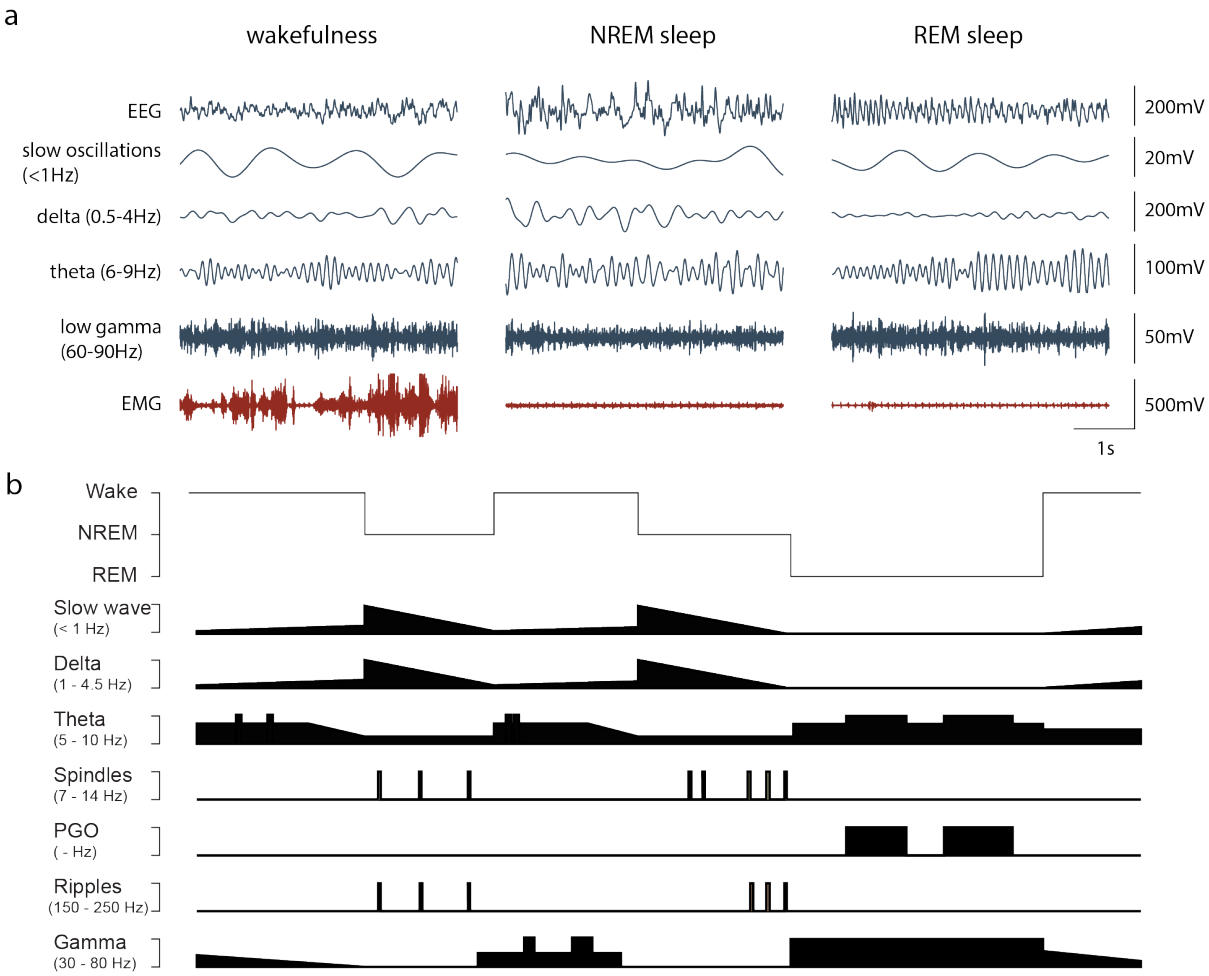

Figure 1.2. Typical EEG characteristics of wakefulness, NREM, and REM sleep in mice

a) Exemplary EEG traces from a recording obtained from mice, which represent the three vigilance states that can be described in these animals (from left to right): wakefulness, which is characterized by fast oscillations with low amplitudes, NREM sleep which is dominated by high amplitude slow waves, and REM sleep which displays very rhythmic theta oscillations. Additionally, the same traces can be seen bandpass filtered in the most relevant frequency bands for vigilance assessment. The bottom traces represent the electromusculogram (EMG), which reveals a lot of muscle activity during wakefulness and very little during the sleep states.

b) The top trace shows a representative hypnogram of the mouse sleep-wake-cycle. The bottom traces represent the presence of the ascribed oscillatory bands and events. Note, that oscillations in all frequency bands are never fully absent but just more or less present. However, spindles and sharp-wave ripples can be defined as distinct events due to their well described cellular and circuit mechanisms with quite specific characteristics. Sleep slow waves are quite densely present in mice, which is why they are represented as a continuum with diminishing amplitudes in the course of a NREM bout. Additionally, as sleep pressure builds up, increasing presence of 0.5-4Hz oscillations can be observed already during wakefulness. Adapted from Adamantidis et al. 2019 [31].

SPW-R and this pattern was even stronger after acquisition of a new rule, which suggests that hippocampal SPW-R's might provide an important impact to target cortical regions to consolidate memory [100].
As for REM sleep, it could be shown that hippocampal theta rhythm and gamma rhythm are coupled as well: Gamma power is greater during the positive slope of a theta cycle in REM sleep. However, this does not occur not in a fixed manner but rather in intermittent bursts of greater so called "phase-amplitude-coupling" [101]. Functionally, theta-gamma-coupling during wakefulness has been implicated in working memory [102], memory retrieval [103] and decision making [104] but its role during REM sleep remains to be investigated further. Furthermore, replay of neuronal firing sequences have been reported for REM sleep as well. However, instead of a temporal compression, replay occurred at a similar time scale as during wakefulness with sequences representing tens of seconds to a few minutes of wake experience. Theta power was elevated during the replay [105].

1.3 Function of sleep

As mentioned above sleep is a necessary behavior even though some species can go without it for extended periods of time during mating season, migration or birthing [106]. Consequently, the most severe consequence of total sleep deprivation during a sufficiently long period is death [107]. Studies in humans showed that less severe short term consequences of acute lack of sleep include reversible decreases in cognitive and motor performance [108], feelings of tiredness and irritability [109], and intrusions of sleep-like patterns in electroencephalographic measurements [110]. Long term consequences of chronic lack of sleep include increased blood pressure, increased mediators of inflammation and metabolic disturbances, such as slowing of glucose metabolism and increased appetite [111]. Sleep loss has also been implicated in the development of neurodegenerative diseases, such as Alzheimer's disease, possibly due to the brain clearance function of sleep [112], [113] (see section on brain clearance). Thus, it can be concluded that the organism performs certain functions exclusively or primarily during sleep and they seem not to be restricted to the brain but include the entire organism. The most important ones will be presented in this section, including metabolism, memory consolidation, brain maturation, brain clearance and immunity.

Metabolism
Metabolism can be described as the total range of biochemical processes occurring in the living organism that maintain the organism and produce energy. It can be roughly divided into anabolic processes which build up bio matter and catabolic ones which break it down [114]. These processes appear to be quite strongly regulated by sleep as many studies in both humans and animals demonstrated changes in weight, food intake and hormonal dysregulations upon sleep deprivation. Early hypotheses on possible functions of sleep stated that it is a behavioral state which conserves more energy as compared to quiet wakefulness [115], [116]. However, the rather small amount of energy that is effectively saved and the uptick in slow wave activity after torpor and hibernation make it appear unlikely that it is sleep's *primary* function [117], [118]. More modern hypotheses proposed that sleep and wakefulness are accompanied by a partition of metabolic processes into ones which occur primarily during wakefulness and ones which

occur during sleep [106]. The authors roughly divided the processes in ones that *run the machine* (active behaviors that the organism executes) and ones that *maintain the machine* (processes which maintain the biological integrity of the organism). A simple example related to the brain is provided by Tononi and Cirelli [119] who propose that during wakefulness net synaptic strength increases as a result of active behavior and acquisition of information (organism executes behaviors) and sleep allows a net synaptic downscaling that reflects the "elimination" of useless information (i.e. forgetting or synapse weakening) and restores plasticity of the brain to maintain the ability to gather information in the next wakefulness period (biological integrity is preserved).

Metabolic processes are tightly regulated through hormonal control and indeed, studies found effects of sleep loss on the ones responsible for metabolic regulation: Human growth hormone (GH or HGH) is a peptide hormone produced and secreted by the anterior pituitary. Its main metabolic functions include the promotion of protein synthesis, lipolysis, suppression of gluconeogenesis from amino acids, and slowing of glucose usage [6]. Its secretion is elevated during sleep with a maximum during SWS [120].

Cortisol is another hormone that shows strong changes in secretion across the sleep-wake cycle. As a glucocorticoid it is produced and secreted by the adrenal glands and is thus part of the *"hypothalamic-pituitary-adrenal axis"* (HPA). It is involved in stimulation of lipolysis, peripheral protein breakdown, and suppression of uptake and usage of glucose [6]. Cortisol levels are first strongly reduced during sleep and then start to rise again in the second half of the night in humans, mainly during REM sleep and reach a peak in the early morning after awakening [121], [122].

Interestingly, acute sleep deprivation leads to a reduction in nocturnal GH release with a subsequent compensatory increase during recovery sleep with reversed observations for cortisol [123], [124]. However, recurrent partial sleep restriction did not show such compensatory effects in hormone secretion [125], [126].

Ghrelin is released from mucosa of the stomach as a response to fasting and contributes to the feeling of hunger [127]. Leptin is produced by adipose tissue and suppresses appetite. It has been shown to rapidly respond to caloric shortages or surpluses with a decrease or increase, respectively[128]. Both ghrelin and leptin act on neurons in *nucleus ventromedialis hypothalami* [124] and the hypothalamic *nucleus arcuatus*, which in turn regulates the activity of other hypothalamic regions such as the *nucleus paraventricularis hypothalami* which regulates hormonal systems such as the thyroid and adrenal gland. Additionally, ghrelin and leptin also have an influence on orexins/hypocretins-expressing neurons which thus serves as a potential link between sleep and metabolism [114], [129]. Human studies showed increases in leptin and ghrelin levels during sleep with ghrelin levels falling during the latter part of the night [130], [131]. In further studies, leptin levels dropped and ghrelin levels increased during sleep restriction [132], [133].

Effects on insulin in relation to sleep loss have been described as well. Namely, several studies found a slowed glucose metabolism after sleep loss [134], decreased glucose tolerance [135] and reduced insulin sensitivity [136], which is consistent with the observation that chronic sleep loss is associated with an increased risk of developing type 2 diabetes [137]. It is speculated that these effects might be due to increased inflammatory mediators under chronic sleep loss which have been shown to alter insulin sensitivity [138].

However, the effects of these disturbances in metabolic hormones on measurements such as food intake, weight change and energy expenditure show quite different results in humans and

animals that make it difficult to interpret: total sleep deprivation in rodents showed quite consistently that the animals progressively lost weight in the course of the experiment despite strong increases in food intake (up to 100% of baseline levels) [107], [139]–[143] which could not be explained by reduced efficiency of gut function. Additionally, it could be demonstrated that total energy expenditure strongly increased by measuring caloric value of food, CO_2 production and decline of core body temperature towards the end of the experiment, which was defined either by death of the animal or euthanasia when imminent death was obvious [139]. Sleep restriction instead of total sleep deprivation showed similar results with weight loss despite hyperphagia. Additionally, the authors found lengthening of the small intestine in sleep restricted rats which reflects the increased demand for nutrients [144], [145].
The effects of sleep deprivation on energy expenditure in humans are less clear though. The hormonal disturbances described above [132], [133] were followed by reported increases in hunger and appetite, as well as a preference for high carbohydrate and salty foods. However, the authors suggested that this increased food intake might be rather due to an emotional rather than a caloric need. Furthermore, some studies found either no changes in energy expenditure [146], [147] or even reported weight gain [114], [148] under conditions where food was *ad libitum* accessible. However, a study under moderate calory restriction found greater weight loss in the sleep restricted group [149] and one study found an increase in energy expenditure in humans under sleep restriction [150].
When comparing results in humans and rodents, it is important to keep in mind that lab animals underwent much more severe conditions of sleep deprivation (e.g., up to the point of major weight loss or death) and many observations concerning weight and energy expenditure in animals occurred only in the latter parts of the experiments. Experiments on humans on the other hand were conducted either under comparatively mild sleep restrictions or only short periods of total sleep deprivation. Therefore, it is possible that the increases in appetite and food intake are adaptive responses that kick in early and counterbalance the increase in energy expenditure under milder conditions at least for a while.

Memory consolidation
One of the most highly investigated aspects of both NREM and REM sleep is their involvement in memory consolidation as part of learning. This term is here somewhat loosely used to cover a process occurring after the *acquisition* of new information whereby the information is stored for long term usage, thus becoming a *memory*. Consolidation refers to a process whereby a newly formed memory becomes less and less susceptible to interference from other similar memories and normally occurring amnesia [151].
Quintilian, a roman rhetorician from the first century AD, once noted: "*What could not be repeated at first is readily put together on the following day; and the very time which is generally thought to cause forgetfulness is found to strengthen the memory"* [152], albeit it is not quite obvious if he referred to sleep precisely or broader circadian rhythms. And indeed, several scientific studies have demonstrated the beneficial effects of sleep (both regular nocturnal sleep and naps) on both procedural memory (pertaining to trained motor behavior) and declarative memory (pertaining to episodic memory and explicit knowledge) [153]–[156].
Importantly, the complete process of memory consolidation can be divided further in the *stabilization of memories* and *enhancement of memories*. Stabilization refers to a process where the memory trace becomes stable so that it is retained for long term storage. Stabilization has been demonstrated to also occur during wakefulness and it is effective enough that sleep is not

required [151], [157]–[161]. Enhancement occurs mainly during sleep and it encompasses a process where acquired skills and memories become more strongly encoded, resulting in the recovery of previously lost memories, additional learning, and better understanding of underlying "rules" of a cognitive task [162], [163]. Importantly, it seems that especially difficult tasks and information, tasks that are behaviorally relevant, and emotionally salient information benefit from sleep [163], [164].

Studies in humans have shown quite consistently that consolidation of procedural memory is a process that is linked to sleep specifically and not just a function of time ([163], [164] for reviews of some of these studies). It could even be demonstrated that different parts of sleep contributed differently depending on the type of task. Meaning, depending on whether the task involved visual discrimination, motor learning etc. either SWS, NREM 2 or REM sleep showed the strongest correlations with performance [157], [165]–[167]. An important caveat to keep in mind is the fact that studies with humans often showed correlation which is not necessarily saying something about causation.

The effect of sleep on hippocampus-dependent declarative learning on the other hand is not so clear and data are a bit inconsistent in humans [163], [164]. Many early studies operating with word-pair association tests did not show benefits of sleep on memory performance or alterations in sleep architecture. However, more recent studies found associations between memory performance and SWS-rich early night sleep with stabilization and potentially enhancement of memories [163], [164], [168]. Data from studies on REM sleep suggest that during REM sleep weak associations were strengthened [169] and creative, flexible information processing was enhanced [170]. Furthermore, several studies in mice could show that not only sleep but specifically the cross-frequency coupling between hippocampal ripples and cortical spindles and slow waves was crucial for memory consolidation in either fear conditioning or novel object recognition tasks [77], [93], [171]. Additionally, REM sleep seems to play an important role in these two tasks as well [172].

The mechanism of how this consolidation of newly acquired information works is still under a lot of investigation and several hypotheses have been developed that try to give a cohesive explanation on how the brain stores information during sleep. Early theories on memory consolidation speculated that there was a first early storage for memories in the hippocampus that then transferred recent information to other parts of the brain, i.e. neocortex, for long term storage [173], [174]. The *Systems memory consolidation hypothesis* [175] is in a way an extension of the early theory. As the field gained more understanding of circuit and cellular mechanisms, the hypothesis was modified to state that the cortex and other brain areas host the different aspects of a memory trace from the beginning. Neurons across different parts of the neocortex fire together in an ensemble to form an "engram" which can be roughly described as a bit or cognitive unit of a memory. They are “bound together” in the beginning by the hippocampus as a short-term memory [176]–[178]. The cortical engram then becomes independent of the hippocampus through replay during sleep which strengthens the connections between the cortical areas forming the engram. The engram is thus stored in a long-term fashion. During sleep, replay of the neuronal representation of the memory in the form of sharp-wave ripples (see section on sharp-wave ripples) occurs first in the hippocampus and triggers downstream neocortical replay of neuronal assemblies there [168], [175]. Two fMRI studies in humans found increasing activity in the respectively involved PFC regions during memory recall over time and one of them even found a decrease in hippocampal activity. These results can indeed be interpreted as readouts of the aforementioned process, also called *“corticalization”*.

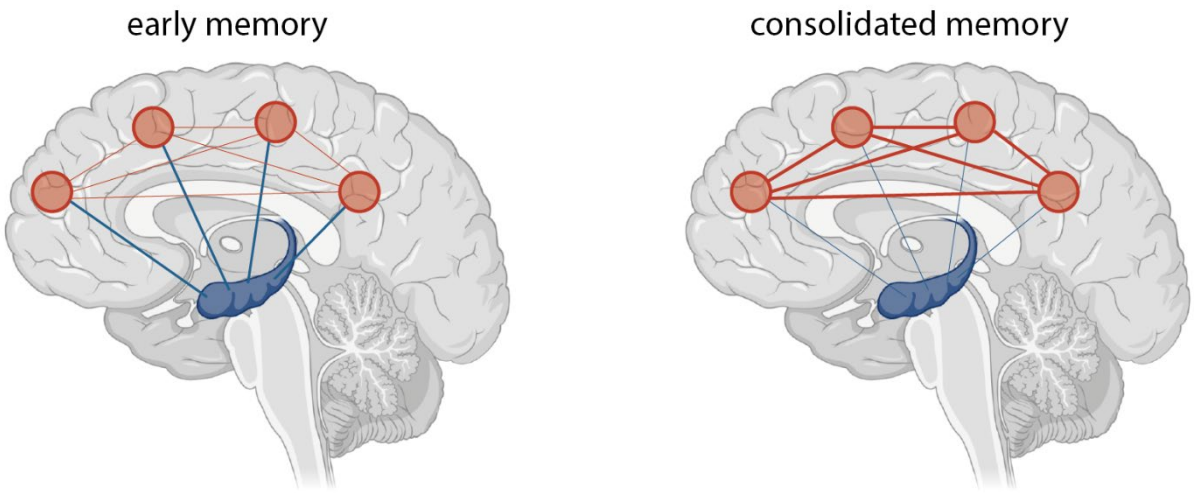

Figure 1.3. Abstract schematic of systems memory consolidation

When new memories are acquired, different parts of the cortex encode certain aspects of the whole engram and the hippocampus connects them all together. As the memory is replayed during sleep, connections between the cortical engram parts get reinforced and the hippocampal connections are not needed anymore. Adapted from Klinzing et al. 2019 [169]; made with BioRender ("https://biorender.com"; template name: "Brain (sagittal, hippocampus only)").

Importantly, the current data available suggest that the flow of information seems to be bidirectional between hippocampus and cortex. Early data suggested a flow from hippocampus to cortex during sharp-wave ripples in NREM [179], [180]. More recent data found that cortical activity influenced hippocampal activity patterns during sharp-wave ripples [181]. As a result, a part of the field proposed a bidirectional dialogue between the two brain areas [168], [182], [183].

Replay of neuronal ensembles has been described in different cortical regions, such as prefrontal [46], parietal [184], motor [185], visual [99], entorhinal [186] as wells as subcortical regions like ventral striatum [187] and the ventral tegmental area (VTA) [188]. Furthermore, hippocampal replays occur in coordination with neuronal sequence firing in other brain areas during SWS, usually with a delay of ~40-50ms [46], [99], [187], [189], [190], suggesting that the hippocampus is the initiator of ensemble replay, which is in line with the seemingly spontaneous nature of ripple occurrence (see section on sharp-wave ripples). Slow waves and spindles seem to play an important role during cortical replays. Spindles tend to nest in the UP states of slow waves and a study using two-photon imaging could show that during this compound event cortical pyramidal neurons and parvalbumin-positive interneurons that inhibit pyramidal somas showed maximum activity, whereas somatostatin-positive neurons which inhibit pyramidal dendrites showed very low activity, suggesting that this was a time window where pyramidal cells in the cortex were very sensitive to receive input activity, from the hippocampus for instance, but did not generate much output activity. This mechanism is potentially supportive of memory consolidation [191]. On a broader view, NREM shows signs of a general decrease in

synaptic strength [192], [193] but it was also reported that newly formed spines were preserved during SWS and formation of new spines was even promoted in motor neurons that were involved in a newly learned motor task [194]. Similarly, anatomical imaging in mice showed that newly formed synapses were generally pruned during REM sleep, while preserving and even strengthening the ones involved in a newly learned task [195]. It is speculated that REM sleep and NREM serve complementary functions in memory consolidation: one study investigated the triplets of NREM-REM-NREM bouts and found that hippocampal firing rates were reduced in the second NREM period while the intra-ripple synchrony increased. Synchrony was defined as pair-wise correlation between hippocampal spikes which putatively represents replay. These findings also correlated with theta power in the interleaved REM episode [196]. Further studies showed correlations between incidences of spindles and ripples with REM-related decreases in hippocampal firing rates. Another one found correlations of spindle incidences with REM sleep-related increases in immediate early gene expression involved in synaptic plasticity after exposure to a novel environment [197], [198].

