Year : 2024

Neural circuits and mechanisms of transitions in vigilance states

Broglia Gianandrea

Broglia Gianandrea, 2024, Neural circuits and mechanisms of transitions in vigilance states

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_134594B07C044

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département des Sciences Biomédicales

Neural circuits and mechanisms of transitions in vigilance states

Thèse de doctorat en Neurosciences

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Gianandrea Broglia

Master of Science in Neuroscience, University College London, UK

Jury

Prof. Anita Lüthi, Présidente
Prof. Mehdi Tafti, Directeur de thèse
Dr. Mojtaba Bandarabadi, Co-directeur de thèse
Prof. Vladyslav Vyazovskiy, Expert
Prof. Denis Burdakov, Expert

Thèse n° 396

Lausanne
(2024)

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

LEMANIC NEURO SCIENCE DOCTORAL SCHOOL

UNIL | Université de Lausanne

UNIVERSITÉ DE GENÈVE

UNIL | Université de Lausanne

UNIVERSITÉ DE GENÈVE

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Madame	Prof.	Anita Luthi
Directeur·trice de thèse	Monsieur	Prof.	Mehdi Tafti
Co-directeur·trice de thèse	Monsieur	Dr	Mojtaba Bandarabadi
Expert·e·s	Monsieur	Prof.	Vladyslav Vyazovskiy
	Monsieur	Prof.	Denis Burdakov

---

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Gianandrea Broglia

Titulaire d'un Master en Neurosciences,
University College London, UK

intitulée

Neural circuits and mechanisms of transitions in vigilance states

Lausanne, le 19 janvier 2024

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Anita Luthi

¿Qué es la vida? Un frenesí.
¿Qué es la vida? Una ficción,
una sombra, una ilusión,

y el mayor bien es pequeño;
que toda la vida es sueño,
y los sueños, sueños son.

Pedro Calderon de la Barca, 1635

Contents

Acknowledgement

My profound gratitude to my supervisors Prof. Mehdi Tafti and Dr. Mojtaba Bandarabadi who made this thesis possible by critically guiding my projects. Thanks also for their kind and unlimited support during these years of investigative research life. Similarly, all my appreciation for sharing their incredible and vast knowledge of the scientific world of sleep research.

A special thank also to the president and the expert members of this thesis, Prof. Anita Lüthi, Prof. Vladyslav Vyazovskiy and Prof. Denis Burdakov who accepted to be involved in this project and enhanced the level of my engagement to the research work.

Thanks to all the Tafti's lab members who were present during these years. In particular to Mme Anne-Catherine Thomas who took care of genotyping our experimental lines, to Mme Corinne Pfister who managed all the lab furniture and equipment, and to Dr. Ali Seifinejad who taught me how to score EEG/EMG data for polysomnography. Another important person that I would like to acknowledge is Dr. Anne Vassalli who was always present with her beautiful and very on-point insights on lab investigative issues, overall on genetics. Moreover, I would like to thank Dr. Pierre-Hugues Prouvot-Bouvier for his firm kindness in helping in every possible situation, Dr. Giorgio Corsi for his high-level knowledge and technique, Prof. Stamatina Tzanoulino for her strong academical presence, Dr. Meriem Haddar, Dr. Li-Yuan (Debby) Chen, Dr. Mergim Ramosaj, Dr. Marie-Laure Possovre, and Dr. Sha Li for their dedication and passion on work and for some few nice break-times during the working-day.

Also special thanks to my former mentors Prof. Andrew Batchelor, Prof. Stephen Price, Prof. Per Roland, Prof. Davide de Pietri-Tonelli, Dr. Jakob Neef, and Dr. An Dau.

In addition, great thanks to Prof. Pierre-Hervé Luppi, to Prof. Christelle Peyron, Prof. Luis de Lecea and Prof. Antoine Adamantidis for their comments and suggestions on the ongoing work.

Lastly, GRAZIE, to my friends, my family, my beloved who had been around ever since and that are the persons to whom I dedicate the passion I put on this work.

To the loving memory of my father.

Summary

Long-term recordings have revealed that vigilance state transitions are not stochastic processes, but rather involve deterministic substrates, allowing the brain to maintain stable sleep-wake patterns and regulate the number and duration of episodes in a given state across day and night. The hypocretin system plays a critical role in the regulation of the sleep-wake cycle in the current theoretical models, e.g., the flip-flop switch model. However, sleep-wake cycling still operates in patients with narcolepsy and in animal models of narcolepsy with hypocretin deficiency. How the transitions emerge in the absence of hypocretin and how these are related to clinical conditions (narcolepsy with cataplexy) is unclear. To untangle this controversy, we investigated the circuit mechanisms of spontaneous transitions in vigilance states in wild-type mice and a mouse model of cataplexy with loss of the two hypocretin neuropeptides (*Hcrt*^{KO} mice). Several important observations were made in relation to the activity of wake-promoting and sleep-generating nuclei, which are important in determining sleep architecture and the physiological interplay between vigilance states.