The exact mechanisms regulating which networks and synapses are consolidated during sleep are not well understood yet, but they potentially involve dopaminergic activity and theta oscillations. Namely, one study in rats found increased theta coherence between prefrontal cortex and hippocampus at the decision point of a Y-maze. It additionally suggested that this increase in theta coherence was caused by dopaminergic inputs to prefrontal areas [199]. Furthermore, dopamine has been shown to “tag” synapses for later strengthening [200]. Additionally, hippocampus receives input from the VTA and stimulation of these inputs during encoding increases replay of hippocampal neuron ensembles during sleep [201]. A study investigating the role of theta activity found that place cells that encoded a novel location reactivated during the peak of theta phase and that this phase preference changed to the trough as the location became familiar. The authors suggest that reactivation at the peak strengthened a memory trace while the troughs weakened it [202].

Notably, hippocampal replay seems to be involved even in consolidation of memories that are traditionally considered 'non-hippocampus dependent'. Studies in rats and one in mice found that silencing hippocampus activity impaired consolidation of novel-object recognition performance, which is considered dependent on the perirhinal cortex but not necessarily on the hippocampus and a study in humans found that hippocampal activity in fMRI predicted performance in a motor-skill task [203], [204]. However, the notion that these types of memories are not requiring hippocampal activity for acquisition and retrieval still hold to date, whereas hippocampus-dependent memories rely on it also in these stages.

Recent data suggested even an implication of the thalamus in memory consolidation through information transfer. The authors could show that when mice were exposed to a novel visual stimulus, the neurons in the lateral geniculate nucleus showed a potentiation of their response to the stimulus during the encoding phase, while a similar response potentiation appeared in neurons of the primary visual cortex (V1) only after sleep. The authors showed that spike-field coherence of thalamic neurons to cortical slow waves and spindles increased in this sleep period. Additionally, when corticothalamic V1 neurons were optogenetically silenced, the increase of spike field coherence disappeared and V1 neurons did not show a response potentiation [205]. Thus, information transfer from thalamus to cortex occurs during sleep and it depends on sleep spindles and slow waves. However, studies regarding the other thalamic nuclei which encode other sensory modalities are lacking to this day, as well as a potential involvement of the hippocampus in this process which would be expected according to the

hypothesis.
While replay during sleep of neuronal assemblies that have been formed during wakefulness has been now well established, as well as the fact that neuro-oscillatory events such as slow waves, sleep spindles and sharp-wave ripples are involved in memory consolidation, the concept of the engram has not been fully demonstrated. While several studies successfully identified cell populations which participate in the expression of memory traces in animals (see [206] for an extensive review), full engrams consisting of neurons from remote regions which encode different sensory modality information and are "bound together" by the hippocampus, and become independent of it remain to be demonstrated. The precise role of replays has also not been fully understood to this day and it is not clear that they promote synaptic plasticity or long-term memory formation. However, two studies could demonstrate that NMDA receptor antagonism (which blocks LTP) could disrupt the formation of neuronal assemblies which formed replays [207], [208]. Nonetheless, the fact that replays occur also during wakefulness [49] raised the question from the authors of one review [209] why then animals should risk going to sleep at all if replays can also occur during wakefulness. Another issue is the fact that replays do not occur during the entire time of sleep but there are rather distinct and short time windows when they occur. Thus, one question is then what the function of neuronal activity outside of replays is and how the right replays are selected for potentiation. Also, it could be argued that since the brain is spontaneously active during sleep, the occurrence of replays and preplays can be expected by default and that the brain could also replay different combinations of already present memories [209]. Furthermore, preplays have been reported to occur *before the actual experience* of the represented trajectory [210] and a study found that preplay occurrence might just be a statistical anomaly [208].
The *synaptic homeostasis hypothesis* (SHY) has been posited by Giulio Tononi and Chiara Cirelli. They stated that synapses overall increase their strength during the day and during sleep, especially during slow waves, overall synaptic strength decreases again to restore brain plasticity. Recently formed engrams are stored if the strength of a synapse remains greater after sleep induced down-scaling than before it partook in the engram [209].
As the animal is awake, it gathers information, which is stored at least temporarily in the brain. Information storage in the brain is understood to occur through the strengthening of synapses during different behaviors such as exploration [211], association learning [212], contextual memory formation [213], fear conditioning [214], visual perception learning [215], cue-reward learning [216], and avoidance learning [217]. But there a few exemptions to this rule where *learning by depression* occurs in the hippocampus in spatial reversal learning [218], in the amygdala during fear extinction [219], and in perirhinal cortex during familiarity recognition [220]. However, strengthening synapses comes at a cost: stronger synapses usually require more proteins to enable the strengthening (e.g. more receptors in the post-synaptic membrane) and more energetic supplies [221], [222]. Furthermore, stronger synapses mean that the chance of the post-synaptic neuron to fire generally increases as suggested by two models [223], [224]. This means that the neuron will spend more energy on action potentials, which are already the greatest source of energy consumption in neurons [225], [226], and that the neurons will lose part of its selectivity as stronger synapses mean that a wider range of inputs can initiate a post-synaptic action potential [223], [224]. However, both energetically as well as from a computational point of view neurons fire ideally sparsely and when a *suspicious coincidence* occurs, which describes the case when inputs occur together more frequently than would be expected by chance, thus indicating regularities in the input and therefore in the

environment [209], [223], [224]. A way to deal with these costs of increased synaptic strength would be to reduce synaptic strength at times again to allow a partial loss of gathered information while preserving the most important ones. The proponents of the SHY hypothesis posited that the brain is doing that during sleep. As the brain goes *offline*, spontaneous neuronal activity arises instead of activity in response to external stimuli, which allows the brain to obtain a comprehensive sampling of its overall knowledge of the environment without biases introduced by inputs to neurons stemming from outside [209].
In order to not lose all stored information during synaptic *down scaling*, not all synapses are depressed equally. Computer simulations suggested models where either all synapses decreased in strength proportionally but those below a certain threshold become ineffective [227], a specific *spike-timing dependent plasticity* rule protected strong synapses from being depressed [228], or higher spiking rates during sleep of post-synaptic neurons served as an indicator to spare associated synapses from down scaling [224], [229]. Another simulation could demonstrate that overall synaptic down scaling increased signal to noise ratios, promoted memory consolidation, gist extraction and integration of new memories with established knowledge (see below) [229]. Indeed, experimental data described potential mechanisms that could explain how strongly activated synapses depress less or not at all. Namely, high levels of calcium (which enters the postsynaptic intracellular space upon receptor activation) could block calcineurin, a phosphatase that promotes synaptic depression and that is upregulated during sleep [230]. Additionally, high calcium levels reduced the inhibitory activity of CamKII inhibitor, which decreases synaptic strength by preventing CamKII from binding to NMDA receptor [231]. Furthermore, the alpha isoform of the CamKII inhibitor was upregulated during sleep [230] and was in turn inhibited by calcium [232].
One potential mechanism through which overall synaptic downscaling could be achieved is via the influence on *spike-timing dependent plasticity* which describes the process of inducing either long term potentiation or depression of a synapse based on frequency and timing of spike activity at that synapse [233]. It could be shown for example that levels of AMPA receptors with GluR1 subunit were higher after wakefulness than after sleep and phosphorylation statuses of these receptors, CamKII, and GSK3β were in line with net synaptic depression during sleep and net potentiation during wakefulness [193]. Additionally, 6h sleep deprivation led to an increase of GluR1 containing AMPA receptor as compared to a similar period of sleep. However, a similar study found the same circadian phase of AMPA receptor expression but failed to reproduce the results after sleep deprivation [234]. Furthermore, one study found insertion of GluR1-containing AMPA receptors into synapses during wakefulness, another the removal thereof during sleep, and a third found increases in dephosphorylation of these receptors at the Ser845 peptide during sleep [235] which is a molecular hallmark of synaptic depression [236].

Electrophysiological data could show that the amplitude and slope of evoked response potentials increased with time spent awake and decreased after sleep again. It even showed a correlation of the amplitude reduction with slow wave activity [193]. Additionally, mean cortical firing rates increased after prolonged hours of wakefulness [193], as well as amplitude and frequency of miniature excitatory postsynaptic currents [237], which are thought to indicate the current elicited by single transmitter vesicles. However, it is worth noting that the amplitude differences between controls and sleep deprived animals were rather small.
The proponents of the *SHY hypothesis* also place a special emphasis on slow waves, as they see them as both an index and a contributor to synaptic homeostasis. The feature as an index is based on studies, which showed that general slow wave activity (SWA) increased when rats

were placed in an enriched environment and the increase correlated with the induction of cortical BDNF which is a marker of synaptic potentiation [238]. Local SWA increases in the respective cortical areas after learning a task in both rats [239] and humans [240], [241]. The feature as a contributor is supported by studies, which showed that burst firing, which is common during slow wave sleep, led to depression of excitatory post-synaptic potentials [242], possibly through removal of AMPA receptors [243]. The relationship between SWA and synaptic strength can be thought of as a control loop where synaptic strength is the regulated variable and SWA serves to regulate it. In this loop, synapses are first upregulated, which leads to higher firing rates and synchrony during SWA, which thus becomes higher. As burst firing during SWA leads to synaptic depression, firing rates and synchrony decrease in the course of slow wave sleep again and thus SWA reduces as well. Consequently, the decline of synaptic strength and SWA would be self-limiting according to a computational study [228]. However, it is important to keep in mind that many SWA-like stimulation protocols were able to induce LTD *in vitro* but failed to do so *in vivo* in both hippocampus [244], [245] and cortex [246], [247]. Nonetheless, one study could demonstrate that LTD was *more reliably* induced *in vivo* when the animal was anesthetized [247] which could be blocked again by stimulation of the pedunculopontine nucleus (PPT). Since PPT activity is low during NREM as compared to wakefulness [248] and since thalamocortical activity is generally at the lowest level during NREM, some authors speculate that NREM simply provides an ideal (and maybe necessary) state for LTD to occur [210].

Neuromodulators serve potentially an important role in synaptic up- and downscaling as well. Studies demonstrated that changes of cholinergic and noradrenergic tone during sleep favored a shift towards depression on the STDP curve, thus promoting general down-scaling [249], [250].

A second potential mechanism to achieve synaptic downscaling, besides STDP, would be through *homeostatic synaptic plasticity.* This is a process which describes the maintenance of total synaptic strength of a single neuron in a cell-autonomous manner [251]. The main variable that is supposedly regulated by this process is the neuron's average firing rate. Indeed, there is data from *in vivo* and *in vitro* experiments showing that neurons maintain average spike activity around a homeostatic set point. When neurons are induced to fire more or less, they will come back to baseline levels over a period of several hours by adjusting postsynaptic strength respectively [252]–[254]. Importantly, this *scaling* of synaptic input affects the entirety of synaptic contacts a neuron receives [253]. Increased or decreased somatic calcium levels, respectively, lead at the end of the signal induction chain to adjustments in AMPA receptor density at the postsynaptic membrane [255], just to name one example of the cellular mechanisms. While synaptic scaling involves mainly postsynaptic changes in receptor accumulation [251], [253], presynaptic changes have been observed as well, such as increases in transmitter vesicles and increased probability of transmitter release. These mechanisms are induced under different circumstances and via different pathways than long term potentiation or depression. Another important difference is the time scale: while LTP and LTD occur within minutes to hours, homeostatic synaptic plasticity occurs over several hours [251], [256]. An attractive property of this mechanisms is the fact that neuronal activity is stabilized without affecting the *relative* strength of synaptic inputs, thus preserving stored information.

The benefits of this mechanism can be found on both a cellular level as well as on a network level. The brain already accounts for 25% of the body's glucose consumption while only making up 2% of the body mass in humans [257]. Not only is the neuron's energy usage high but it

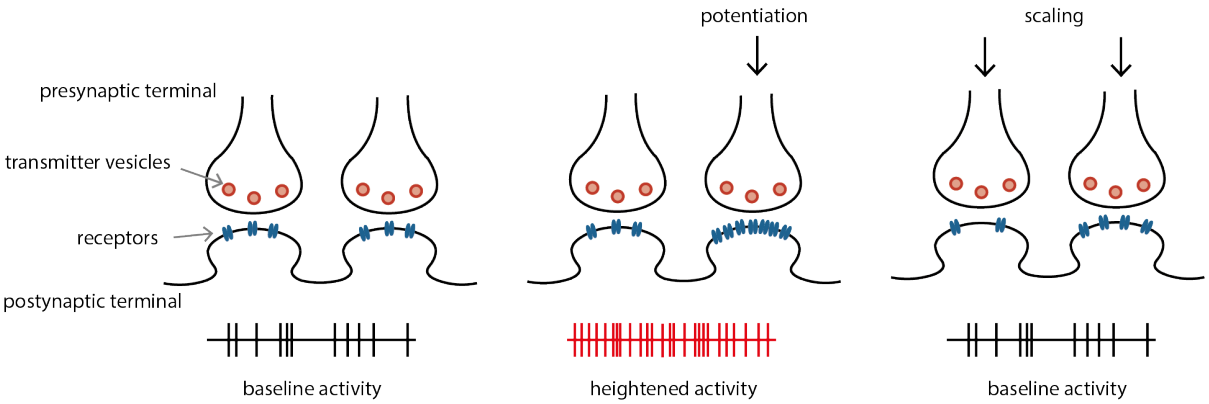

Figure 1.4. Schematic representing homeostatic synaptic scaling

Neurons are regulating their action potential activity around a homeostatic set point. When synapses are potentiated, incoming input will more easily lead the neuron to reach the threshold for action potential initiation. Thus, the neuron scales all synapses down but preserving the relative strength between the inputs and the action potential activity is back to baseline levels. Adapted from Turrigiano 2012 [245].

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appears to be also quite fixed, as a study could show that glucose usage of the brain increased linearly with the number of neurons [258]. More precisely, synaptic activity makes up the biggest part of the brain's energy usage because the involved processes are energetically highly demanding such as release of neurotransmitter vesicles, action potential initiation and propagation, and the restoration of the membrane potential after an action potential [209]. Thus, a net increase in synaptic strength would necessarily lead to higher energy consumption. Additionally, synapses require a lot of cellular constituents such as mitochondria, synthesized lipids, and proteins which are often transported across the entire axon. These energetic costs get even higher if the synapse is strengthened, as that process requires more lipids and proteins for production and recycling of transmitters and vesicles [221], [222].

As for actual evidence concerning overall synaptic downscaling during sleep, structural studies in *Drosophila* showed that protein levels of pre- and post-synaptic components were high after wakefulness and declined during sleep [259]. Size of synapses and number of dendritic spines in giant tangential neurons in the visual system followed the same trajectory [260]–[262]. Furthermore, a two-photon microscopy study in 1-month-old mice demonstrated that spines formed and disappeared at all times, but wakefulness showed a net gain while sleep showed a net loss in spine counts and synaptic contact area. Importantly, the largest synaptic contacts remained unaffected. However, adult mice had a smaller spine turnover that was not impacted by wakefulness and sleep [263] and other studies showed the emergence of new spines and *local* increases in the mentioned synaptic measures co-occurring with the net decrease during sleep [192], [194], [195], [234]

On the network level, simulations could show that net synaptic depression increased the signal to noise ratio of stored information, given that weaker synapses were depressed more than stronger ones [228]. Additionally, the authors claimed that SHY accounts for *gist extraction*,

integration, and *protection from interference* as well. Gist extraction refers to the brain's ability to extract general patterns and rules of the environment and remember high-level abstract invariants like faces and places, rather than low-level details and particularities of an environment. This process was demonstrated to be supported by sleep [264], [265]. However, the authors referred to computational rather than empirical data to make that claim [229].
Integration describes the feature whereby new memories are incorporated into a preexistent cohesive body of memories which helps to remember the new ones better. Once again, sleep facilitated this process in a few studies [264], [265]. Computer models showed that new memories which fitted well with old ones were preserved from down scaling while new ones, which did not fit, underwent depression and were thus forgotten [229].
Interference stands for a "computational" mistake where information from different memories enter a non-related one and thus "corrupt" it. Sleep shelters especially declarative memories from these mistakes [266], [267]. Potential mechanisms which ensure this may occur via blockade of LTP-like potentiation and thus prevent false new learning [268], [269]. Another mechanism could involve a similar idea as mentioned above which says that synapses are tagged during wakefulness for stabilization. More concretely, wakefulness supposedly promotes the early induction phase of synaptic potentiation while sleep promotes the maintenance phase [236]. However, no data so far could definitely prove this concept and it remains a hypothesis to this day.
The synaptic strengthening hypothesis is a very elegant and simple explanation for how memories are consolidated during sleep. However, clear evidence for *overall* synaptic weakening is not present and there is even data pointing towards synaptic strengthening during sleep (see below) as two important studies showed that changes in cortical and hippocampal firing rates across sleep bouts changed in a non-uniform manner [198], [270]. The spike activity changes across sleep states and the changes of cortical excitability as read out by evoked potentials can be well explained through other explanations as well. For example, increased inhibitory activity during sleep or cell-intrinsic forms of plasticity rather than synaptic plasticity may underly the mentioned observations [271]. Indeed, a study found increasing GABA release rates in the cortex across periods of sustained wakefulness [272] and another one found decreasing extracellular glutamate levels in the cortex during sleep deprivation after an initial rise [273]. Consistent with these findings, it was shown that periods of overactivity led to pre- and postsynaptic enhancements of inhibitory synapses on pyramidal neurons such as increased $GABA_A$ receptor expression and increased presynaptic GAD65 [274], [275].
Furthermore, the SHY hypothesis can't exactly explain the purpose of the partition of sleep in NREM and REM sleep and it does not propose very concrete purposes of other sleep oscillations such as spindles and sharp-wave ripples, apart from their potential influence on modulating STDP. Also, the fact that the proportion of time spent in REM is linked to brain size [276], [277] ,unlike NREM, raises the question whether it could be during REM sleep when synaptic weakening occurs. Indeed, firing rates in cortex [270], [278] and hippocampus [196] decreased during REM sleep in one study. Furthermore, the decreases were proportional to REM bout duration [270] and to local theta rhythm in hippocampus [196]. Additionally, an *in vivo* imaging study showed that elimination of newly formed spines was blocked by REM sleep deprivation [195]. A possible mechanism for synaptic weakening during REM sleep might be provided by interneurons which spike at significantly higher rates during REM, as compared to NREM sleep and wakefulness [271]. Lastly, the SHY hypothesis also does not explain what the exact role of the hippocampus is, which seems to be specifically involved in contextual aspects of

memory consolidation.
The last hypothesis on memory consolidation that shall be mentioned has been proposed by Sara J. Aton and Carlos Puentes-Mestril and states that during sleep, both synaptic strengthening and weakening occur heterogeneously during sleep [210]. Whether STDP then tilts towards strengthening or weakening depends on the prior experience during wakefulness. For this, the authors point towards many studies on contextual fear memory which is one of the most intensely studied forms of memory in animals. It relies on neuronal activity in the CA1 area of the hippocampus and it could be demonstrated that the first couple of hours after conditioning were crucial for the consolidation phase [279]. First, neuronal firing and amplitude of network oscillations were enhanced during both NREM and REM over the entire 24h span between conditioning and recall, as compared to baseline. Additionally, connectivity of networks in CA1 which was based on spike timing between recorded neurons increased after conditioning as well [280]. Then, a follow-up study by the same group showed that pharmacogenetic inhibition of parvalbumin expressing interneurons (PV+ neurons) in CA1 disrupted the described connectivity patterns and memory performance during recall. On the other hand, optogenetic stimulation at 8 Hz strengthened the connectivity networks between hippocampal neurons [281]. Taken together, the authors speculated that these results, especially the network results, could be interpreted as suggestive of synaptic potentiation rather than weakening in the hippocampus. Furthermore, several other studies showed that consolidation of contextual fear memory is mechanistically linked to long-term potentiation of glutamatergic synapses: behavioral manipulations like sleep deprivation not only disrupt memory consolidation [282] but also LTP of Schaeffer collaterals in CA1 [283]. It could also be demonstrated that intracellular pathways required for LTP were activated in the hippocampus following contextual fear conditioning [284] and in concordance with that, another study found disruption of these pathways also disrupted memory consolidation [283]–[285]. Lastly, experimental enhancement of LTP in the hippocampus led to increased memory consolidation [286].
Amassing data on STDP during sleep comes from studies on the visual system. *Ocular dominance plasticity* describes a response plasticity in the primary visual cortex (V1) caused by loss of sensory input through stitching up one or two eye lids for example. Importantly, this response is most robustly elicited during a short time window after birth of the animal. The main result of the adaptive response is that the affected V1 neurons start responding to input from the spared eye. It has been well established that sleep promotes this process. An early study found changes in visual responses of V1 neurons after 6 hours of monocular visual experience which were reversed by 6 hours of sleep deprivation (still with monocular experience) but augmented after 6 hours of unperturbed sleep [287]. This sleep dependent enhancement in responsiveness to input from the other eye was mediated through NMDA receptor activation via LTP-mediating kinase pathways [288]–[290]. This process was further associated with a reduction in firing activity of fast-spiking interneurons and an increase in pyramidal neuron firing during sleep, as well as with an increase of spike coherence with slow waves and spindles during NREM sleep [290], [291].
Furthermore, experiments in adult mice could demonstrate *orientation-specific response potentiation* which describes enhanced firing of V1 neurons in response to specific patterns of visual stimulation [292]. After repeated exposure to an oriented grating stimulus for 30-60 minutes, V1 neurons showed an enhanced response to the stimulus but only after 6-12 hours of sleep, not immediately after the experience. Furthermore, sleep deprivation after the experience blocked the potentiation process, and the potentiation was proportional to NREM and

REM sleep time. Like observations in ocular dominance plasticity, V1 neurons showed higher firing rates in the first hours after the experience in NREM and REM sleep and increased coherence of neuronal firing to sleep oscillations in NREM [278], [293]. Both observations were associated with consolidation of stimulus response as well. When looking at the cellular mechanisms of this type of potentiation, it was found that blocking cellular pathways for LTP in glutamatergic pathways led to a loss of the response potentiation. Additionally, *in vivo* stimulation of the lateral geniculate nucleus in the thalamus which induced thalamocortical LTP occluded induction of response potentiation and vice versa, likely through reaching a ceiling of potentiation and thus suggesting a common pathway between thalamocortical LTP and orientation-specific response potentiation [294].

As mentioned above, the authors of the hypothesis proposed that synaptic potentiation or depression depends on the previous experiences during wakefulness. Indeed, one study looked at the spiking activity of V1 neurons after dark exposure (or no visual experience so to speak) versus patterned visual stimuli. The authors found that while dark exposure led to an increase of firing rates during subsequent bouts of wakefulness, the patterned stimuli led to increases during NREM and REM sleep but not during wakefulness [197].

There is also data suggesting that sleep oscillations specifically can potentially induce long term potentiation. Namely, a study showed that synchronous optogenetic stimulation in mouse somatosensory and motor cortex at 2 Hz during NREM sleep (mimicking SWA) could rescue perceptual learning of a somatosensory perceptual task in mice that underwent sleep deprivation after learning [295]. To clarify the effects of slow wave-like oscillations on STDP, another study showed that the amplitude of cortical evoked potentials increased when applied during NREM sleep but not during REM sleep or wakefulness [296], which is suggestive of LTP promoting effects of SWA. Furthermore, the authors found increased EPSP amplitudes in cortical neurons upon presynaptic stimulations that mimicked SWA. An LTP promoting effect of sharp wave ripples was observed in hippocampal neurons *in vitro* [297].

As for REM sleep, there is somewhat less data on the topic but nonetheless there are some studies indicating that LTP occurs during REM sleep as well. Namely, REM sleep deprivation disrupted induction and maintenance of LTP in CA1 in one study [298]. Additionally, REM sleep deprivation after learning could disrupt hippocampus-mediated memory consolidation which has been described above as likely depending on LTP [298], [299]. These effects were mediated by changes in PKA and CREB signaling, as well as expression of Arc and BDNF [300], [301]. REM sleep also plays a critical role in ocular dominance plasticity which has been described before. The shift of V1 responses in favor of the spared eye was disrupted when REM sleep was deprived [302]. Furthermore, a study in orientation-specific response orientation found that changes in the firing rate of V1 neurons increased more during REM than NREM [278]. In concordance, another study found that REM sleep deprivation disrupted enhancements in LTP-mediating ERK kinase in V1 which was otherwise induced after a visual experience [302].

The hypothesis of memory consolidation through synaptic strengthening is not so much a fully fleshed out model that tries to give a full explanation on how the brain stores information but rather seeks to give a counterweight to the SHY hypothesis which claims a very uniform process that all synapses undergo during sleep. Accordingly, the authors do not go into depth about sleep oscillations and their functions, the differences between each other, or why sleep is divided into NREM and REM sleep.

Brain maturation

As the other functions performed by the brain undergo a development and become more sophisticated, so does sleep show a maturation process where its features become established. Furthermore, not only does sleep undergo a maturation process but sleep is itself also important for the maturation of the entire brain and proper development of the entire central nervous system.

Anyone who has children will notice that they sleep more than adults. This observation holds true for the entire animal kingdom. At birth, human babies sleep about 16 hours per day and as they become older a gradual drop occurs down to ~8 hours at adulthood [303]. Additionally, sleep architecture undergoes changes as well. REM sleep drops from 50% of total sleeping time after birth to 25% in adults and to 15% in the elderly. Preterm babies have been reported to spend up to two thirds of their sleeping time in REM [304]. But not only the total amount of the different states changes, but also their bout durations. The sleep-wake cycles of infants goes from brief and fragmented bouts with short cycle durations to more consolidated and longer bouts over the first several postnatal months [305]. Circadian variations in sleep-wake behavior start to emerge by 5-6 weeks in human infants, mainly by decreasing the amount of REM sleep during the day and increasing the amount NREM during the night. By 3-4 months of age, NREM dominates in the first third of the night and REM in the last third [306].

It is not quite clear when the dichotomy of sleep and wakefulness starts to arise but from animal data (mainly sheep) we know that it happens already in fetuses. Fetal electrocorticograms, measurements of cerebral blood flow and leucine metabolism could show that even NREM and REM can be differentiated *in utero* [307]–[309]. After birth, babies and rodent pups already show periods of active wakefulness, behavioral quiescence which corresponds to NREM sleep and periods where twitching of limbs, head, and tail (in animals of course) occurs during quiescence, which corresponds to REM sleep [310]. Though, in these early stages REM sleep is generally referred to as *active slee*p and NREM as *quiet sleep* due to the lack of the usual distinctive EEG features. EEG studies in preterm babies showed that, unlike in sheep fetuses, active and quiet sleep in babies younger than 30-weeks of gestational age are not quite distinguishable based on the EEG [311]. From 30 weeks onward, rapid eye movements occur and the EEG shows continuous delta activity, mainly above occipital electrodes, during active sleep, and quiet sleep shows high amplitude bursts (≥3s) with quiescent inter-burst intervals (≥15-20s). From approximately 36 weeks onwards, two active and two quiet sleep stages can be distinguished. Active sleep 1 shows mixed frequency activities with so called *delta brushes* (see below for description) and active sleep 2 low amplitude alpha and theta activity. One quiet sleep stage displays a so-called *tracé alternant* where the mentioned high amplitude bursts are of equal length to the inter-burst intervals. This stage is initiated by the second quiet sleep stage, called *High Voltage Slow Wave Pattern*, which consists of continuous high voltage delta waves, predominantly above occipital and central areas. At 48 weeks of gestational age true electroencephalographic REM and NREM start to emerge, the *tracé alternant* becomes continuous, true sleep spindles become apparent above central midline electrodes, and the transition to sleep will most likely start with NREM, whereas before sleep could often start with active sleep [311].

EEG measurements in rat pups showed the emergence of delta activity from the 11th postnatal day onwards (P11) in quiet sleep. Hippocampal theta begins to emerge around P8 in short bursts, right after twitches, which develops into a continuous rhythm in active sleep by P12 [312], [313].

Cortical EEG and LFP of rat pups show so called *spindle bursts* with a peak frequency of 12-

15Hz and are the rodent analogue to human delta brushes, which have a frequency of 8-20Hz and are riding on top of a delta wave. Both are speculated to be the precursors of sleep spindles and they can occur in every sleep-wake state in early infants and pups and become more and more restricted to sleep with maturation. Also, similar to sleep spindles they are thought to be implicated in brain plasticity. Interestingly, studies in rats show that they occur in the primary somatosensory (S1) and motor (M1) cortex in response to muscle twitches and in primary visual cortex (V1) in response to spontaneously occurring retinal waves [314], [315].
These last observations have led to a prominent hypothesis which states that sleep in infants, especially REM sleep, serves as a state which provides endogenous brain activation. As the infant is physically not able to engage with the external environment in an extensive degree, the organism generates spontaneous stimuli to the sensory system through muscle twitches and retinal waves for example, in order to stimulate structural brain maturation [306], [314], [316]. Scientific studies especially from the Blumberg group have sought to understand this endogenous stimulation and have mainly investigated how muscle twitches during active sleep may be involved in maturation of cortical and cerebellar circuits. They showed that M1 of rat pups is not strongly involved in generating movement until at least the second postnatal week and that motor control is executed by brainstem structures until that point [314], [317], [318]. The muscle twitches during active sleep are controlled to a big extend by the mesencephalic *nucleus ruber* (or colloquially "red nucleus"), whose neurons show an increase in activity before twitches and pharmacological inactivation shows a reduction of twitching [319]. So, when the red nucleus induces a muscle twitch, proprioceptors and tactile receptors in the respective limb are consequently stimulated as well. This sensory "reafference" is transferred via dorsal horn of the spinal cord to the *nucleus cuneatus* (ECN) in the brainstem. From there, the signal travels further and activates cerebellum, S1 and M1 in the cortex, and finally the hippocampus as well through projections from cortex to entorhinal cortex [314]. This activation is often visible in cortical LFP as spindle bursts which occur shortly after twitches. Additionally, neurons adjacent to the red nucleus project to the cerebellum via the inferior olive and induce neuronal activation simultaneous with a twitch. Lastly, the *nucleus reticularis lateralis* in the brainstem receives inputs from both red nucleus and reafferences from the spinal cord and, in turn, contacts the cerebellum [314].
The consequences of the reafferent activations in cortex and cerebellum are not yet fully understood. However, it was observed that at P8 about 90% of M1 neurons responded exclusively to twitches during active sleep and not to reafferent input during wakefulness, which was gated at the ECN at this time point. Then, at P12 80% of M1 neurons exclusively responded to wakefulness related reafferent input [318] as the sensory gating at ECN had opened. Similar observations have been made in visual processing [320]. Furthermore, it could be demonstrated that the loss of M1 responses to twitches stemmed from local inhibitory networks which seem to have developed by this time. This finding is consistent with the known role of inhibitory circuits in sharpening and tuning cortical sensory responses [321], [322]. Altogether, the current data suggest that during active sleep a sensory foundation for M1 is laid before it is involved in motor function, suggesting that this sensory foundation is crucial for proper execution of motor control [314].
The aforementioned consolidation of sleep architecture with the establishment of a circadian cycle and longer state bouts indicates that structures in hypothalamus and brainstem which regulate the sleep-wake cycle undergo a maturation process as well. Indeed, a lagging of sleep-wake consolidation in orexin knockout-mice indicates that the orexin system undergoes a

development which might underlie the process of sleep-wake consolidation [323]. Furthermore, the establishment of the circadian influence on sleep-wake behavior in rats seems to rely on the development of retinal input to the *nucleus suprachiasmaticus* (SCN, the *"master clock"* of the brain [324]) during the first postnatal week. Before that, the rhythmicity of SCN which is already present at birth is entrained by the mother [325], [326]. Lastly, connectivity between SCN, *Nucleus dorsomedialis hypothalamic* (DMH) which receives input from almost all hypothalamic nuclei and integrates their information [327], and the wakefulness-promoting *locus coeruleus* (LC) has been demonstrated to strongly increase in the first postnatal week of rat pups [328], [329].

Brain clearance

A sleep function that was already proposed in 1913 [330] but only quite recently gained some track in terms of evidence is the idea of a *glymphatic function* of sleep, meaning the clearance and removal of toxins and metabolic waste through convective flow in the brain. It is speculated that big molecules such as glycogen are broken down during wakefulness because of cell activity into smaller molecules such as H^+ and CO_2 which lead to heightened cellular osmotic pressure and volume. Since the brain is a closed compartment, wakefulness related cell activity causes the ratio of cell volume to extracellular fluid to change during the day. Additionally, higher cell activity causes increases in local blood flow, which further increases extracellular fluid volume [331].

One study found that amyloid-β (Aβ) levels in the interstitial fluid of mice paralleled the sleep-wake cycle with higher levels during wakefulness and lower levels during sleep. Furthermore, they showed that sleep deprivation increased Aβ depositions in amyloid-precursor-protein-transgenic mice, while pharmacologically induced prolonged sleep decreased them [332].

A potential mechanism for the lower levels of Aβ during sleep might be due to increased interstitial space during sleep which consequently increases the convectional exchange between the interstitial space fluid and the cerebrospinal fluid. Indeed, two-photon imaging showed increased clearance rates of fluorescent Aβ constructs during sleep. Additionally, ketamine anesthesia and application of norepinephrine receptor antagonists in the cortex showed the same effects. [333]. Whether the increase of the interstitial space is a passive result of the biochemical processes mentioned above or if it is an active process is an open question to date.

A recently discovered system of lymphatic vessels in the dura might provide an additional anatomical route for clearance of interstitial space fluid with corresponding metabolites in it [334]. However, the precise anatomical area of drainage for this system and the quality of the fluid that is being drained remain to be investigated.

Gene repair

An aspect that only quite recently got attention is the influence of sleep on gene repair and maintenance. A 2014 study in sleep deprived rats [335] found among increases in protein damage and lipid peroxidation also increases in oxidative stress on DNA in liver, lung and intestine, which came back to baseline levels after recovery sleep. A small study in humans found lower levels of DNA repair gene expression and more DNA strand breaks in blood lymphocytes of physicians after a nightshift as compared to baseline levels [336].

Groups investigating DNA dynamics in the brain [337], [338] found that double-stand breaks of DNA (DSBs) increased in neurons during wakefulness and they could even show that it was specifically due to neuronal activity as optogenetic activation of neurons, visual stimulation

and enrichment of environment were sufficient to cause a greater increase of DSBs as compared to normal baseline wakefulness. It is important to keep in mind that these DSBs are not thought to happen accidentally but because of induction of immediate-early genes which causes DSBs in a type 2 topoisomerase-dependent process. Many of these genes in neurons are associated with synaptic plasticity [339], [340].
A further study could then show that chromosome dynamics were increased during sleep in zebrafish larvae [341]. This means that movement of fluorescently marked telomeres and centromeres increased which has been shown to be a readout of gene expression and DNA repair [342]. Indeed, the authors found that sleep reduced DSBs and that increased chromosome dynamics were necessary for this process. Furthermore, DSBs in neurons are a potential factor which increases sleep pressure because the authors of the same study chemically induced DSBs and found increased sleeping time together with increased chromosome dynamics compared to controls. These findings were specific to neurons and were not detected in Schwann cells and endothelial cells. However, similar studies in other types of cells and tissues are lacking to this date.
Ca^{2+} could potentially play an important role in these processes, both in causing and repairing DSBs: as it enters via glutamate ion channels during wakefulness it can induce activation and transport of cytosolic topoisomerases to the cell nucleus. At the same time, a lot of Ca^{2+} enters neurons during burst firing which occurs extensively during sleep and could regulate DNA damage sensors such as PARP-1 which facilitates DNA repair [343].
Even though I refer to only five studies and one opinion article in this section, I still deemed this topic important to mention here because it is an exciting new area of study as it offers a lot of potential in furthering our understanding of the link between disturbances of sleep and increased risk of developing chronic diseases [344].

Immunity
Current evidence suggest an interesting relationship between sleep and the immune system that seems to be bidirectional as sleep influences cytokine secretion and cytokines can have both somnogenic effects as well as inhibitory effects on sleep [345].
In an early experiment in the 70s [346], cerebrospinal fluid was taken from sleep deprived animals, injected into non-sleep deprived ones which then fell asleep shortly after. The experimenters called the substance in the fluid *endogenous factor S* (S standing for *"sleep promoting"*) and identified it later to be a bacterial cell wall peptidoglycan fragment, called muramyl peptide. Later, they found out that it induced Interleukin-1-beta secretion (Il-1β) which was the actual sleep promoting factor [347]. Furthermore, a human study showed that a challenge with endotoxin increased NREM sleep and delta power while decreasing the amount of REM sleep [348]. Another study showed that better sleep was associated with survival from an infectious disease in rabbits [349]. So, challenges to the immune system led to increased sleep, a mechanism that is important for the hosts immune response to an infectious challenge. Besides Il-1β, tumor necrosis factor α (TNF-α) has been most extensively investigated in its effects on sleep and other cytokines include Il-1α, Il-2, Il-4, Il-6, Il-8, Il-10, Il-13, Il-15, Il-18, TNF-β, interferon-β, interferon-γ and macrophage inhibitory protein 1β. The ways through which cytokines interact with the central nervous system (CNS) include cytokine receptors present on the cell surface of neurons, peripheral stimulation of the vagus nerve, active transport of cytokines from periphery into the CNS but also *de novo* synthesis by both neurons and glia in the CNS [350].
Both Il-1β and TNF-α have been shown to increase NREM sleep, but Il-1β caused sleep

fragmentation [351]. Additionally, REM sleep was suppressed [352]. It follows that these sleep alterations are also observable in the course of an infection that the organism has to fight [350]. Furthermore, inactivation of the cytokine effects reduced spontaneous NREM sleep, the increase of NREM sleep after sleep deprivation [352], and excessive food intake was lost as well [353].

An important mechanism which leads to these effects of cytokines is through interaction with the serotonergic system of the brainstem in the *Nucleus raphe dorsalis* (DRN) which regulates wakefulness and sleep behavior. DRN neurons are mainly firing during wakefulness and are important for promoting wakefulness [354] but are also involved in the induction of NREM sleep. The dual effect works through fast acting effects which promote wakefulness and delayed effects which promote NREM sleep at the respective phase of the light-dark cycle when sleep usually occurs [355]–[357]. NREM sleep promoting effects of serotonin secreting neurons are likely to occur through action on the preoptic area and basal forebrain [350]. Indeed, it has been demonstrated that Il-1β recruits $GABA_A$-receptors on neurons [358], increases Cl^--uptake by acting on $GABA_A$-receptors [359] and it induces a delayed potentiation of GABA-elicited Cl^- currents [358]. Il-1β has also been shown to reduce firing rates of serotonergic neurons in DRN [360], likely through the aforementioned mechanisms. Cytokine effects in the preoptic area (POA) and basal forebrain (BF) haven been investigated as well. Il-1β directly inhibits wakefulness-promoting neurons and stimulates sleep promoting ones [361]. Additionally, it stimulates serotonin (5-HT) release from axon terminals in the in the POA/BF which in turn inhibit cholinergic neurons that are involved in cortical activation [362]. Taken together, this might be a potential mechanism by which Il-1β induces NREM sleep and suppresses REM sleep via 5-HT.

A possible reason to induce the described sleep pattern during an infection might be tied to the generation of fever. Imeri and Opp [350] proposed that the increase of sleep reduces energy expenditure as compared to wakefulness (see section on metabolism) and the saved energy is then invested in raising body temperature. However, since body temperature usually decreases during NREM sleep and thermoregulation ceases during REM, NREM sleep becomes fragmented and REM sleep is reduced in order to minimize heat dissipation.

As implied in the beginning of the section, sleep also has an influence on the immune system. However, it is important to keep in mind that it is difficult to tell with certainty whether the phenomena that will be presented are truly regulated by sleep or the circadian rhythm, as only a few studies were designed to be able to make this distinction.

An interesting discovery in relation to the influence of sleep on the immune system was the positive effect of sleep on adaptive immunity after vaccination [363]. Precisely, the authors found 4 weeks after vaccination a twofold higher concentration of antigen-specific antibody concentration in the blood of subjects who slept normally after vaccination as compared to subjects who stayed awake during the night following vaccination. Furthermore, a follow-up study found also more antigen-specific T-helper cells and more T-cells which produced cytokines driving Th1 lymphocytes, which are involved in cellular adaptive immune response, in subjects that slept regularly in comparison to subjects which stayed awake during the night following inoculation [364]. Chronic sleep loss has also been associated with increases in inflammatory markers (see below) and with immunodeficiency. One study found a higher susceptibility to the common cold in association with poor sleep efficiency and rats died from bacteremia after prolonged sleep withdrawal despite a high pro-inflammatory state and general immune activation [365], [366].

Studies of blood samples across the sleep-wake cycle found that several cytokines and immune

cells showed fluctuations across the sleep-wake cycle. As mentioned in the section on metabolism, the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system are strongly downregulated, while GH and Leptin are increased. Additionally, prolactin and melatonin are increased during sleep [367]. These hormones have been shown to impact immune parameters and thus form a link between sleep and the immune system [368]–[375]. Namely, they support the production of pro-inflammatory cytokines like Il-1, Il-12, TNF-α and TNF-γ, promote the activation of the complement system [367], as well as immune cell activation and proliferation. Additionally, monocytes, macrophages and CD4+ T-cells show an autonomous circadian rhythm in their cytokine expression governed by clock genes, which parallel the rhythm generated by the aforementioned hormones across the sleep-wake cycle [376]–[378]. A possible *zeitgeber* for this rhythm might be given by reactive oxygen species, nucleotides and heat shock proteins which accumulate over the course of the waking period [379]. Taken together, various immune indices show a diurnal rhythm such as phagocytic activity, activity of regulatory T cells, and cell proliferation in peripheral blood, lymph nodes and spleen with peaks during the sleeping period [370], [380]. In addition, lymphocytes tend to migrate to the lymph nodes during sleep and to the bone marrow during wakefulness. Lastly, sleep likely enhances the interaction between antigen presenting cells and T cells through enhanced Il-12 production [364], [379]. Taken together, the current evidence suggests that sleep promotes a Th1-immune response and enhances the adaptive immune response against invading agents.
Wakefulness on the other hand, shows an increase in anti-inflammatory cytokine activity, mainly by Il-10 production [379], [381]. The stress hormones cortisol, epinephrine and norepinephrine are released via the activation of the HPA and the sympathetic vegetative system. As a consequence, more terminally differentiated cytotoxic T-lymphocyte, natural killer cells, neutrophils, monocytes, and dendritic cells are found in the blood [379]. This *adrenergic leukocytosis* is thought to serve as a rapid response to injury and arising infectious challenges as it is composed mainly of differentiated cells of innate immunity with developed effector functions. The additional adaptive immune response is then later booted up during sleep [379].
Interestingly, chronic sleep loss has been shown to be associated with increased pro-inflammatory markers, such as Il-6 and C-reactive protein, albeit clear cause-effect relationships have not been established so far. The differences in cytokine induction during SWS and chronic sleep loss are not quite clear, but it is speculated that while the cytokines released during SWS support adaptive immune responses, the ones released under chronic sleep loss support innate immunity. Even though the observed increases are small, they are still thought to be implicated in metabolic disturbances such as type 2 diabetes and cardiovascular disease (see section on metabolism) [379], [382], [383].