State dynamics and coordination of multiple brain sites during transitions were also analyzed in depth and a novel and unexpected scenario was drawn from the results of the experiments. Importantly, electrophysiological components of the sleep EEG were strongly affected by our manipulations, as was the intrinsic firing activity of several components of the brain sleep networks. This work highlights novel findings that will broaden the comprehension of the neural circuitries implicated in sleep generation and arousal maintenance and would be of importance to further expand the knowledge on the regulatory mechanisms of hypocretin that has been accumulated since the discovery of this incredible neuropeptide 25 years ago.

Résumé

Des enregistrements à long terme ont révélé que les transitions entre les phases du sommeil ne sont pas des évènements stochastiques, mais impliquent plutôt des substrats déterministes permettant au cerveau de maintenir des schémas veille-sommeil stables et de réguler le nombre et la durée des épisodes dans une phase donnée, de jour comme de nuit. Le système hypocrétinergique joue un rôle essentiel dans la régulation du cycle veille-sommeil dans les modèles théoriques actuels, par exemple dans le modèle de l'interrupteur à bascule (flip-flop switch). Cependant, le cycle veille-sommeil fonctionne toujours chez les patients atteints de narcolepsie et dans les modèles animaux de narcolepsie présentant un déficit en hypocrétine. La façon dont ces transitions émergent en l'absence d'hypocrétine et comment elles sont liées aux affections cliniques (narcolepsie avec cataplexie) n'est pas claire. Afin de clarifier ces questions, nous avons étudié les mécanismes des circuits de transitions spontanées entre les états de vigilance chez des souris de type sauvage et dans un modèle murin de cataplexie avec perte des deux neuropeptides hypocrétines (souris $Hcrt^{KO}$). Nous avons ainsi pu faire plusieurs observations importantes concernant l'activité des noyaux impliqués dans la détermination de l'architecture du sommeil et l'interaction physiologique entre les états de vigilance.

Nous avons également étudié en profondeur la coordination de plusieurs noyaux cérébraux au cours des transitions et un scénario nouveau et inattendu a émergé de nos résultats. Il est important de noter que les caractéristiques de l'electroencephalogramme du sommeil ont été fortement affectés par nos manipulations, tout comme l'activité intrinsèque de plusieurs composants du réseau cérébral du sommeil. Ce travail souligne de nouvelles découvertes en mesure d'élargir la compréhension des circuits neuronaux impliqués dans la génération du sommeil et le maintien de l'éveil et sont importants pour élargir davantage les connaissances sur les mécanismes de régulation de l'hypocrétine et qui ont été accumulées depuis la découverte de cet incroyable neuropeptide il y a 25 ans.

1. Introduction

1.1. Vigilance states

Vigilance states, also known as arousal states, refer to the different levels of wakefulness and alertness that an organism experiences [1, 2]. These states reflect a continuum of consciousness and attentiveness, ranging from full wakefulness to deep sleep. Humans and animals show different identifiable vigilance states because of brain cortical rhythms, muscular tone, behavioral and expressed physiological engagement [3]. In humans, as in laboratory rodents, sleep is divided into two main categories of non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) [4]. Various techniques including electroencephalography (EEG), electrooculography (EOG), and electromyography (EMG) are used to identify sleep stages by inspection and detection of specific criteria, such as changes in EEG activity, eye movements, muscle tone, or heart rate [2].

The regulation of sleep involves complex interactions between neural circuits, neurotransmitters, neuropeptides, and various physiological and environmental factors [5]. The brain contains multiple wake-promoting and sleep-generating areas that are located in different regions. Brain structures like the hypothalamus and the brainstem play a critical role in controlling sleep-wake cycles in rodents and humans [6, 7]. In the hypothalamus, hypocretin (orexin) neurons are particularly important for promoting wakefulness and sleep-wake transitions [8-10]. Furthermore, the reticular activating system (RAS), which is located in the brainstem and consists of several aminergic and cholinergic nuclei, is important for promoting wakefulness and arousal and for regulating transitions in vigilance states [11]. Another important brain structure in controlling vigilance and determining state transition is the thalamus [12]. The thalamo-cortical system can generate and propagate rhythms at the level of the cortex and the most important nuclei related to this function are the sensory relay nuclei, the midline nuclei and the thalamic reticular nucleus [13, 14].