2. Hypothesis and aim

The hypothesis of my PhD research project is that thalamic nucleus reuniens is responsible for the synchronization of sleep oscillations in hippocampus and medial prefrontal cortex during NREM sleep and that this effect plays a key role in consolidating memory traces during NREM sleep. To test this hypothesis, I have combined diverse expertise in *in vivo* multi-site high-density neuronal recordings, optogenetics and behavioral neuroscience. This interdisciplinary approach was expected to provide fundamental insights into the "master orchestrator" mechanisms through which sleep oscillations support cognitive functions in mammals. The aim was to observe how different forms of oscillations are orchestrated between the thalamus, hippocampus, and medial prefrontal cortex, i.e., how sharp wave ripples, spindles, δ delta waves relate to one another, to find out what are the anatomical substrates supporting these functional connections and how these are involved in memory consolidation during sleep. Furthermore, I aimed at interfering with the process of memory consolidation by influencing the action potential activity of neurons in the nucleus reuniens using optogenetics.

3. Results

"Coupling between the prelimbic cortex, nucleus reuniens, and hippocampus during NREM sleep remains stable under cognitive and homeostatic demands"

Contribution: The experiments for this study were designed by Antoine Adamantidis, Carlos del Rio Bermudez and me. Most of the surgeries, recordings and behavioral procedures were performed by me, with help from Carlos del Rio Bermudez. Analysis tools were either custom written by myself or Thomas Rusterholz, Mojtaba Bandarabadi, Christian Mikutta, or adapted from previous works for the needs of this study by me. I made all the figures. The manuscript was written by me and Antoine Adamantidis with inputs from all contributing co-authors.

Coupling between the prelimbic cortex, nucleus reuniens, and hippocampus during NREM sleep remains stable under cognitive and homeostatic demands

Running title: Nucleus reuniens modulates coupling of cortical slow waves and spindles to hippocampal ripples during NREM sleep

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Abstract

The interplay between the medial prefrontal cortex and hippocampus during non-REM (NREM) sleep contributes to the consolidation of contextual memories. To assess the role of the thalamic nucleus reuniens (Nre) in this interaction, we investigated the coupling of neuro-oscillatory activities between prelimbic cortex, Nre, and hippocampus across sleep states and their role in the consolidation of contextual memories using multi-site electrophysiological recordings and optogenetic manipulations. We showed that ripples are time-locked to the Up state of cortical slow waves, the transition from Up to Down state in thalamic slow waves, the troughs of cortical spindles, and the peaks of thalamic spindles during spontaneous sleep, rebound sleep, and sleep following a fear conditioning task. In addition, spiking activity in Nre increased before hippocampal ripples and the phase-locking of hippocampal ripples and thalamic spindles during NREM sleep was stronger after acquisition of a fear memory. We showed that optogenetic inhibition of Nre neurons reduced phase-locking of ripples to cortical slow waves in the ventral hippocampus while their activation altered the preferred phase of ripples to slow waves in ventral and dorsal hippocampi. However, none of these optogenetic manipulations of Nre during sleep after acquisition of fear conditioning did alter sleep-dependent memory consolidation. Collectively, these results showed that Nre is central in modulating hippocampus and cortical rhythms during NREM sleep.

Introduction

Non-rapid eye movement (NREM) sleep is characterized by behavioral quiescence and heightened response threshold to sensory or environmental stimuli. During this state, key electroencephalographic (EEG) features in humans and rodents include a shift towards high-amplitude, low-frequency oscillatory activities (<4Hz) and the occurrence of discrete phenomena such as slow waves [384], sleep spindles [25] and sharp-wave ripples [49]. While sleep spindles and slow waves occur within thalamo-cortical networks [8], [16], [25], sharp-wave ripples are generated within the hippocampal formation [32], [49]. Crucially, cortical slow waves and spindles have been shown to co-occur with ripples in the hippocampus (HPC) [89], [92], [96], [385], [386]. Indeed, some of the ripples are temporally locked to the troughs of spindles [98], [386], [387] and the UP state of slow waves [95], [386] in the medial prefrontal cortex (mPFC) in rodents. Studies in humans revealed that coupling between ripples and slow waves or spindles also occurred between the HPC and many other cortical areas including parietal and occipital regions, with a preference for frontal regions [89], [92], [385].

Neural oscillatory activity is thought to support brain plasticity [168], [209], [210] and memory consolidation. In particular, the coupling of hippocampal ripples to slow waves and spindles in the prefrontal cortex (PFC) during NREM sleep is thought to be implicated in the consolidation of fear memories through replay processes during sleep [174], [175]. Indeed, the temporal locking of hippocampal ripples to UP states of prefrontal slow waves in rats has been shown to increase after a learning task [93], [95]. Furthermore, mice that were sleep deprived immediately following a hippocampus-dependent conditioning task did not show an increase in sharp wave ripple activity during recovery sleep compared to mice that were allowed to sleep immediately after the conditioning task [388], supporting the idea that neural oscillatory activity facilitates memory consolidation. Along this line, while the suppression of hippocampal ripples during NREM sleep impaired performance in a radial-maze task in rats [77], enhancing coupling of ripples to both cortical slow waves [93] and spindles [171] improved contextual memory performance.

Both anatomically and functionally, different aspects of memories are encoded by different parts of the HPC and PFC. The prelimbic part of the PFC is implicated in the expression of fear memory [389, p.], [390] and the encoding of danger- versus safety-associated cues [391]–[393]. The dorsal HPC is implicated in spatial aspects during environmental exploration or goal directed tasks, while the ventral portion of HPC is implicated in emotional and social aspects [394], [395]. On an anatomical basis, while neurons in the ventral parts of the HPC and subiculum project directly to the PFC [396], neurons in the dorsal HPC are indirectly connected to the PFC via relays located in the basal ganglia and thalamus [397].

Connections from the PFC to HPC are primarily relayed through the peri- and enthorhinal cortices [398] and the midthalamic *nucleus reuniens* (Nre) [399], [400]. Anatomically, the Nre is uniquely located to play a role in the coordination of cortico-hippocampal activity as it is reciprocally connected to mPFC (including prelimbic and infralimbic cortices) and the hippocampal formation through axonal projections to the CA1 stratum lacunosum moleculare and inputs from the subiculum [399]–[401]. In addition, the Nre is reciprocally connected to the amygdala, which plays a key role in the expression of fear behavior [402].

Because of the aforementioned anatomical features, Nre neurons support associative behaviors through their influence on HPC and PFC networks, including the acquisition, consolidation, and recall of fear memories [403]. Indeed, inactivating Nre neurons using local injections of the GABA agonist muscimol prior to a fear extinction procedure impaired the acquisition of both short- and long-term extinction memories in rats [404]. Muscimol inactivation of Nre neurons prior to fear conditioning in rats also impaired the acquisition of a fear memory in contextual

fear conditioning while its administration before recall led to generalized freezing to a novel context with no effects on auditory discrimination during a fear conditioning task [405]. In addition, local muscimol injection in Nre of rats impaired retrieval of spatial memory in a cross-word-like maze looking for a reward [406]. In contrast, the role of Nre in memory consolidation remains unclear. Inhibition of Nre neurons either has no effect [406] or leads to generalization of contextual memory after fear conditioning in mice [407], [408]. Finally, several studies demonstrated Nre's implication in consolidation of remote memories (i.e., after at least 21 days) [407], [409]–[411].
At circuit level, studies have shown that Nre neurons modulated the coherence of delta frequency oscillations [412], [413], the synchrony of gamma-bursts [414] between PFC and HPC in urethane anesthetized rats, as well as firing sequences of neurons in the HPC and PFC during slow waves [415]. In addition, Nre neurons mediate theta coherence, theta-gamma coupling and directionality between cortex and HPC during a spatial working memory task and support performance in the task [416]. Altogether these studies suggested the involvement of Nre neurons in behaviors implicating cortico-hippocampal communication during both wakefulness and sleep.
Thus, in this study we assessed the involvement of Nre neurons in the temporal coordination of cortical slow waves and spindles with hippocampal ripples during NREM sleep, and associated memory consolidation in mice using electrophysiological recordings and optogenetics.

Results

First, we quantified the densities and coordination of sleep oscillations, i.e., synchrony of slow waves, spindles, and ripples in mPFC, Nre and CA1 after sleep deprivation or a learning task. Changes in spindle density during sleep after learning and sleep deprivation have been demonstrated for cortical regions [43], [45], [417] but not for Nre. Higher ripple densities during sleep were described after learning [90] but not after sleep deprivation. As mentioned above, ripples co-occur with cortical spindles and slow waves with a preference of ripples to be nested in the UP state of slow waves [95], [386] and the troughs of spindles [98], [386], [387] while coupling of these oscillations increased after learning [93], [95]. However, the densities and stability of phase-locking of hippocampal ripples to UP states and spindle troughs after sleep deprivation and learning remain sparsely described [95]. To test this, we simultaneously recorded neural activities between mPFC, HPC and Nre across different sleep-wake states and experimental conditions. We stereotactically implanted male mice with multi-site electrodes in Nre, ventral and dorsal portion of CA1 (vCA1 and dCA1), and the prelimbic portion of the mPFC (Prl) on the left hemisphere. To assess sleep-wake states, EEG and electromyographic electrodes (EMG) were chronically implanted (Fig.1a-b, supp. fig. 1a). After recovery, mice underwent three experimental sleep conditions, namely unperturbed sleep ('baseline', BL), sleep after deprivation ('rebound sleep', Reb), and sleep after memory acquisition in an auditory contextual fear conditioning paradigm ('post-learning sleep', PL, Fig.1c-d, supp.fig.1b-c, supp. table 1, see methods for details). These conditions aimed at testing possible effects of heightened homeostatic and mnemonic demands on the dynamics of the PFC-Nre-HPC network. Local field potential (LFP), EEG and EMG signals were recorded during natural sleep for 5 hours following experimental procedures (Fig.1e).

Slow waves and spindles in prelimbic cortex and Nre are coupled to and drive hippocampal ripples

One aim of the study was to characterise the temporal coupling between spindles and slow waves in thalamo-cortical networks (Fig.1f, g), and sharp-wave ripples in the vCA1 and dCA1

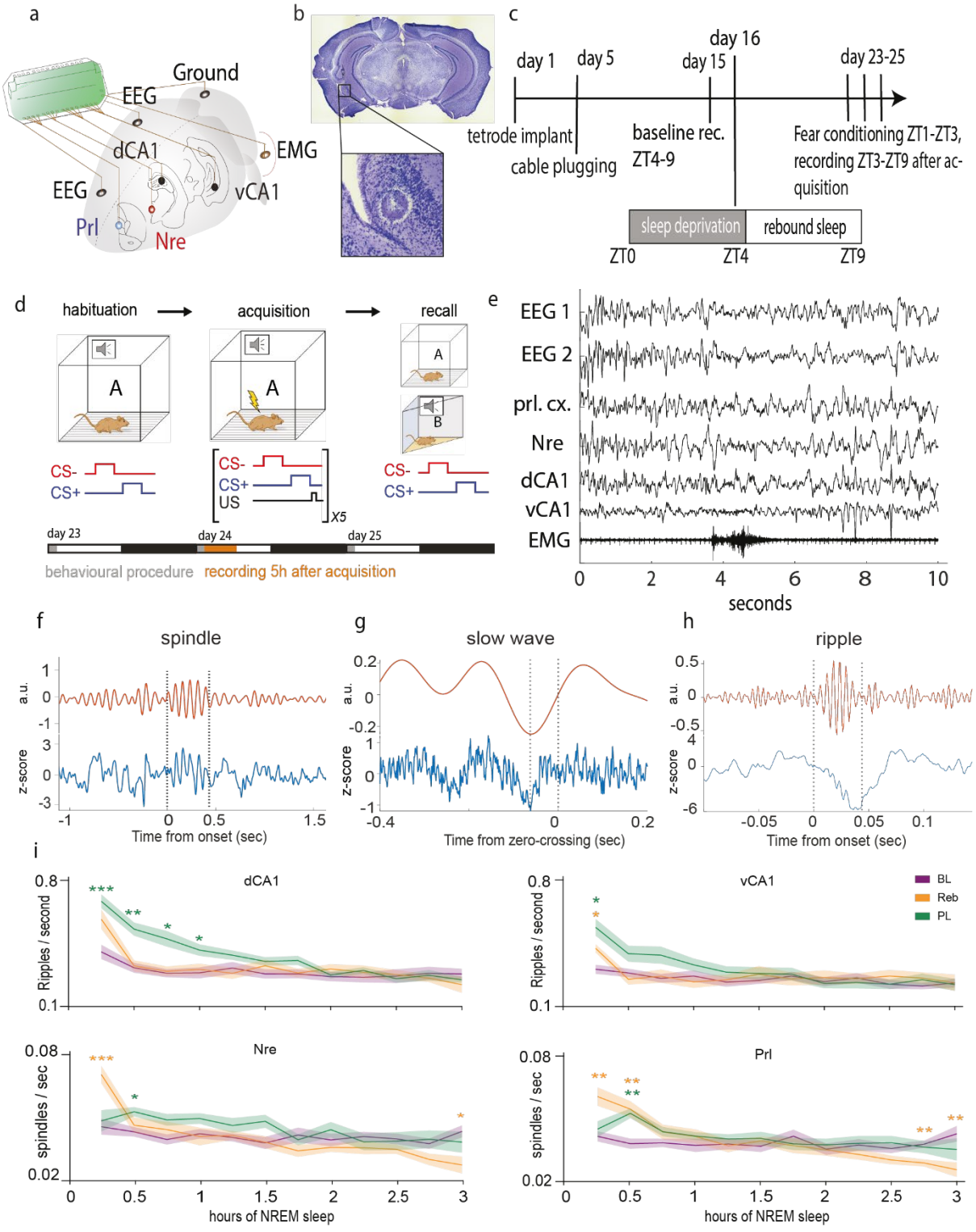

Figure 3.1: Experimental design and event detection.

a) Schematic showing tetrode and electrode placement. Tetrodes were placed in prelimbic cortex (Prl), nucleus reuniens of the thalamus (Nre), dorsal CA1 (dCA1) and ventral CA1 (vCA1).

b) Example of gliosis marking tetrode placement in vCA1 after electric current was driven through the tetrode.

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c) Timeline of the experimental procedure marking days of tetrode and electrode implantation, tethering to recording cables, baseline sleep recording, sleep deprivation and fear conditioning.

d) Schematic showing the fear conditioning procedure over three days: mice were first habituated to context A, CS- and CS+, followed by acquisition of fear memory and later recall of fear memory.

e) Example trace of EEG, LFP and EMG recordings, showing a period of NREM sleep with a short arousal approximately at second 4.

f) Example of a detected spindle during NREM sleep. Top shows the filtered trace (9-16Hz, artificial unit) and bottom shows the raw LFP trace (z-scored).

g) Example of a slow wave centered at the negative peak and the following zero-crossing with the filtered trace at the top (0.5-4Hz, artificial unit) and raw LFP trace at the bottom (z-scored).

h) Example of a hippocampal ripple with filtered trace (150-250Hz, artificial unit) on the top and raw LFP trace at the bottom (z-scored).

i) Time-course of ripples and spindles +/- S.E.M. during NREM sleep (NREM sleep bouts were concatenated and cut to 3h, as the minimum duration of sleep for all animals across conditions) in dCA1 ripples (n= 11 in BL, n= 8 in Reb, n= 9 in PL; top left), vCA1 ripples (n= 8 in BL, n= 8 in Reb, n= 8 in PL; top right), Nre spindles (n= 14 in BL, n= 11 in Reb, n= 9 in PL; bottom left) and Prl spindles (n= 14 in BL, n= 11 in Reb, n= 8 in PL; bottom right) during baseline (BL), rebound sleep (Reb) and post-learning sleep (PL). *P<0.05, **P<0.01, ***P<0.0001 using two-way ANOVA with Dunnett's multiple comparisons.

(Fig.1h). In a first step, we quantified the occurrence of spindles and ripples over the course of NREM sleep (i.e., densities) as an indication of potential changes in cross-frequency coupling (see methods for concatenation of NREM sleep episodes; Fig.1i). During the first half hour of 'rebound sleep' both spindles in Nre ($F_{condition}$= 1.5, F_{time}= 9.2, $F_{time\ x\ condition}$= 3.6; Fig.1i, bottom left) and Prl ($F_{condition}$= 0.4, F_{time}= 9.2, $F_{time\ x\ condition}$= 4.1; Fig.1i, bottom right), and ripple events in vCA1 ($F_{condition}$= 0.5, F_{time}= 16.1, $F_{time\ x\ condition}$= 3.7; Fig.1i, top right) showed higher occurrence densities than during baseline which were not present in spindles measured from frontal EEG (supp. fig. 1d), suggesting a potential homeostatic response for these events. As compared with baseline, post-learning sleep showed higher ripple densities in dCA1 for the entire first hour of NREM sleep ($F_{condition}$= 2.3, F_{time}= 35.1, $F_{time\ x\ condition}$= 5.7; Fig.1i, top left). This result suggested the involvement of hippocampal ripples in sleep-dependent memory consolidation consistent with previous findings describing higher ripple densities after learning [90], suggesting the involvement of hippocampal ripples in sleep-dependent memory consolidation [93], [95]. Since the event densities were significantly elevated for the first half hour of rebound sleep and for the first hour of post-learning sleep, these time windows were selected for the subsequent LFP and spike analyses of this experiment and compared to total sleep time during baseline sleep. Thus, we first calculated the total event density during total baseline sleep, the first 30min of rebound sleep and the first hour of post-learning sleep. We found a significant increase of thalamo-cortical slow waves and cortical spindles after both rebound sleep and post-learning compared to baseline conditions (Prl slow waves: F= 11.1, P for Reb= 0.0002, P for PL= 0.0139. Nre slow waves: F=5.7, P for Reb= 0.009, P for PL= 0.0274. Prl spindles: F=24.7, P for Reb <0.0001, P for PL= 0.0385) as well as an increase in dCA1 ripples in post-learning sleep (F= 2.97, P= 0.0494; Fig.2a, supp. table 2). Next, we investigated the amount of hippocampal ripples that occur at

the time of thalamo-cortical slow waves and spindles (see methods). We found that the amount of ripples coupled to slow waves during rebound sleep and post-learning sleep was also increased as compared to baseline conditions (Nre-vCA1: F= 16.8, P for Reb= 0.0017, P for PL<0.0001; Nre-dCA1: F= 14.97, P for Reb<0.0001, P for PL= 0.0018; Pfl-vCA1: F=17.1, P for Reb<0.0001, P for PL= 0.003; Prl-dCA1: F=6.684, P for Reb= 0.0042; Fig.2b top, supp. table 3). The coupling of hippocampal ripples to spindles remained stable across conditions (Fig.2b bottom, supp. table 3). To rule out whether the increased co-occurrence rates of slow waves and ripples in all electrode pairs in rebound and post-learning sleep resulted from the increase of oscillatory events, we calculated the rate of co-occurrence using data, where we shuffled the time stamps of ripples, slow waves, and spindles 100 times and calculated the ratio of observed and shuffled occurrence rates. We found that coupling of ripples to slow waves in both Nre and Prl occurred at rates that were between chance level and 1.4 times above chance level (Fig.2c top, supp. table 3). Coupling of thalamic spindles to ripples occurred at a rate that was 2-3 times higher than chance level. (Fig.2c bottom, supp. table 3).

However, the aforementioned increases in the co-occurrence rate of slow waves and ripples observed during rebound sleep and post-learning sleep as compared to baseline sleep were not present anymore, suggesting that they resulted from the higher densities of slow waves, spindles, and ripples.

Next, we tested whether hippocampal ripples occur at specific phases of slow wave and spindle oscillatory activity in Prl and Nre, as shown for cortical recordings in humans and rats [92], [95], [98]. Specifically, we performed phase-amplitude coupling analyses for ripples that co-occur with a slow wave or a spindle by first extracting the phase of Prl and Nre traces that were filtered in slow wave range (0.5-4Hz) or spindle range (9-16Hz), respectively. Next, we extracted the phase of the slow wave or spindle at which each ripple occurred and calculated the mean circular vector of all extracted phase values to quantify the coupling of ripples to the phase of thalamic and cortical slow waves and spindles (see methods). We found that ripples in both ventral and dorsal CA1 were preferentially locked to the UP state (shortly after 0°, positive slope) of slow waves in the cortex. As for the coupling to thalamic slow waves, ripples from both parts of CA1 occurred at the transition from UP to DOWN state (around 90°). Similarly, to previous studies [98], [387], ripples fell into the trough of spindles in Prl and onto the peak of spindles in Nre. We observed a significant increase in the phase-locking of vCA1 ripples to Nre spindles in post-learning sleep and a shift in the preferred phase in rebound sleep (F=14.5, P<0.0001 in Reb, P= 0.0192 in PL). These were accompanied by a shift in the preferred phase of vCA1 ripples to Nre slow waves in post-learning sleep and a reduced phase-locking during rebound sleep (F=110.1, P <0.0001 in both Reb and PL). To a lesser extent, we also found a shift in the preferred phase of dCA1 ripples to Nre slow waves in post-learning sleep (F=77, P<0.0001), a shift in the preferred phase of vCA1 ripples to Prl slow waves in post-learning sleep and a reduced phase-locking during rebound sleep (F=44.9, P=0.0109 in Reb, P<0.0001 in PL). These changes were associated with a reduced phase-locking and shift in the preferred phase of dCA1 ripples to Prl slow waves in post-learning sleep (F=31.7, P<0.0001), shifts in the preferred phase of dCA1 ripples to Nre spindles in rebound and post-learning sleep as well as an increased phase-locking in post-learning sleep (F=19.8, P= 0.0002 in Reb, P<0.0001 in PL), and an increased phase-locking of vCA1 ripples to Prl spindles in post-learning sleep (F=4.8, P=0.0316, Fig.2d, supp. table 3). These findings suggested that subtle changes in oscillations synchrony are associated with increased homeostatic or mnemonic needs.

To assess the directionality (i.e., flow of information) of these oscillatory events, we next calculated the phase slope index (PSI), see methods for further details. We found that both slow waves and spindles in cortex and thalamus drove hippocampal ripples (negative values indicate that ripples drove slow waves/spindles; Fig.2e, supp. table 3). Interestingly, even though

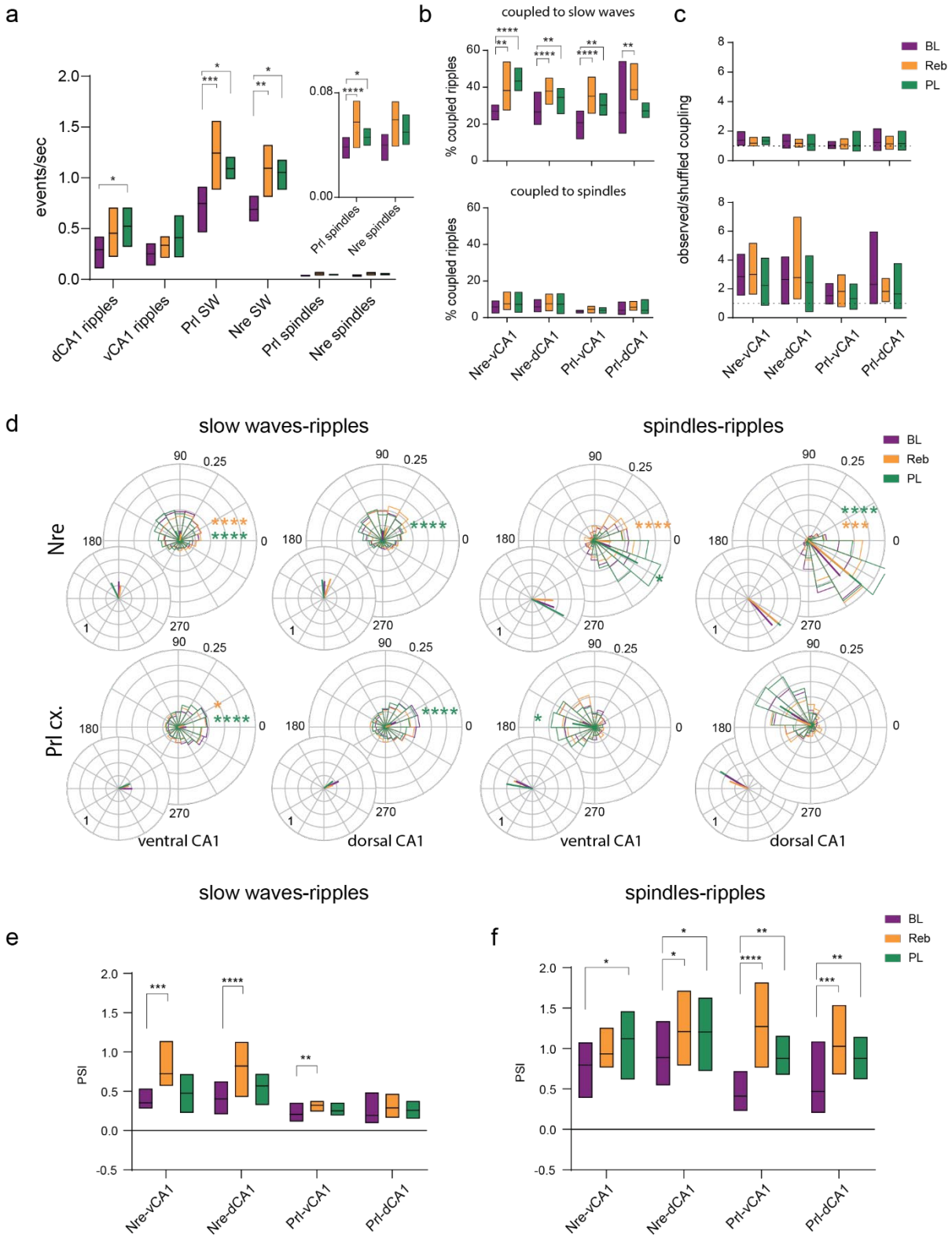

Figure 3.2: Coupling of cortical and thalamic slow waves and spindles and hippocampal ripples.

a) Box plots from minimal to maximal value with indicated mean showing the average densities of slow waves, spindles, and ripples during baseline sleep (BL), rebound sleep (Reb) and post-learning

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sleep (PL). Inset shows zoomed in values of spindle densities. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 using one-way ANOVA followed by Dunnett's test for multiple comparisons.

b) Box plots from minimal to maximal value with indicated mean showing the average percentage of co-occurrence of ripples coupled with slow waves (top) or spindles (bottom) for all detected ripples. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 using One-way ANOVA followed by Dunnett's test for multiple comparisons.

c) Box plots from minimal to maximal value with indicated mean showing the average ratios of co-occurrence of ripples coupled with slow waves (top) or spindles (bottom) between observed data sets and shuffled data sets. No significant differences were measured using one-way ANOVA followed by Dunnett's multiple comparisons.

d) Normalized circular histograms showing the phase-amplitude coupling of hippocampal ripples to slow waves and spindles during baseline sleep (BL), rebound sleep (Reb) and post-learning sleep (PL). Thick lines represent mean circular vectors. The phase in the plots follows the cosine, so 0° marks the positive peak (UP state) and 180° marks the peak of the negative slope (DOWN state). Insets show a magnification of mean circular vectors with unit circle. All vectors were tested for non-uniformity with the Rayleigh test for non-uniformity. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 using Parametric Watson-Williams multi-sample test for equal means as a one-way ANOVA test. If the assumptions of the test were not met, a non-parametric multi-sample test for equal medians was employed with Bonferroni correction of P-values.

e, f) Box plots from minimal to maximal value with indicated mean showing the average phase slope index (PSI) +/- S.E.M. between ripples and slow waves (e) and ripples and spindles (f). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 using One-way ANOVA followed by Dunnett's test for multiple comparisons.

the phase-locking of ripples to slow waves during rebound sleep did not change, we observed significant increases in the directional strength of slow waves and spindles onto ripples. This was marked by a significant increase of the drive of Nre slow waves onto vCA1 and dCA1 ripples during rebound sleep. To a lesser extent, Prl slow waves showed an increase of the drive on vCA1 ripples as well (Nre-vCA1: F= 12.5, P=0.0001; Nre-dCA1: F= 14.2, P <0.0001, Prl-vCA1: F= 6.2, P = 0.0043). Additionally, Prl spindles showed a robustly increased drive onto all ripples during rebound sleep, while this was also observed for Nre spindles onto dCA1 ripples as well. Intriguingly, both thalamic and cortical spindles showed an increased drive onto all ripples during post-learning sleep (Nre-vCA1: F= 3.2, P for PL= 0.0376; Nre-dCA1: F= 4.03, P for Reb= 0.0471, P for PL= 0.042; Prl-vCA1: F= 3.4 P for Reb <0.0001, P for PL= 0.0088; Prl-dCA1: F= 2.4, P for Reb= 0.0003, P for PL= 0.0057). These results suggested an influence of homeostatic and mnemonic needs on the directionality between slow waves or spindles and ripples. Collectively, these results confirmed the coupling of cortical slow waves. Furthermore, both thalamic and cortical slow waves and spindles exert a drive on hippocampal ripples, consistent with a previous study [418].

Nucleus reuniens single-unit activity is locked to hippocampal ripples

To investigate the firing characteristics of Nre neurons across behavioral states in baseline, rebound sleep and post-learning sleep, single units from Nre neurons were sorted from multi-unit recordings (MUA) and their spike rate in all three conditions was calculated (see Meth-

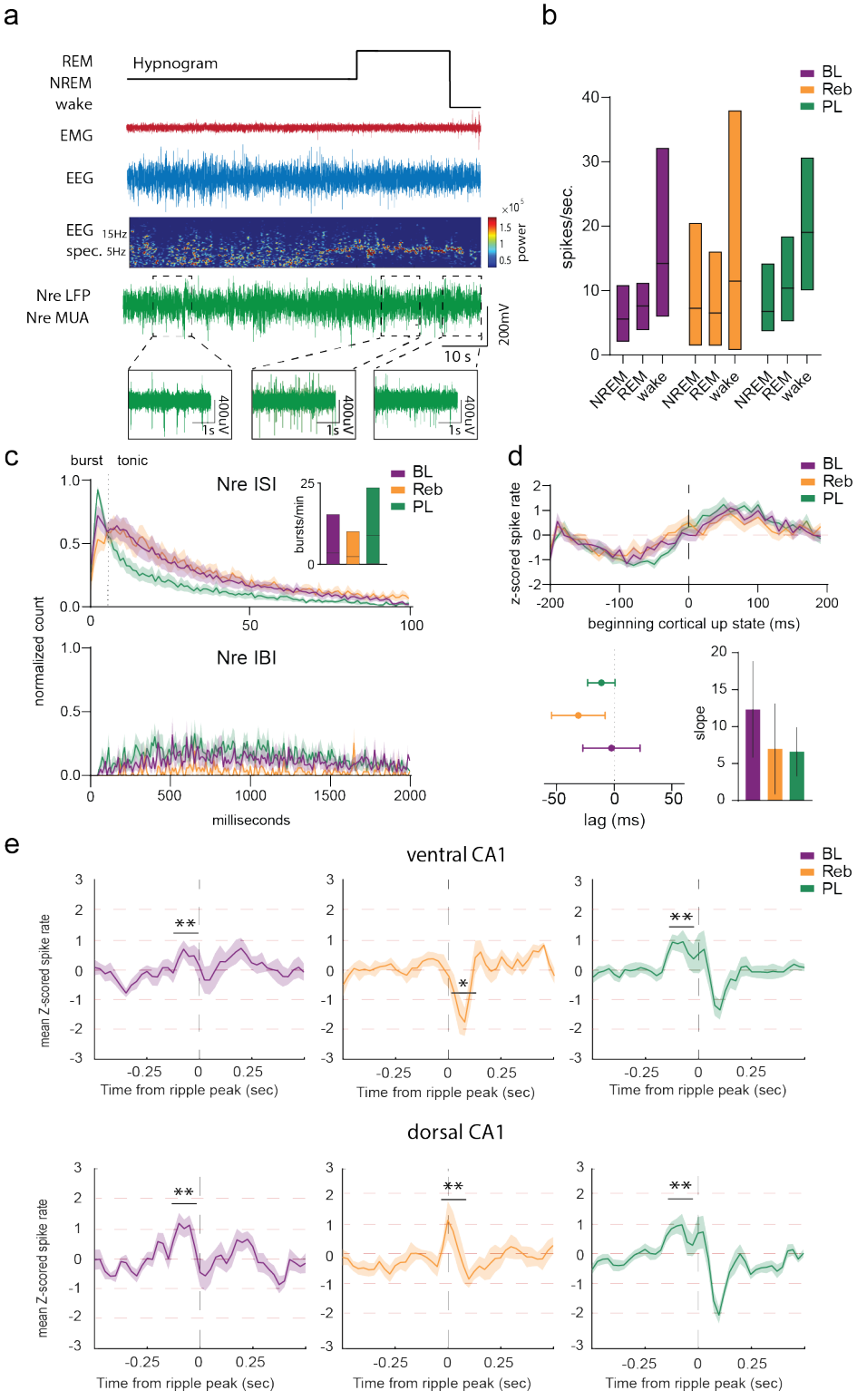

Figure 3.3: Nre spiking behaviour during different vigilance states and in relation to cross-regional neuro-oscillations during baseline sleep (BL), rebound sleep (Reb) and post-learning sleep (PL).

a) Representative hypnogram, EMG and EEG recordings, power spectrogram of the cortical EEG and multi-unit recordings from Nre across sleep-wake cycle. Insets: highpass filtering showing NRE neu-

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ron spiking activity during wakefulness, NREM and REM sleep. Totally recorded units: n=11 cells from 8 animals in baseline sleep, n= 11 cells from 6 animals in rebound sleep, n= 10 cells from 6 animals in post-learning sleep.

b) Box plots from minimal to maximal value with indicated mean showing the average showing Nre spike rates across vigilance states during baseline sleep (BL), rebound sleep (Reb) and post-learning sleep (PL). No significant differences were measured using Two-way ANOVA with Dunnett's multiple comparisons test.

c) Distribution of inter-spike (top) and inter-burst intervals (bottom) across vigilance states during baseline sleep (BL), rebound sleep (Reb) and post-learning sleep (PL). Nre cells show bursting behavior during NREM sleep. Inset, Box plots from minimal to maximal value with indicated mean showing the average of Nre cells bursts per minute during baseline sleep (BL), rebound sleep (Reb) and post-learning sleep (PL). No significant difference using One-way ANOVA with Dunnett's multiple comparisons.

d) Average spike rate +/- S.E.M. (z-score) of Nre neurons during a cortical UP state start (top). Time-lag +/- S.E.M. of Nre spike rate in relation to cortical UP state (bottom left) and Average slope +/- S.E.M. of Nre spike rate (bottom right) are shown. No significant difference One-way ANOVA followed by Dunnett's test for multiple comparisons.

e) Average spike rates +/- S.E.M. (z-score) of Nre neurons during vCA1 (top) and dCA1 (bottom) ripples during baseline sleep (BL), rebound sleep (Reb) and post-learning sleep (PL). During rebound sleep, a decrease after the ripples was observed for vCA1 ripples, indicative of the end of the UP state. For dCA1 ripples, the peak was co-occurring with the ripples. Significance was tested by comparing peaks, or troughs, to baseline level firing at the beginning of the time window using a paired sign-rank test (see methods). $*P<0.05$, $**P<0.01$.

ods; Fig.3a). We found that during baseline sleep, Nre cells fired at 14.2 ±2.6Hz during wake, 5.6 ±0.8Hz during NREM sleep and 7.6 ±0.8Hz during REM sleep (Fig.3b). During rebound sleep, Nre cells showed spike rates of 11.5 ±3.7Hz during wake, 7.2 ±1.8Hz during NREM sleep and 6.5 ±1.3Hz during REM sleep. During post-learning sleep, spike rates were 19.02 ±2.4Hz during wakefulness, 6.8 ±1.03Hz during NREM sleep and 10.4 ±1.5Hz during REM sleep (baseline sleep: n=11 cells from 8 animals, rebound sleep: n= 11 cells from 6 animals, post-learning sleep: n= 10 cells from 6 animals; Fig 3b). Note that differences in spike rates across experimental conditions were not significant.

Next, we assessed the bursting activity of Nre neurons by calculating their inter spike intervals (Fig3c top). We found that in all conditions Nre neurons displayed bursting activity patterns. Indeed, Nre neurons exhibited 3.6 ±1.6 bursts per minute during baseline sleep, 2.5 ±1.1 bursts/min during rebound sleep and 8.8 ±2.8 bursts/min during post-learning sleep (Fig.3c inset). The occurrence of bursts did not show a clear rhythmicity or significant differences between the experimental conditions (Fig.3c bottom).

To assess the influence of Nre spiking on Prl, which in turn could affect the connectivity between cortex and HPC, we calculated Nre spike rates time-locked to the beginning of UP states in Prl (Fig.3d top). We found a significant increase of Nre spike rates occurring immediately before the beginning of the UP state (lag during baseline sleep: -2.4 ±7.5ms, rebound sleep: -31.1 ±6.99ms, post-learning sleep: -11.1 ±3.8ms; Fig.3d bottom left). The timing and steepness of

the increase did not change across conditions (slope during baseline: 12.3 ±6.5 (a.u.), rebound sleep: 7 ±6.1, and post-learning sleep: 6.6 ±3.3; Fig.3d bottom right).
Next, we investigated the spiking behavior of Nre in relation to hippocampal ripples by calculating spike rates time-locked to ripples (Fig.3e) and found a significant increase preceding ripples during baseline sleep (vCA1 P= 0.0078; dCA1 P= 0.0078 using paired sign rank test) and post-learning sleep (vCA1 P= 0.0098; dCA1 P= 0.002), consistent with the observation that ripples occurred at the end of thalamic UP states (Fig.2d). During rebound sleep, the increase of Nre neuron spiking shifted closer towards a later time point and thus occurred together with ripples in dCA1 (P= 0.0078). At the same time, Nre spikes did not show an increase of spike rate locked to ripples in vCA1 but rather a decrease immediately after the ripple (P= 0.0391), likely corresponding to the end of the UP state (Fig.2d). Lastly, we explored Nre spike rates during ripples that were either locked to the UP state of cortical slow waves or troughs of cortical spindles; this analysis did not show any clear patterns (supp. fig.2). The results showed that sleep deprivation potentially leads to a change of Nre spiking behavior in relation to hippocampal ripples through an increase of the spike activity increase during dCA1 ripples and a decrease of spike activity during vCA1 ripples.

Optogenetic manipulation of nucleus reuniens alters cross-frequency coupling between hippocampus and prelimbic cortex

To test whether Nre plays a causal role in the functional connectivity between prelimbic cortex and HPC, we used optogenetics to perturb the firing of Nre neurons. To genetically express opsins in Nre neurons, AAV2-CamKII-ChR2-EYFP, AAV2-CamKII-ArchT3.0-EYFP or AAV2-CamKII -EYFP were stereotactically injected in the Nre of male wildtype mice for optogenetic manipulation of Nre neurons in freely moving mice (n=6 per group, see methods, Fig.4a). After 20 days, mice were implanted with chronic multi-site tetrodes in Prl, Nre (except for mice injected with AAV2-CamKII -EYFP), dCA1 and vCA1, EEG and EMG electrodes, and an optic fiber placed above the Nre as previously described (see methods) [419]. After recovery and habituation to the recording conditions, mice underwent an auditory contextual fear conditioning task to test for a possible Nre involvement in both hippocampus-dependent and independent memory consolidation (see methods, Fig.4b). After acquisition, separate groups of ChR2 and ArchT mice underwent either a NREM sleep-specific optogenetic stimulation or silencing protocol over the first 4h of NREM sleep following conditioning (ChR2: 140 trains of 5-ms pulses delivered at 7Hz; ArchT: continuous 20-second-long pulse; Fig.4c). As shown in the raster plot, our methods were proven effective of silencing (ArchT) and stimulating (ChR2) neurons in Nre. Control mice (EYFP control mice) were evenly assigned to either the stimulating protocol or the silencing protocol. Only LFP data in NREM during stimulation/silencing were considered for the subsequent analyses.
First, a coherence analysis was performed across the relatively short trials with optogenetic stimulation/inhibition permitted it. Note that this was not possible during the hour-long recordings from the previous experiments. We found that optogenetic silencing of ArchT-expressing Nre neurons selectively during NREM sleep led to a significant decrease of coherence of low delta frequency oscillations (1-2Hz) between dCA1 and Prl as compared to control conditions (Fig.4d top). Stimulating tonically at 7Hz led to a decrease in higher frequencies from 6Hz onwards (Fig.4d top). Interestingly, coherence between vCA1 and Prl was not affected neither by stimulation nor silencing of Nre (Fig.4d bottom).
Based on previous results (Fig.2), we next characterized the phase-amplitude coupling of hippocampal ripples to cortical slow waves and spindles upon optogenetic activating or silencing of Nre neurons during sleep. Both activating and silencing Nre neurons altered the preferred phase of ripples locked to cortical spindles (vCA1 in ArchT P= 0.0074; vCA1 in ChR2 P= 0.0015,

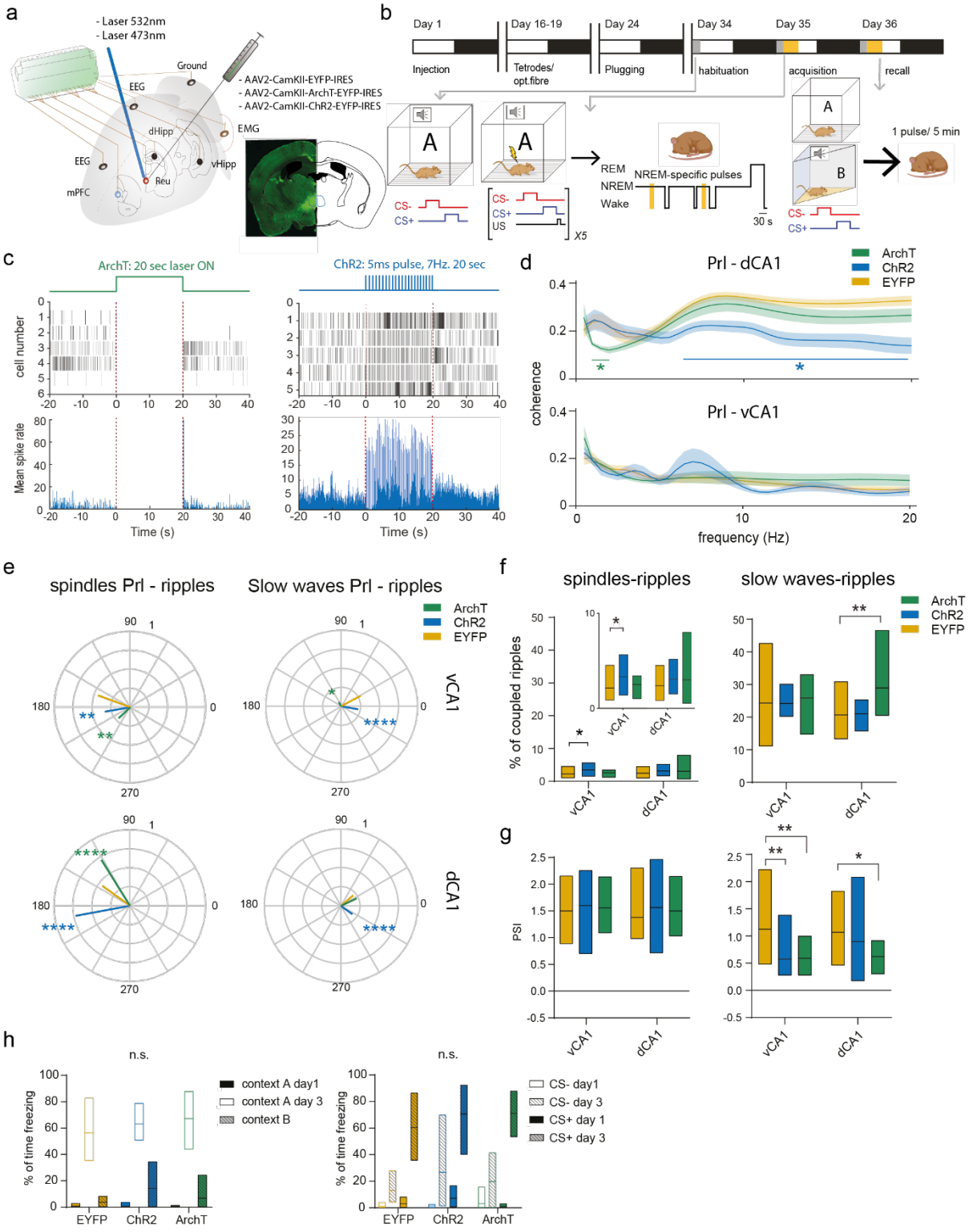

Figure 3.4: Optogenetic manipulation of nucleus reuniens modulates cortico-hippocampal cross-frequency coupling.

a) Schematic of the experimental setup showing viral transfection of Nre neurons, placement of tetrodes in Prl, Nre, vCA1 and dCA1, EEG/EMG electrodes, and optic fibre. Inset, Representative illustration of AAV2-CamKII-E1fa-ChR2-EYFP transfection in Nre.