1.1.1. Brief history of sleep research

Baron Costantin Von Economo was the first to provide evidence in 1917 for the existence of the sleep centers in the brain [15, 16]. A pandemic (*encephalitis lethargica*) spread in Europe for almost a decade in the first quarter of the 19th century, and affected people developed severe hypersomnia and rarely insomnia. Based on postmortem examinations of these patients, Von Economo postulated a sleep-generating area in the anterior hypothalamic region and a wake-promoting area in the posterior hypothalamus. Indeed, histological analysis confirmed that patients who developed insomnia had inflammatory lesions in the anterior preoptic hypothalamus, whereas in patients who developed hypersomnia symptoms, the lesioned tissue was found in proximity of the posterolateral hypothalamus.

Another essential contribution to sleep research in neuroscience came in 1924 when Hans Berger, a German psychiatrist, recorded electrical activity from the human scalp using his invention, electroencephalography [17]. Berger showed differences in brain rhythms when individuals were awake or asleep, where NREMS EEG signal is characterized by synchronized high-amplitude slow waves, while wakefulness is primarily characterized by desynchronized low-amplitude fast waves. He was also the first to use Greek letters to classify the different frequency bands that were related to different behaviors. EEG is at the basis of nowadays predominantly used polysomnography, which is used as a screening technique of different physiological parameters and thus permits to investigate and define vigilance states.

In the beginning of 1950, Nathaniel Kleitman's group recorded the eye motility of subjects using electrooculography over the whole night and noticed sporadic bursts of EOG activity during sleep. Because of this sustained and unexpected ocular activity, they hypothesized that a new sleep state existed and coined the name REMS [18]. The progressive mutual alternations between sleep stages, known as sleep architecture, are very peculiar since the succession and length of each sleep state are extremely similar in all healthy subjects [19]. Sleep architecture

abnormalities are detrimental for an individual, as sleep could not be restorative or permit recovery of cellular homeostatic functions, and sleep could intrude at moments of the day when it could be dangerous to lose awareness (e.g., while driving) [20]. Therefore, clinical conditions where sleep architecture is defectively regulated are a burden for the health of individuals and society, and their causes are currently under investigation to prevent and cure these adverse conditions [21-25].

1.1.2. Functions of sleep

The function of sleep is complex and multifaceted, and it continues to be the subject of extensive scientific research. While several important theories on the role of sleep have been proposed, the real purpose of this physiological state is still largely debated. However, a universally accepted and fundamental function of sleep is mental restoration and physical recovery [26]. During sleep, the body undergoes various biomolecular processes to repair cells and maintain tissues, in addition, immune system activity is enforced [27]. Conversely, inflammation or infection are capable of influencing sleep patterns, building up sleep pressure, or creating arousal disturbances such as sleepiness [28]. At the brain level, sleep helps to clear metabolic waste products inside and outside of cells and also to remove the excess of neurotransmitters in the extra-synaptic space, which is important for the efficient communication of neural circuits [29].

Sleep is also indicated to have a prophylactic action on neuronal activity. If neurons are active for extended periods of time, they will accumulate reactive oxygen species that could be tolerated over a certain amount, however when they reach a threshold, oxidative stress could damage cells and even cause apoptosis. To prevent this oxidative damage, neurons will reduce their firing and induce a state of slow-wave activity (SWA) to allow waste-clearing mechanisms to sustain their function [26, 30]. Related to this preventive function of sleep, recently Maiken

Nedergaard group discovered presence of the glymphatic system in the brain [31]. This system is composed of an astrocyte network mostly active when neurons are oscillating in SWA, therefore the glymphatic system is strongly active during NREMS [32]. Astrocytes of the glymphatic system direct flow and volume of cerebrospinal fluid around the brain and this permits the removal of cellular debris, metabolic by-products and waste substances like β-amyloid plaques that accumulate in the interstitial space and that, if not removed, could be noxious for neurons [33].