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b) Experimental timeline showing the time points of injection, implantation, habituation, fear conditioning and optogenetic activation or silencing. Activation protocol consisted of 140 laser pulses à 5ms seconds duration at 7Hz for 20 seconds. Silencing protocol consisted of turning the laser ON for 20 seconds.

c) Representative rasterplot (top) and average spike rates (bottom) of Archt (left)- and ChR2 (right)-expressing Nre neurons during optogenetic silencing (20s continuous illumination) and activation (5-ms pulses at 7Hz over 20 seconds), respectively.

d) Average coherence +/- S.E.M. between Prl and dCA1 (top) or vCA1 (bottom) during optogenetic activation (blue) or silencing (green) and control mice (yellow, n= 6 mice in each group). *P<0.05, using Two-way ANOVA with Dunnett's multiple comparisons.

e) Circular histogram showing the normalized phase-amplitude coupling of hippocampal vCA1 (top) and dCA1 (bottom) ripples to spindles (left) and slow waves (right) in Archt (green), ChR2 (blue) and control mice (yellow, n= 6 mice in each group). Colored lines represent mean circular vectors. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 using Parametric Watson-Williams multi-sample test for equal means as a one-way ANOVA test. If the assumptions of the test were not met, a non-parametric multi-sample test for equal medians was employed with Bonferroni correction of P-values.

f) Box plots from minimal to maximal value with indicated mean showing the average percentage of co-occurrence of vCA1 and dCA1 ripples with spindles (left, inset shows magnification) or slow waves (right) during optogenetic stimulation, silencing, and control conditions. *P<0.05, **P<0.01, using One-way ANOVA with Dunnett's multiple comparisons.

g) Box plots from minimal to maximal value with indicated mean showing the phase slope index (PSI) between vCA1 and dCA1 ripples and cortical spindles (left) or slow waves (right). *P<0.05, **P<0.01, using One-way ANOVA with Dunnett's multiple comparisons.

h) Box plots from minimal to maximal value with indicated mean showing the average percentage of freezing behavior of ArchT (green), ChR2 (blue) and control (yellow) mice (n= 6 per group) during re-exposure to previous contextual (left), or discrimination (right) cues after acquisition of an auditory contextual fear conditioning test and optogenetic manipulation of Nre neuron activity (see panel b for timeline). No significant differences between the conditions were observed using two-way ANOVA followed by Dunnett's multiple comparisons test.

F= 8.1; dCA1 ArchT and ChR2 P<0.0001, F= 66.8), while the effects on slow waves were more diverse. Optogenetic activation of ChR2-expressing Nre cells shifted the preferred phase of ripple activity in both vCA1 (F=146.4) and dCA1 (F=100.5) (vCA1 and dCA1 P<0.0001). Optogenetic silencing of ArchT-expressing Nre cells had no effect on ripples in dCA1 while the phase-locking of ripples in vCA1 was significantly reduced (P= 0.0234; Fig.4e, supp. table 4). Next, we found that co-occurrence of ripples with spindles or slow waves, respectively, was also influenced by Nre neuronal firing. Optogenetic activation of ChR2-expressing Nre neurons increased co-occurrence of vCA1 ripples with spindles (F= 3.7, P= 0.0459), while optogenetic silencing of ArchT-expressing Nre neurons increased the co-occurrence of dCA1 ripples with slow waves as compared to controls (F= 7.2, P= 0.0037; Fig.4f, supp. table 4). Both findings could not be attributed to higher densities of slow waves, spindles, or ripples, hence the calculation of the ratio of observed/shuffled co-occurrence was irrelevant (supp. fig. 3e-h). Altogether, these results

suggested that Nre neurons were not only involved in the precise phase-amplitude coupling between cortical slow waves and spindles and hippocampal ripples, but they also contributed to the initiation of cortical slow waves and spindles in Prl.
Lastly, we calculated the PSI to assess Nre's influence on the directionality of Prl slow waves and spindles, and hippocampal ripples. Both activation and silencing significantly decreased the drive of slow waves on vCA1 ripples compared to the control condition (F= 5.4, P for ChR2= 0.0032, P for ArchT= 0.0061). Additionally, optogenetic silencing of Nre neurons reduced the drive of slow waves onto dCA1 ripples (F=1.7, P= 0.0268; Fig.4g, supp. table 4). We next tested whether optogenetic activation, or silencing, Nre neurons during post-learning NREM sleep had an effect on memory consolidation of both hippocampus-dependent and hippocampus-independent fear memory. Despite the significant changes in cortico-hippocampal cross-frequency coupling upon optogenetic perturbation, there were no significant changes in neither contextual memory performance nor hippocampus-independent cue learning (Fig.4h, see also supp. table 5).

Discussion

The Nre has been implicated in a variety of functions that involve prefrontal-hippocampal communication, such as acquisition, retention, and recall of contextual memories [404]–[406], [420]–[423], as well as spatial working memory [416], [424]–[426]. Additionally, the Nre is thought to play a role in re- and destabilization of fear-related memories as well as in their specificity [408], [427], [428]. However, its involvement in consolidation of hippocampus-dependent memories is less clear. One study found no effect in a maze task after inhibiting Nre during the consolidation phase [406], while another reported a more generalized expression of fear and stronger persistence of fear memory after contextual fear conditioning and Nre inhibition with muscimol during the consolidation phase [407]. A third study found weaker performance in a passive avoidance task when Nre was inhibited using tetracaine immediately after acquisition [420].
In the present study, we showed that neural oscillations in the Nre and prelimbic cortex are time-locked to sharp-wave ripples in the hippocampus and that this coupling remains stable during sleep after sleep deprivation and after acquisition of a fear conditioning task (learning). We showed that only a fraction (20-40%) of hippocampal ripples co-occur with cortical or thalamic slow waves, and with spindles (3-8%). These results extended the quantification of discrete events as compared to Siapas and Wilson in 1998 which provided correlation-coefficients of events in rats [96] and the root mean square of ripple activity in relation to slow waves and spindles in humans [385]. Even though the observed increases in co-occurrence were mainly due to higher densities of all events, higher co-occurrence of these oscillations may contribute to information transfer from HPC to cortex, as proposed by the *systems memory consolidation* hypothesis [175]. Our findings are complementary to the findings of Maingret et al. that showed an increased co-occurrence after a fear conditioning task relative to a baseline recording, and possibly a similar increase in their respective densities [93]. Furthermore, we confirmed that ripples tend to occur during a cortical UP state [89], [95], [97], [386], [429] and during troughs of spindles [98], [386], [387]. We also showed that ripples are coupled to the end of the Up state (reflected in both LFP and potentially also spike analyses) and the peak of spindles in thalamic Nre. The most striking changes were the increased phase-locking of vCA1 ripples to Nre spindles during post-learning sleep, a shift towards the Down state in the locking of vCA1 ripples to Nre slow waves during post-learning sleep and a reduced phase-locking during rebound sleep. However, we did not confirm the increased phase-locking of dCA1

ripples with prefrontal slow waves after a learning task as described before [95]. This discrepancy is possibly due to the usage of EEG instead of LFP electrodes and a different type of learning task, i.e. odor-learning task vs. fear conditioning. Additionally, despite low sampling, Nre spiking shows a peak before the occurrence of hippocampal ripples. This constellation of ripples locking to thalamic spindle peaks and Nre neurons showing a peak in their spiking activity right before ripples supports the findings of a previous study which showed that precise timing of Nre spikes and spikes of pyramidal CA1 neurons modulates spike-timing related plasticity in PFC [430]. Despite the low sampling, we still observed, that the activity of Nre neurons is modulated in relation to hippocampal ripples and cortical slow waves. Yet, these observations remain to be confirmed in future investigations using higher throughput experimental approaches.

Both rebound sleep and post-learning sleep displayed elevated densities of spindles, ripples, and slow waves in the first 30min or in the first hour, respectively. The elevation of spindle densities contrasted with the drop in spindle densities after sleep deprivation observed in humans [417], [431] and in mice [432]. This discrepancy may be explained by our shorter time of sleep deprivation as Vyazovskiy et al. used a sleep deprivation protocol of 6 hours. Nonetheless, the fact that spindles were elevated on a local level warrants the consideration that sleep deprivation causes spindles to be less spatially synchronized and possibly not detected from cortical EEG electrodes. Ripple densities after sleep deprivation have not been extensively investigated so far except for one study that found that it did not elevate during recovery sleep in mice that were sleep deprived after a hippocampus-dependent conditioning task as compared to mice that were allowed to sleep immediately after the task [388]. The elevation of ripple densities after sleep deprivation in our study points to a possible homeostatic function. Given that CA1 pyramidal cells do not show similar OFF, or bistable, states as thalamic and cortical neurons do during sleep [433], it is possible that other oscillations than slow waves serve homeostatic functions in the HPC [198].

On the other hand, the higher event densities after post-learning sleep on the other hand are in line with previous results described for spindles [40], [45] and ripples [90], [388], [434] and further demonstrate the importance of spindles and ripples for memory consolidation. Furthermore, our results showed a change in the drive of cortico-thalamic slow waves and spindles of hippocampal ripples. The drive of cortical spindles and slow waves on ripples during NREM sleep has been described in humans before [418]. The higher drive after sleep deprivation may be in part explained through higher synchrony of neuronal ensembles during slow waves, reflected by the higher slope of slow waves after sleep deprivation [30], [119]. Thus, increasing synchronous cortical and thalamic neural assemblies may induce a stronger downstream cascade which in turn influences and drives hippocampal ripples more effectively. We theorise, that the higher synchrony slow waves may have an influence on the spindle nesting [435], [436]. The higher drive during post-learning sleep would be consistent with higher mnemonic demands of involved cortical and thalamic areas, which may support the process of memory consolidation and replays of neuronal ensembles [175]. However, the exact initiation of coordinated replays in cortical and hippocampal areas remains to be fully understood. Our results suggest a cortical drive of ripple events, which is consistent with a previous study [418] and the nesting of ripples in Up states of slow waves and spindle troughs, both of which start before the onset of ripples.

Optogenetic manipulations of Nre during natural NREM sleep showed that Nre plays a key role in coordinating sleep oscillations between Prl and HPC. Particularly, it regulated the coherence of delta frequencies as well as theta and beta frequencies between cortex and the dorsal portion of CA1, which confirms previous findings in anesthetized animals [412], [413] as well as awake rats during a working memory task [416]. Furthermore, our observation confirmed

that Nre neurons coordinated phase-locking between hippocampal ripples and cortical slow waves and spindles. Precisely, optogenetic silencing or stimulating did not affect the strength of phase-amplitude coupling (except for vCA1 ripples and slow waves) but rather changed the preferred phase of ripple occurrence with preserved mean vector length. Additionally, optogenetic silencing or stimulation of Nre increased co-occurrence of cortical slow waves with dCA1 ripples and of cortical spindles with vCA1 ripples, respectively. Additionally, stimulation reduced the drive of slow waves on vCA1 ripples and silencing reduced the drive of slow waves on all ripples.

Despite these changes in cortico-hippocampal communication during NREM sleep, optogenetic manipulation of Nre did not result in any significant changes in sleep-dependent memory performance. This finding contrasts with previous studies assessing the role of Nre in memory consolidation. Troyner et al. [407] observed relatively small but significant levels of generalization in contextual memory performance when silencing Nre with muscimol during the consolidation phase. Similarly, Xu and colleagues reported a strong contextual generalization upon silencing of Nre projections to PFC [408]. In the latter study, however, Nre neurons were either chronically silenced using tetrodotoxin *before* fear conditioning or optogenetically stimulated during *acquisition* in a phasic fashion (15 pulses at 30 Hz every 5 seconds). A potential explanation for these discrepancies is the different intervention protocol used in both studies. We used a mild optogenetic perturbation approach based on 20-second-long train of stimulation/silencing every second NREM episode for 4h of NREM sleep in order to restrict perturbation of Nre neural networks that may affect sleep and avoid the triggering of compensatory mechanisms. Thus, Nre cell activities, and associated sleep oscillations, were altered only during a restricted number and duration of NREM sleep episodes (supp. fig. 3d). This contrasts to the methods used in other studies where tetrodotoxin expression in Nre neurons [408], or muscimol infusion in Nre area [407] abolished Nre neuron activity for hours, or an unlimited period of time, respectively, irrespective of the sleep-wake states of the animals after acquisition. Aside from a likely role of Nre cells in cognition during wakefulness, an alternative silencing protocol would possibly involve targeting every Up state of Nre with a closed loops system to disrupt Nre firing before ripple occurrence. Additionally, several studies have demonstrated Nre's involvement in retention of remote memories [407], [409]–[411]. Thus, a promising approach would be to either stimulate or silence Nre neurons during NREM, but also REM, sleep for several days to manipulate the consolidation of remote memories, which may strengthen its implication in the process of '*corticalization'* where memories become independent from the HPC, as proposed by the *systems memory consolidation hypothesis* [175].

To summarize, our analyses revealed a role of Nre in orchestrating interactions between PFC and HPC during sleep. This interaction is not only crucial for memory consolidation [175] but also for episodic memory retrieval [176], [437] and working memory [438], [439]. Thus, further investigations on the role of Nre neurons in sleep-dependent memory consolidation, or weakening, are warranted. Furthermore, the clear role of Nre neurons in mediating prefrontal-hippocampal communication suggested its potential involvement in psychiatric diseases including schizophrenia where disruption of Nre neuronal connections has been reported. In this context, spatial working memory relies on theta and gamma oscillation coherence between PFC and HPC [440]. Deficits in this cognitive aspect or anatomical defects in medial thalamic regions [441]–[443] all have been associated with schizophrenia [444] or dementias.

Methods

Animals

We used adult male C57Bl6 mice that were 6-12 weeks old at the time of surgery form Janvier labs, France. Animals were treated according to protocols and guidelines approved by the veterinary office of the Canton of Bern, Switzerland (license no. BE 119/2020). Up until tetrode implantation, mice were housed in groups of 2-5 in IVC cages at constant temperature (22± 1°C), humidity (40-50%) and circadian cycle (12h light-dark cycle with lights on at 08:00). Food and water were provided *ad libitum*. After tetrode implantation, mice were housed individually in custom made hard plastic cages with an open top under the same conditions. After 5 days, mice were chronically tethered to recording cables (and optic fibers, respectively) and mice were given another 10 days of habituation before the start of the experiments. Treatment guidelines and protocols were approved by the Veterinary Office of the Canton of Bern, Switzerland (license number BE129/2020).

Stereotaxic injection of AAV

At six weeks of age, mice were anesthetized with isoflurane (5% for induction, 1.25-1.75% for maintenance) in oxygen and mounted in a stereotaxic frame (Model 940, David Kopf Instruments). Before incision, saline (10ml/kg) and meloxicam (5mg/kg) were given subcutaneously. The skin above the skull was shaved and aseptically prepared with Betadine, followed by a longitudinal midline incision along the skull from the level of the eyes to the insertion of the trapezoid muscle at the back of the skull. Bregma and the point 5mm caudally to it were aligned to ensure proper positioning of the skull. Injections were done with a Hamilton syringe (7000 series, model 7000.5, 0.5µL volume) and a syringe pump (Pump 11 Elite Nanomite Infusion/Withdrawal Programmable Syringe Pump, Harvard Apparatus). Animals were randomly assigned to receive either AAV2-CamKII-E1fa-ChR2-EYFP, AAV2-CamKII-E1fa-ArchT-EYFP or AAV2-CamKII-E1fa-EYFP. The virus was injected in Nre (-0.66mm AP, 0.3mm ML, -4.29mm DV, 4°, 200nl) according to Paxinos' and Franklin's mouse brain atlas at an injection rate of 50nl/min. After the virus was delivered, the needle was left in the current position for min. 10 minutes to allow diffusion into the brain tissue. After injection, mice were given at least 20 days of recovery before tetrode implantation. All plasmids came either from University of North Carolina Vector Core or University of Zurich Viral Vector Facility.
pAAV.CamKII(1.3).eYFP.WPRE.hGH was a gift from Karl Deisseroth (Addgene plasmid # 105622 ; http://n2t.net/addgene:105622 ; RRID:Addgene_105622)
pAAV-CaMKIIa-hChR2(H134R)-EYFP was a gift from Karl Deisseroth (Addgene plasmid # 26969 ; http://n2t.net/addgene:26969 ; RRID:Addgene_26969) (Lee et al., 2010)
pAAV-CaMKIIa-eArchT3.0-P2A-EGFP-WPRE-hGHpA was a gift from Jonathan Ting (Addgene plasmid # 51110 ; http://n2t.net/addgene:51110 ; RRID:Addgene_51110)

Tetrode implantation

Electrodes for EEG signals and grounding were obtained with stainless steel screws and bare-ended steel wires were used for EMG signals. Tetrodes were made of four strands of 10µm tungsten wire (CFW0010954, California Fine Wire) which were twisted and connected to an electrode interface board by gold pins (EIB-36-PTB, Neuralynx). Anesthesia was induced using isoflurane in oxygen and maintained using a mix of medetomidine (0.27 mg/kg), midazolam (5 mg/kg), fentanyl (0.05 mg/kg). The animals were prepared in the same manner as described above and the screws for the EEG signal ware placed in the skull above the frontal lobe and the parietal lobe, the ground screw was placed above the cerebellum and the EMG wires were sutured to the trapezoid muscle. Tetrodes were implanted into Prl (+1.7mm AP, 0.4mm ML, -2.4mm DV), Nre (-0.66mm AP, 0.4mm ML, -4.38mm DV, 2°), dCA1 (-2.2mm AP, 1.5mm ML, -

1.15mm DV) and vCA1 (-2.9mm AP, 3.38mm ML, -4.5mm DV). In the optogenetic experiment, optic fibers of 200µm diameter were additionally implanted in nucleus reuniens via attachment to the respective tetrode in ChR2 and ArchT mice. EYFP control mice were only implanted with the optic fiber. After tetrodes were placed in the right position, they were fixed with Tetric EvoFlow cement by *Ivoclar vivadent*. Once all tetrodes were placed and fixed, the EMG wires and wires attached to the EEG and ground screws were accordingly connected to the interface board and the surgery was finalized by fixing the implant with Paladur methacrylate cement by *Kulzer*. Anesthesia was terminated by injecting Atipamezole, Naloxone and Flumazenil subcutaneously and the mice were given time to recover in their home cage which was placed on a heating mat.

Data acquisition

For all recordings, mice were connected to a tethered, digitizing head stage (RHD2132, Intan Technologies) and recordings were done at 20kHz using an open-source software from Intan Technologies (RHD2000). In the optogenetic experiment, optical fibers were connected to patch cords which were coated with black tubing and the connections were covered in black varnish to avoid disturbing the mice via visual stimulation. First mice underwent recording of baseline sleep (ZT4-ZT-9), followed be sleep deprivation on the following day and fear conditioning one week after sleep deprivation (see below). When sufficient data was acquired in one of the first two experiments, the respective experiment was omitted.

Sleep deprivation

At the onset of light at 08:00 (ZT0) mice were kept awake for the next 4h by gently handling them when stationary. At ZT 4 the procedure was stopped, and subsequent sleep was recorded from ZT4-ZT9. See supp. fig. 1b and c for power spectral density analysis, showing the increase in delta frequency after sleep deprivation.

Fear conditioning

Before the experiment was started, mice were habituated by gently handling them for 5min on 5 consecutive days. On the first day of the procedure (habituation) at ZT0, a foreign cage with a metal grid as a floor (context A) was wiped with 70% ethanol and the mice were placed in it. The walls were marked with stripes to provide additional contextual information. The mice were first given 3min of time to explore the novel environment, followed by playing a first auditory stimulus (CS-) consisting of 27 beeps of 50ms duration at 2000 Hz played over 30 seconds for 5 times with a variable interstimulus interval (ISI) between 10s and 30s. Then, a second auditory stimulus (CS+) was played under the same conditions but at 8000Hz. 24h later, acquisition of fear memory was performed by wiping context A with alcohol again and subsequently placing the animals in the same cage with the metal grid. After 3 min, CS+ and CS- were played in an intermixed manner. An unconditional stimulus (US) was paired with CS+ by applying an electric foot shock through the metal grid on the floor at 0.5 mA for 1 second, starting when CS+ ends. Interstimulus interval was set between 15 and 90 seconds. CS-, CS+ paired with US were played 5 times. Another 24h later (recall), a novel cage (context B) was wiped with 1% acetic acid and mice were placed in it. After 3 min of exploration, CS- and CS+ were presented to the mice with the same protocol as during habituation. Approximately 30min later, the mice were placed in context A again to assess contextual fear memory. Freezing behavior was measured as a measure of learning performance. It was scored manually as absence of any movement except breathing. Freezing during context was measured from the end of the first minute onward for two minutes. Freezing to CS+/CS- was measured during the time the tone was playing, and only the first two occurrences of CS+ and CS- were considered. Freezing was quantified as the total time of freezing during the total playing time of the tones,

or during the total 2 minutes in context A or B, respectively. If mice generalized in the first series of experiments (Fig.1-3) (i.e., they did not discriminate between CS+ and CS-) they were excluded from the analysis. See supp. fig. 1e and supp. table 1 for results of memory performance.

Optogenetic stimulation protocol

After acquisition, laser pulses between 20-30mW were delivered during every second NREM episode for 4h of NREM sleep. After recall, laser pulses were delivered every 5 minutes for 4 hours of NREM sleep. ChR2 mice and respective EYFP control mice received 5ms pulses à 7Hz for 20 seconds resulting in 140 pulses per stimulation train. ArchT mice and respective EYFP control mice received a single 20-second-long laser pulse for every train. Only trains delivered during NREM were considered for subsequent analysis and only the LFP signals during the stimulation/silencing trains were considered for analysis concerning phase-event coupling, event co-occurrence and phase slope index analysis. The LFP signals were concatenated for coherence measures. The stimulus was not interrupted in case of state transition. After recall, mice underwent another stimulation or silencing protocol to increase the relevant electrophysiological data samples. The stimulation trains were the same, but a train was delivered every 5 min, irrespective of vigilance state for 4h. Only trains occurring during NREM sleep were considered for data analysis.

Sleep scoring

Sleep scoring was performed manually, based on frequency and amplitude characteristics of the EEG and EMG in custom software written in Matlab. NREM sleep was identified by high-amplitude, synchronous activity in the EEG with a delta (0.5- 4 Hz) frequency dominating the signal and low EMG activity, REM by highly synchronous theta (6- 9Hz) especially in the parietal electrode and flat EMG and wake mainly by increased EMG activity. The start of wake was defined as the onset of rapid increase in muscle tone concurrent with a low-amplitude, high-frequency (>6Hz) EEG, the start of NREM was defined as the first visible slow wave of more than 200µV amplitude and the start of REM was put at the point of a consolidated theta:delta ratio of more than 1.

Single unit activity

Single units were detected using the *Offline sorter Application* by *Plexon Neurotechnology Research Systems* (Version 4.4.1). Raw LFP data was first band-pass filtered (500-4000Hz, Butterworth filter) and a threshold for multiunit activity was set manually. Single unit activity was then extracted using principal components analysis and manually extracting resulting clusters.

Burst firing of single units was defined as a minimum of three consecutive action potentials with ISI <6ms which were preceded by a quiescent hyperpolarized state of at least 50ms. The interburst interval (IBI) was defined as the distance between the centers of two consecutive bursting activities during NREM episodes. IBI histograms during NREM sleep were created for individual units using a 200ms bin width [419], [445].

As described in a previous publication [419], the average spike rate during vigilance state was calculated as the total number of action potentials during a state divided by total time spent in that state and reported in Hz for every unit separately. Mean spike rates for each unit during transition from DOWN to UP states were calculated by averaging spike rates during all detected DOWN to UP transitions using a non-overlapping moving window of 10ms. Mean spike rates were then fitted with a Boltzmann sigmoidal curve in GraphPad Prism with the following equation:

$$y = minimum + \frac{maximum - minimum}{1 + \frac{t_{\frac{1}{2}} - x}{slope}}$$

Where *y* is the neuronal spike rate and *x* is time. The half-times of the curves ($t_{½}$) were used for comparison of lag and phase. The slopes of the curve fits at the half-time point were used as a measure of neuronal spiking modulation and therefore synchrony [446]. Spike rate of Nre cells locked to ripples was calculated using a non-overlapping 50ms window with a total window of 1 second around ripple peak. Increases or decreases around ripple center were tested for significance by comparing the mean spike rate during 150ms around the peak or trough, respectively, to the mean spike rate during a 150ms baseline at the beginning of the 1 second window using a paired signed-rank test.

Preprocessing of LFP data

After acquisition, LFP data was down sampled to 1000Hz and was re-referenced with a common average reference to reduce possible volume conduction. Then, data were z-scored and the EMG trace was subtracted with linear regression.

Slow wave detection

Individual slow waves were detected during NREM sleep in MATLAB using the SWA-MATLAB toolbox [447], with detection parameters adjusted to rodents from settings described by Panagiotou et al. and Facchin et al. [448], [449]. First, the negative envelope across the LFP signal was calculated, filtered between 0.5 and 4 Hz (Chebyshev Type II filter design), and consecutive zero-crossings were detected. If the duration between successive downward (negative going) zero-crossing and upward zero-crossing was between 100ms and 1s, then the peak negative amplitude was examined and was required to be at least 3 deviations from the median amplitude of all negative peaks in the recording. The amplitude threshold eliminates the potential individual differences of electrode reference type, distance to those references, and electrode depth that would affect the record amplitude. The beginning of the slow wave was marked at the positive to negative zero-crossing before the negative peak and the end of the slow wave was marked at the end of the subsequent positive slope.

Spindle detection

We detected spindles using the wavelet-based method proposed by Wamsley et al. [450] and Bandarabadi et al. [451]. We extracted wavelet energy of the complex frequency B-spline function, which provided the highest normalized power, by considering several criteria. The wavelet energy time series was smoothed using a 200ms Hann window, and a threshold equal to 3SD above the mean was applied to detect potential spindle events. A lower threshold of 1SD above the mean was set to find start and end of detected events. Events shorter than 400ms or longer than 2s were discarded. Using bandpass-filtered LFP signals in the spindle range (9–16 Hz), events with fewer than 5 and more than 30 cycles were discarded as well. To ensure that increases in wavelet energy are spindle specific, and not due to artefacts, we estimated power in the spindle range as well as in 6–8.5 and 16.5–20 Hz frequency bands and discarded those events where power within the spindle band was lower than in the two other bands. We also estimated the central frequency of spindle using the fourier transform. We measured the symmetry of spindles using the position of peak of wavelet energy time-series with regard to the start and end of spindles. The symmetric measure lies between 0 and 1; 0.5 corresponds to complete symmetry, and values lower and higher than 0.5 show a leftward and rightward shift of peak, respectively.

Ripple detection

Ripple detection was based on previously published methods of detecting them [56], [452]. Data was downsampled to 1000Hz, and band pass filtered by first applying fast fourier transform and subsequently filtered LFP and power of frequencies between 150-250Hz were extracted via inverse fourier transform. Ripples were then detected as events where power surpassed 5SD of the mean power for at least 10ms. Start and end of the ripple were marked at the beginning of the uprising towards the threshold and end of the descend below threshold of the power.

Event densities over time

Event densities for spindles and ripples over time were calculated by cutting out wake and REM episodes and concatenating NREM episodes. Next, the event densities were calculated for 15min bouts over 3h as this was the minimum amount of time all animals slept during the recordings in baseline, rebound and post-learning sleep, thus avoiding higher error margins towards latter parts of recordings.

Co-occurrence measuring

Co-occurrence of slow waves, spindles, and ripples was measured by calculating the average length of spindles and slow waves to determine the window length for co-occurrence. Next the windows for co-occurrence were centered at the middle of slow wave and spindle centers. Co-occurrence was then calculated as a percentage of all detected ripples which occur within the windows.

Phase-amplitude coupling analysis

For the following sections, we performed event-locked cross-frequency analysis. Namely, ripples locked to slow waves and spindles were taken as the events and the LFP data of thalamus, cortex and HPC from -1.25 to 1.25 seconds around the ripples were considered for the following calculations. We used finite-duration impulse response filters (FIR) to band-pass filter the data in the respective frequency range (see above) (forward/backward, filtfilt, Matlab, signal processing toolbox, https://www.mathworks.com/products/signal.html). FIR filters used an order equal to three cycles of the lower cut off frequency. The phase was extracted using Hilbert transform. The peak of a coupled ripple was matched to the corresponding phase value of the slow wave or spindle. Next, a phase-amplitude histogram (PAH) was computed where the phase was divided in 18 bins with each covering 20 degrees and the mean circular vector was calculated. All resulting vectors were tested for non-uniformity with the Rayleigh test for non-uniformity. To estimate if slow waves and spindles drive ripples or vice versa, we calculated the cross-frequency directionality (CFD) on the same events as described above. When two or more ripples occurred within the same slow wave or spindle, that time window was only analyzed once. This measure is based on the phase-slope index (PSI) between the phase of slower oscillations and the power envelope of faster oscillations [435], [453]. Values above zero indicate that the lower frequency (slow wave or spindle) drives the higher frequency (ripple), while negative values indicate that ripples drive slow waves and spindles. To prove for the statistical significance of CFD, a non-parametric approach based on randomizations was applied. By that we control for multiple comparisons with respect to the multiple frequency pairs included in the analysis [454]. Lastly, a normalization factor was obtained through fitting an exponential decay function to resulting phase slope index values per number of trials and applied to the PSI value of each subject.

Immunohistochemistry and Histology

After all experiments were done, animals were anaesthetized with isoflurane as described above and electric current was sent through two channels of each tetrode (30µA, 5 pulses à 2s)

in order to induce gliosis at the spot of tetrode placement. After min. 2h, animals were euthanized with 15mg pentobarbital i.p. and the cardiovascular system was transfused with 30ml of cold, heparinized phosphate buffered saline (PBS), followed by 30ml of 4% Paraformaldehyde (PFA) via puncture in the left ventricle of the heart. Brains were removed and kept in PFA overnight. The next day, they were cryo-protected in 30% sucrose for 48h. Then, they were flash-frozen in 2-methylbutane at approximately -80°C. Next, the brains were cut in 40µm thick sections. To confirm virus transfection, sections were washed in PBS + 0.1% Triton X-100 (PBST) three times for 10min each, blocked by incubation with 4% bovine serum albumin dissolved in PBST for 45mim and subsequently incubated with anti-GFP antibodies (AB_221569) for 24h-48h at 4°C. After that, sections were again washed in PBST (three times à 10min) and then incubated with a secondary antibody (ab150073) that binds to the primary antibody for 1h at room temperature. To confirm tetrode placement, the tetrodes were stained with 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanin-perchlorat before implantation. After cutting the brain, sections with visible dye traces were selected and either Nissl-stained or HE-stained to reveal the gliosis.

Statistical methods

We used MATLAB® (R2019b, MathWorks, Natick, MA, USA) and Prism 8 (GraphPad) for all analyses and statistics. The CircStat toolbox was used for circular statistics (no ANOVA tests with multiple comparisons available). To test differences between conditions with circular data, we pooled all obtained phase values for each condition and employed a Parametric Watson-Williams multi-sample test for equal means as a one-way ANOVA test. If the assumptions of the test were not met, a non-parametric multi-sample test for equal medians was employed. Multiple comparisons were subsequently performed by first performing a parametric two-sample test between the experimental condition and the control condition (i.e. baseline sleep in the first experiment or EYFP control in the second experiment). If the test conditions were not met, a non-parametric two-sample test for equal medians was performed. The resulting p-values were then adjusted with Bonferroni correction. No power calculations were performed to determine sample sizes. Data was compared via one-way or two-way ANOVA followed by multiple comparisons tests and *signed rank tests* for non-parametric data, as indicated in the text. Values in the text are reported as mean ± standard error of the means (S.E.M.) unless reported otherwise. Figures were prepared in Adobe Illustrator CC (Adobe).

Acknowledgments and Disclosures

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Parts of Fig.4 were reprinted from "Asleep mouse", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Disclosures

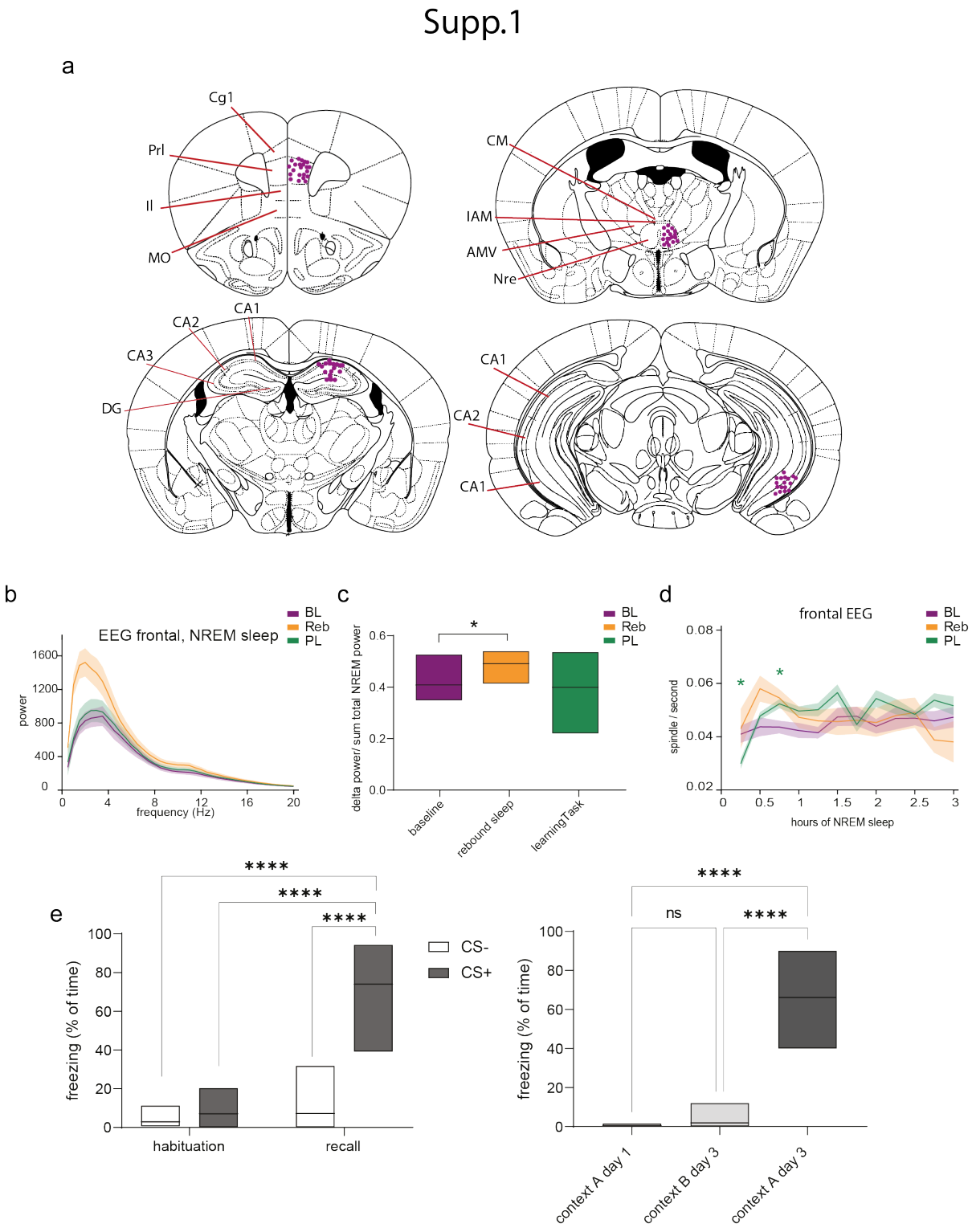
The authors report no potential conflicts of interest.

Data availability
All presented data and analysis scripts, including data files, Matlab scripts and functions are available on GitHub.

List of abbreviations

AP	antero-posterior
ArchT	Archaerhodopsin
BL	baseline sleep
CFD	cross-frequency directionality
ChR2	Channelrhodopsine-2
CS-	conditional stimulus, not paired with US
CS+	conditional stimulus, paired with US
dCA1	dorsal CA1
DV	dorso-ventral
EEG	electroencephalogram
EMG	electromyogram
EYFP	Enhanced yellow fluorescent protein
FIR filter	finite impulse response filter
LFP	local field potential
PL	post-learning sleep
ML	medio-lateral
mPFC	medial prefrontal cortex
Nre	Thalamic nucleus reuniens
PAH	phase amplitude histogram
PBS	phosphate buffered saline
PBST	phosphate buffered saline and triton X-100
PFA	paraformaldehyde
PFC	prefrontal cortex
Prl	prelimbic cortex
PSI	phase slope index
Reb	rebounds sleep
S.E.M.	standard error of the means
US	unconditional stimulus
vCA1	ventral CA1
ZT	Zeitgeber

Supplementary material



(description on next page)

Supp.Fig.1 Confirmation of tetrode placement, sleep deprivation and memory performance of fear conditioning. BL= baseline, Reb= rebound sleep, PL: post-learning task sleep.

a) Purple dots indicate tetrode placement of tetrodes that went into analysis. Cg1: cingulate cortex area 1, Prl: prelimbic cortex, Il: infralimbic cortex, MO: medial orbital cortex, CM: central medial nucleus. IAM: interomedial thalamic cortex, AMV: anteromedial thalamic nucleus, Nre: nucleus reuniens, DG: dentate gyrus.

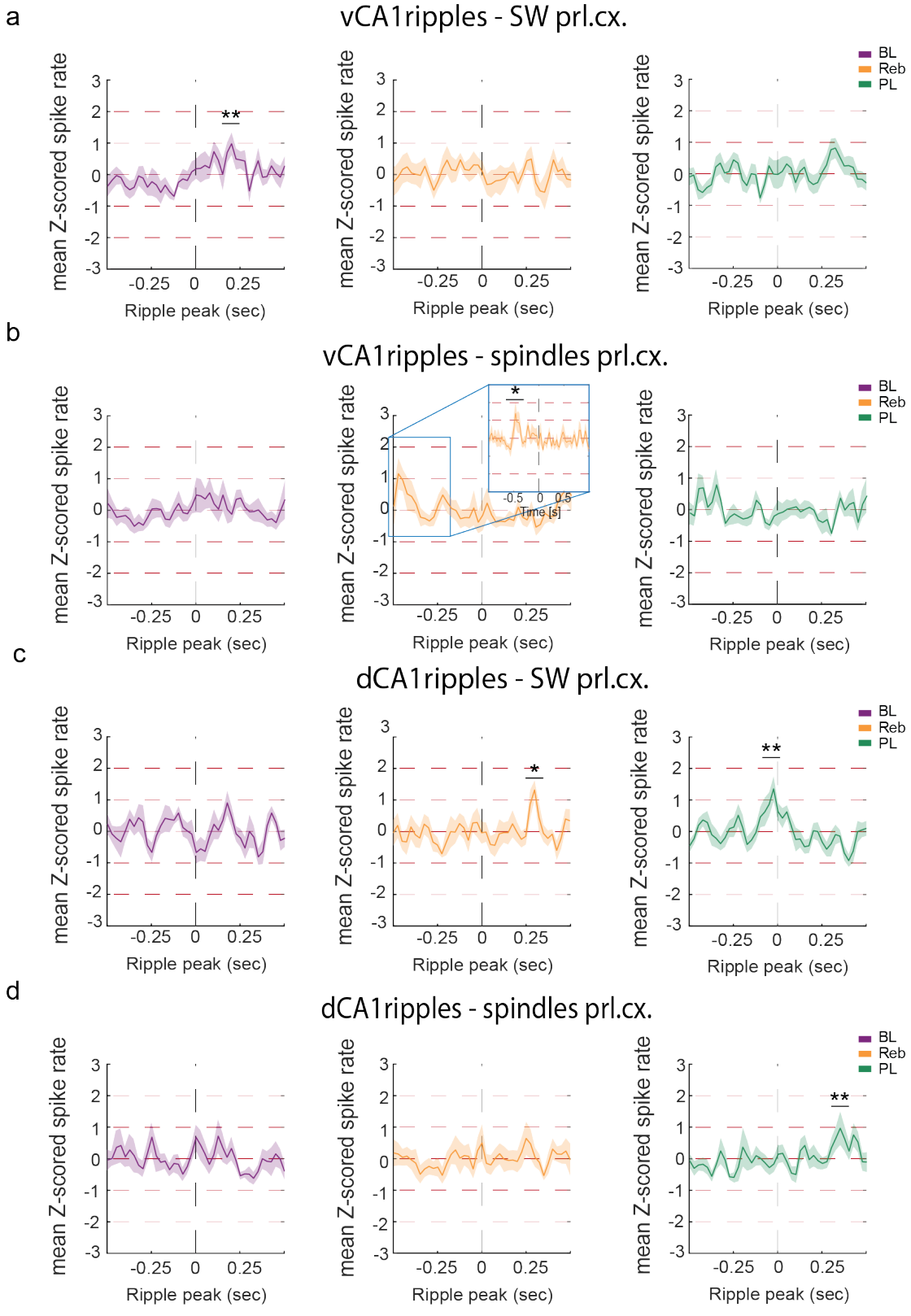
b) Power spectral density of frontal EEG signal during NREM sleep shows the increase in delta power during rebound sleep.

c) Box plots from minimal to maximal value with indicated mean showing the average delta power (0.5-4Hz) divided by the sum of total NREM power to quantify the increase after sleep deprivation: 0.4 ±0.02 during BL (n=12), 0.5 ± 0.02 during Reb (n=9), 0.4 ±0.03 during PL (n=9). One-way ANOVA with Dunnett's multiple comparisons F= 5.3, *P<0.05.

d) cortical spindles densities over time across baseline sleep (BL, N=12), rebound sleep (Reb, N=9) and post-learning sleep (PL, N=9). Two-way ANOVA with Dunnett's multiple comparisons. $F_{condition}$= 0.8, F_{time}= 2.1, $F_{condition\ x\ time}$= 1.7, *P<0.05.

e) Boxplots from minimal to maximal value with indicated mean showing the results of memory performance of fear conditioning assessed by measuring the percentage of time spent freezing (n=9). CS- on habituation: 2.7 ±1.2%, CS- on recall: 7.2 ±3.3%, CS+ on habituation: 7.1 ±2.7%, CS+ on recall: 74.04 ±6.5%. One-way ANOVA with Tukey's multiple comparisons F_{tone}= 93.4, $F_{subject}$= 1.9. Context A on habituation: 0.5 ±0.2%, context A on recall: 66.2 ±6.3%, context B on recall: 1.9 ±1.3%. One-way ANOVA followed by Tukey's multiple comparisons $F_{context}$= 108.2, $F_{subject}$= 1.2. *P<0.05, ****P<0.0001.

Supp.2



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Supp.Fig.2 Reuniens spike activity during ripples that are coupled to cortical spindles or slow waves during baseline sleep (BL), first 30min of rebound sleep (Reb) and first hour of post-learning-task sleep (PL).

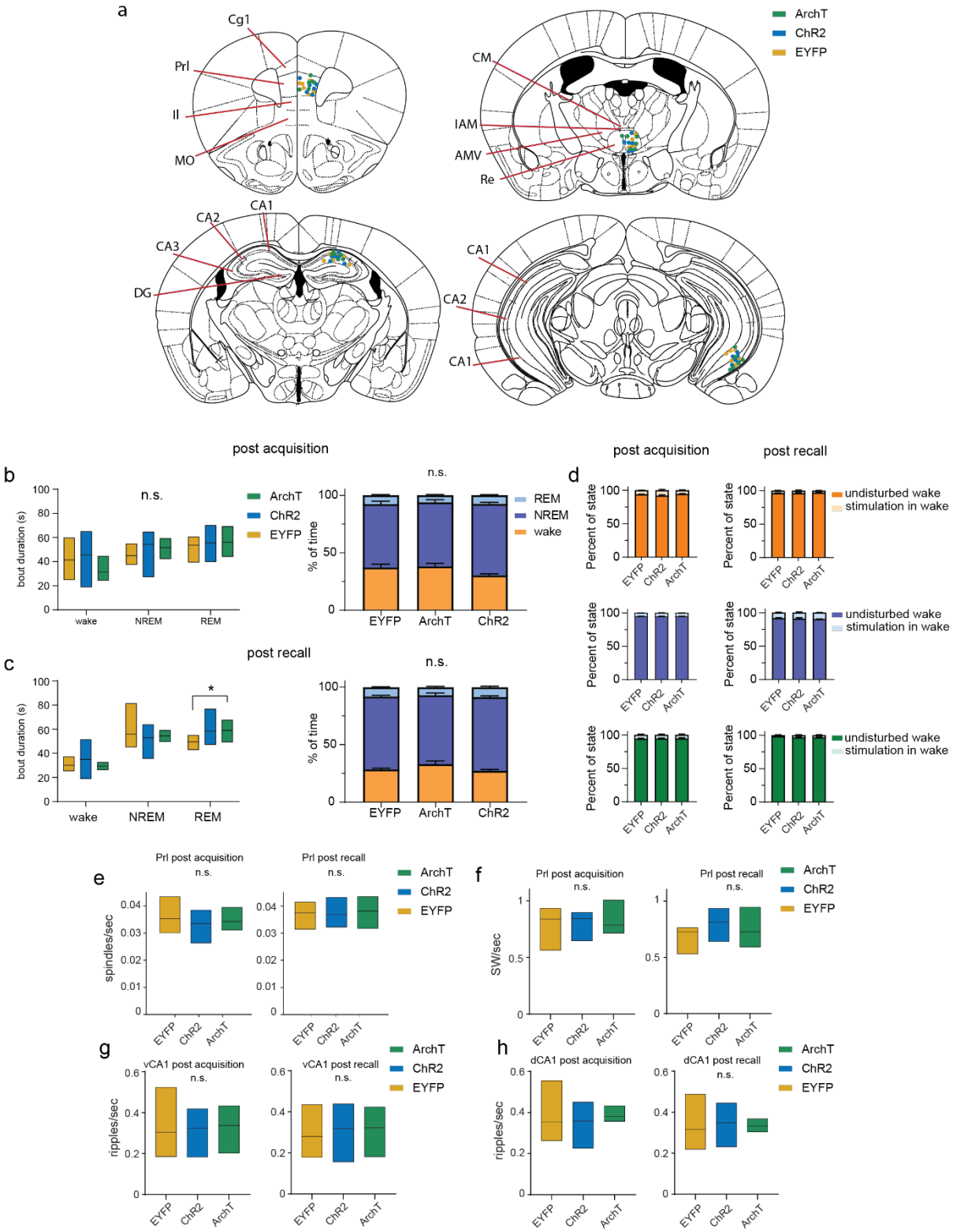
a) Spiking activity of Nre locked to vCA1 ripples that are coupled to cortical slow waves in baseline sleep (BL), rebound sleep (Reb) and post-learning task sleep (PL), P in BL= 0.0039 (Totally recorded units: n=11 cells from 8 animals in baseline sleep, n= 11 cells from 6 animals in rebound sleep, n= 10 cells from 6 animals in post-learning sleep).

b) Spiking activity of Nre locked to vCA1 ripples that are coupled to cortical spindles.

c) Spiking activity of Nre locked to dCA1 ripples that are coupled to cortical slow waves.

d) Spiking activity of Nre locked to dCA1 ripples that are coupled to cortical spindles. Significance was tested by comparing peaks or troughs to baseline level firing at the beginning of the time window with a paired sign-rank test (see methods). *P<0.05, **P<0.01.

Supp.3



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Supp.Fig.3 sleep structure during stimulation/silencing of nucleus reuniens.

a) Placement of tetrodes and optic fibers in the case of Nre in all animals used for optogenetic experiments, indicated with dots in the respective colour. Cg1: cingulate cortex area 1, Prl: prelimbic cortex, Il: infralimbic cortex, MO: medial orbital cortex, CM: central medial nucleus. IAM: interomedial thalamic cortex, AMV: anteromedial thalamic nucleus, Nre: nucleus reuniens, DG: dentate gyrus. All boxplots range from minimal to maximal value with indicated mean.

b) Bout duration of NREM, REM and wake episodes during the recording after acquisition (left). Results for EYFP control mice: 41.4 ±6.4 seconds for wake, 44.98 ±2.6 seconds for NREM, 53.5 ±3.1 seconds for REM. Results for ChR2 mice: 45.4 ±7.7 seconds for wake, 54.4 ±5.9 seconds for NREM, 55.6 ±4.4 seconds for REM. Results for ArchT mice: 31.4 ±3.1 seconds for wake, 51.6 ±2.3 seconds for NREM, 56.02 ±3.5 seconds for REM. No significant differences were measured using two-way ANOVA followed by Dunnett's multiple comparisons. Right panel shows total amount of vigilance states in percentages. Results for EYFP control mice: 37.1 ±2.8% for wake, 55.2 ±2.5% for NREM, 7.7 ±0.6% for REM. Results for ChR2 mice: 37.98 ±2.7% for wake, 55.9 ±2.3% for NREM, 6.1 ±0.8% for REM. Results for ArchT mice: 30.2 ±1.4% for wake, 62.3 ±1.4% for NREM, 7.6 ±0.4₂% for REM. No significant differences were measured using two-way ANOAVA with Dunnett's multiple comparisons.

c) Bout duration of NREM, REM and wake episodes during the recording after recall (left). Results for EYFP control mice: 30.1 ±1.8 seconds for wake, 55.9 ±5.4 seconds for NREM, 49.6±1.99 seconds for REM. Results for ChR2 mice: 34.97 ±7.7 seconds for wake, 52.98 ±3.9 seconds for NREM, 58.4 ±4.2 seconds for REM. Results for ArchT mice: 29.4 ±1.1 seconds for wake, 54.5 ±1.5 seconds for NREM, 59.1 ±2.5 seconds for REM. No significant differences were measured using two-way ANOVA followed by Dunnet's multiple comparisons. Right panel shows total amount of vigilance states in percentages. Results for EYFP control mice: 37.1 ±2.8% for wake, 55.2 ±2.5% for NREM, 7.7 ±0.6% for REM. Results for ChR2 mice: 37.98 ±2.7% for wake, 55.9 ±2.3% for NREM, 6.1 ±0.8% for REM. Results for ArchT mice: 30.2 ±1.4% for wake, 62.3 ±1.4% for NREM, 7.6 ±0.4% for REM. Two-way ANOAVA with Dunnett's multiple comparisons $F_{vigilance\text{-}state}$= 49, $F_{experimental\text{-}group}$= 0.8253, $F_{vigilance\text{-}state\ x\ experimental\text{-}group}$= 1.297, *P<0.05.

d) Quantification of stimulation applied to each state in recordings after acquisition and after recall, described as percentages with S.E.M. of total state duration. Stimulation in wake after acquisition: 3.8 ±0.6% in EYFP control mice, 3.98 ±1.1% in ChR2 mice, 3.1 ±0.4% in ArchT mice. Stimulation in wake after recall: 6.2 ±0.4% in EYFP control mice, 8.03 ±1.3% in ChR2 mice, 5.7 ±0.8% in ArchT mice. Stimulation in NREM after acquisition: 8.4 ±0.8% in EYFP control mice, 8.97 ±1.4% in ChR2 mice, 9.8 ±0.4% in ArchT mice. Stimulation in NREM after recall: 4.95 ±0.4% in EYFP control mice, 5.2 ±0.6% in ChR2 mice, 5.05 ±0.5 in ArchT mice. Stimulation in REM after acquisition: 1.8 ±0.7% in EYFP control mice, 3.6 ±0.9% in ChR2 mice, 3.6 ±0.98% in ArchT mice. Stimulation in REM after recall: 6.1 ±1.4% in EYFP control mice, 5.7 ±1.2% in ChR2 mice, 5.5 ±1.4% in ArchT mice. No significant differences were measured using one-way ANOVA with Dunnett's multiple comparisons. Note that stimulation applied to states other than NREM sleep during sleep after acquisition was accidental, as the stimulation was not interrupted in case of state transition (see methods).

e left) Densities of spindles during sleep after acquisition: 004 ±0.002 events/sec for EYFP control mice, 0.03 ±0.001 events/sec for ChR2 mice, 0.03 ±0.002 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA followed by Dunnett's multiple comparisons. On the right, results for sleep after recall: 0.04 ±0.002 events/sec for EYFP control mice, 0.04 ±0.002

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events/sec for ChR2 mice, 0.04 ±0.004 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA followed by Dunnett's multiple comparisons.

f left) Densities of slow waves during sleep after acquisition: 0.8 ±0.07 events/sec for EYFP control mice, 0.79 ±0.05 events/sec for ChR2 mice, 0.8 ±0.05 events/sec for ArchT mice. One-way ANOVA with Dunnett's multiple comparisons F=0.09. On the right, results for sleep after recall: 0.7 ±0.04 events/sec for EYFP control mice, 0.8 ±0.04 events/sec for ChR2 mice, 0.8 ±0.06 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA followed by Dunnett's multiple comparisons.

g left) Densities of vCA1 ripples during sleep after acquisition: 0.3 ±0.05 events/sec for EYFP control mice, 0.3 ±0.03 events/sec for ChR2 mice, 0.3 ±0.04 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA. On the right, results for sleep after recall: 0.4 ±0.05 events/sec for EYFP control mice, 0.3 ±0.03-events/sec for ChR2 mice, 0.3 ±0.04 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA followed by Dunnett's multiple comparisons.

h left) Densities of dCA1 ripples during sleep after acquisition: 0.4 ±0.05 events/sec for EYFP control mice, 0.4 ±0.1 events/sec for ChR2 mice, 0.4 ±0.04 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA. On the right, results for sleep after recall: 0.3 ±0.04 events/sec for EYFP control mice, 0.4 ±0.03 for events/sec ChR2 mice, 0.3 ±0.01 events/sec for ArchT mice. No significant differences were measured using one-way ANOVA followed by Dunnett's multiple comparisons.

4.General discussion and outlook

The nucleus reuniens (Nre) is positioned to play an important role in a wide range of behaviors and neurophysiologic functions. So far, it has been described in several aspects of working memory [416], [424]–[426], synchrony of sleep oscillations [412]–[415], [455], remote memory consolidation [407], [409]–[411] and stability of sequential neuronal firing in medial prefrontal cortex during slow oscillations as well as in hippocampus during sharp wave ripples [415]. We have provided with this project another piece to the puzzle to understand this thalamic region by showing its properties to synchronize cortical slow waves and spindles with hippocampal ripples.

To date, there is in my opinion enough data available to outline a few future directions for future research and experiments concerned with Nre. For one, its role in working memory and its role in regulating synchrony and communication between prefrontal cortex and hippocampus lend itself to the idea of a potential involvement in psychotic diseases such as schizophrenia which are marked by cognitive deficits that precisely rely on cortico-hippocampal and cortico-thalamo-cortical communication. Some of these deficits include general memory disturbances including working memory, executive functions, attention, and inhibition control, just to name a few [444]. As mentioned in the discussion of the results section, a reduced *adhesio interthalamica* which includes the Nre was described in patients suffering from schizophrenia as well as an overall reduced thalamic volume in relation to total brain mass [456]. Furthermore, other studies found alterations in the volume of Nre and its connectivity with cortex, reticular nucleus thalamic and hippocampus in humans suffering from known genetic risk factors [457] or in genetically modified animals [458], respectively. However, the data on cross-frequency coupling of neuro-oscillations in schizophrenic patients and animal models is rather limited: It's been demonstrated that spindle density in people with schizophrenia is reduced but the cortical coupling of spindles to slow waves is intact [459]. Additionally, a study with an animal model could show that ripple density and power are increased but that the replay during ripples is impaired [460]. Lastly, both cortical [461] and hippocampal [462], [463] theta-gamma coupling were found to be impaired in patients and animal models, respectively.

Taken together a few possible future inquiries present themselves. For one, cross regional coupling of cortical slow waves and spindles with hippocampal ripples during sleep could be investigated for possible alterations both in schizophrenic patients and in animal models, similar to our measurements that were presented in this thesis, including sleep after sleep deprivation and a hippocampus-dependent learning task. As for possible studies with humans, an interesting analysis could be added by correlating the strength of ripple-spindle and ripple-slow wave coupling with Nre volume or connectivity. Studies in animal models would offer the opportunity to examine the electrophysiologic coupling of a developmentally disturbed Nre with prefrontal cortex and hippocampus, similar to our measurements during sleep. It could already be demonstrated that Nre stabilizes neuronal sequences during sharp wave ripples [415] and that these sequences are disrupted in schizophrenic mouse models [460]. Thus, not only would it be worthwhile to investigate Nre's influence on neuronal sequences in prefrontal cortex, but it would also be interesting to conduct an experiment with a schizophrenic mouse

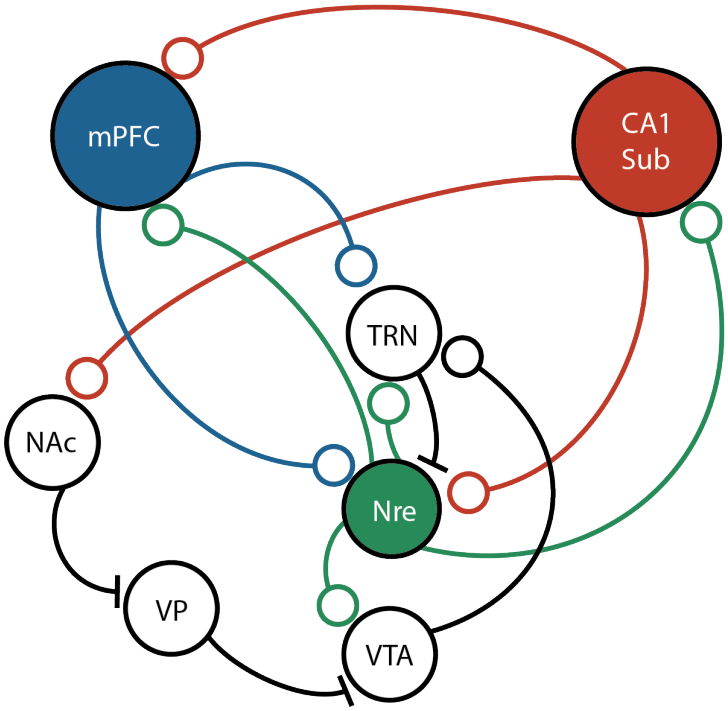

Figure 4.1 Potential circuit that is involved in the pathophysiology of schizophrenia

Hyperactivity in CA1/subiculum (CA1/Sub), which supposedly plays an important role in schizophrenia, leads to activation in the nucleus accumbens (NAc) and via the ventral pallidum (VP) to a dysfaciliation of the ventral tegmental area (VTA) which leads to higher delta frequency bursts in reticular nucleus of the thalamus, resulting in burst firing in nucleus reuniens (Nre) which closes the positive feedback loop. Additionally, proper function between TRN, Nre and medial prefrontal cortex (mPFC) could also be important in in hippocampal hyperactivity. Adapted form Dollemann-van der Weel & Witter 2020 [396].

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model and try to rescue the neuronal sequences during ripples either via optogenetic manipulation of Nre or administration of an antipsychotic drug while monitoring Nre activity. Whether Nre's role in schizophrenia would be via over- or underactivity is at this stage hard to say though: An old study in rats found higher c-Fos expression in midline thalamic regions after administration of antipsychotic drugs [464]. However, more recent studies paint a more complex picture. A hyperactive hippocampus has been hypothesized to be a key structure in the pathophysiology of schizophrenia. Hyperactivity might result from reduced inhibitory activity in the hippocampus, which results in hyperactive pyramidal cells and excessive glutamate release. This leads to excitotoxic damage to interneurons in the hippocampus, thus creating a vicious cycle. But further, this hyperactivity leads to increased excitation of dopaminergic neurons in the ventral tegmental area (VTA) via a polysynaptic path (CA1 → nucleus accumbens→ ventral pallidum→ VTA). This leads to more delta frequency bursts in TRN, which in turn promotes bursting in Nre that in turn projects to CA1 and thus might close a positive feedback loop. This is further supported by study which found that NMDA infusion into Nre increased overall number of active dopamine cells in VTA via the ventral subiculum which receives input from both CA1 and Nre [444]. A further study gave a bit different spin on this

model: It demonstrated that a schizophrenic mouse model where the proper development of TRN was inhibited also led to higher VTA activity and could be lowered by inactivation of infralimbic cortex, which led to lower Nre activity. On the other hand, when infralimbic cortex was inhibited in healthy control animals, VTA activity was increased again because cortical activation of TRN was diminished, which led to Nre disinhibition [465]. Thus, precise interaction between cortex, TRN and Nre and its influence on the hippocampus via tonic or burst firing offer a lot of potential in furthering our understanding of the pathophysiology of schizophrenia.

The second and last future direction worth pointing out is memory consolidation. There are now several studies which link Nre to consolidation of remote memories, i.e. memories that are 20 or more days in the past [407], [409]–[411]. Thus, it may play a crucial role in the *systems memory consolidation* hypothesis. The fact that inactivating Nre during a crucial time window or lesioning it completely disrupts memory performance after several days, points to a potential involvement in *corticalization* of memory engrams and the process of becoming independent of the hippocampus. Possible experiments could involve tracking the stability of neuronal sequences during replay in both hippocampus and cortex in relation to unperturbed Nre activity. Further, the effects of lesions or repeated perturbation of Nre activity on replays could be tracked across several days and correlated to memory performance.

In this context, the consolidation of recent memories deserves further attention. Even though two other studies and ours got negative results, there are still open questions: For one, the other two studies had some potential weaknesses. One study [406] inhibited Nre after acquisition using muscimol. The problem there is that the effectiveness of that compound is not quite clear anymore after a few hours, thus opening a potential time window where compensatory mechanisms can occur. The other [410] lesioned Nre before the learning task permanently, thus disturbing proper acquisition and possibly triggering alternate anatomical pathways as a compensation. Our study has weaknesses in its stimulation and silencing protocol as well, which was described in the results section. Thus, possible future experiments should ideally include a closed loop system with which one could optogenetically perturb Nre's activity upon every UP state during NREM sleep, as we could demonstrate that Nre's shows increased spike activity before a ripple which likely corresponds to the UP state. Importantly, future experiments would ideally cover the entirety of NREM sleep after acquisition of a memory until recall.

To conclude, I hope I could inspire the interested reader and demonstrate the fascinating feature of the brain and the thalamus, specifically, to perform a wide arrange of tasks during different states and in different behavioral situations. It is interesting to consider how man's understanding of the thalamus and sleep have developed over the decades: sleep used to be a passive state of energy preservation and is now considered active with many functions being almost exclusively performed during this state The thalamus used to be a relay station of sensory input to the cortex, now we know that it also plays a role in generation of sleep oscillations, cognition, and emotion. A similar development awaits man's understanding of nucleus reuniens, as so far only the surface has been scratched concerning its role in cognition, memory, and neuro-oscillations.

Ivan Božić, September 2022

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