Sleep may also have an evolutionary function by conserving energy during periods when it is less beneficial to be active [34]. Since sleep is a reversible condition of reduced responsiveness usually associated with immobility, it clearly allows animals to spare energy during non-efficient periods of the day. Consequently, this creates a chance to perform vital behaviors at the most convenient times when animals have their stores of energy replenished and are prompt to use them. Rotation of the Earth every 24 hours exposes living animals to a daily oscillation in light and temperature, but sleep is present in nearly all animals, and a large variation of this behavior exists across species. This high variation could be best explained by adaptation to species-specific ecological, ethological, and energetic demands [35]. For example, humans that strongly rely on vision to perceive and explore the environment are a diurnal species, since at night they would be at a disadvantage by not using this primary sense. Furthermore, if animals sleep in a safe and hidden refuge, this protects them from the risk of possible predation. At present, there are different studies on sleep that are carried out on simpler animal models, including fruit flies (Drosophila melanogaster), worms (Caenorhabditis elegans) and fish (Danio rerio) [36, 37]. Prolonged sleep deprivation eventually has a fatal outcome in rats (Rattus norvegicus), fruit flies and worms [38, 39]. In all cases, the exact cause of lethality is controversial and until now not completely clear, however, because of this extreme

consequence, it is evident that sleep is necessary for the vital functions of living organisms, both vertebrates and invertebrates.

Considering that sleep or sleep-like states are conserved across diverse species and at different orders of the evolutionary scale, from a biological perspective, it could be argued that this vigilance state fulfills a common need that could reside at the cellular level because of the cycling in the expression of genes and proteins and therefore in the intrinsic alternation of activity, growth and expansion versus recovery, healing and conservation that every cell necessarily undergoes [40]. Nevertheless, thanks to neuroscientific studies on animal models, new contributions to sleep research are constantly produced, and the most evident results suggest that sleep function should also be related to energy regulation, memory consolidation, clearance of brain metabolites, dendritic spine remodeling (synaptic homeostasis), and brain plasticity and development [31, 41-44].

1.1.3. Sleep in rodents

Mice are an exceptional model for investigations of sleep-wake neural circuitry. Mice are amenable to genetic manipulation, allowing researchers to create transgenic or knockout animals to study the role of specific genes in sleep-wake regulation [45-47]. In addition, mouse Cre-lines are a powerful tool when coupled to the delivery of cell-specific adeno-associated virus (AAV) vectors carrying neuronal activity fluorescent reporters, such as genetically encoded calcium indicators (GECI) or neurotransmitter-sensing reporters [48-51]. The neuroanatomy and neurochemistry of sleep regulation in rodents share many similarities with humans. Key brain structures and neurotransmitter systems involved in sleep-wake regulation are conserved across species. Indeed, the same brain areas are found to be active or inactive at identical vigilance states in both species.

However, many differences also exist. For example, mice are nocturnal animals and are prey in nature, therefore they show a preferred sleep time during the day and a strong fragmentation in sleep architecture [52]. Another difference is that in humans, it is possible to distinguish NREMS in at least three different substages, while NREMS is usually classified as a unique state in rodents. In mice, duration of a sleep cycle is typically about 10 minutes and in human it lasts about 90 minutes. Moreover, mice have a polyphasic sleep that consists of a continual alternation of sleep and wakefulness during the whole resting period of the day, instead humans tend to have a monophasic sleep every night and, once they are aroused, they are habitually active until the next sleep bout [53]. Despite these discrepancies, rodents and humans share many EEG and regulatory mechanisms of the wake-sleep cycle, and since the already mentioned possibility in these animals of high-invasive recording of brain circuits exists, many experiments are ongoing to foster our knowledge on vertebrate sleep and on their regulatory mechanisms.

Rodents sleep comprises two states of NREMS and REMS that alternate cyclically across a sleep episode. During NREMS, mice and other mammals experience changes in physiological parameters, including reduced heart rate, muscle tone, blood pressure, respiration rate, and core body temperature. NREMS is mainly composed of high-amplitude synchronous cortical delta activity (1-4 Hz) [4]. Higher delta power indicates deeper and more synchronized sleep, and when this rhythm intrudes during wakefulness, it can be used as a quantitative measure of sleepiness and sleep need [54, 55]. During NREMS, the EEG contains thalamo-cortical spindles (11-15 Hz), which are brief waxing and waning bursts of rhythmic high-frequency activity superimposed on the ongoing EEG, and generally last for almost a second [56, 57]. REMS is characterized by sustained theta (6-10 Hz) oscillations and the absence of muscle tone (atonia) with brief movements (twitches) [58, 59].

1.2. Regulation of sleep-wake cycle

A pattern of natural sleep consists of a transition of the animal from an awake state to NREMS and later, when NREMS is consolidated, a transition to REMS can occur. A typical REMS event usually terminates with an awakening in many species. Apart from the full transition to wakefulness after a sleep session, a particular class of brief awakenings called microarousals occurs during NREMS, where the animal is not behaviorally active but the EEG and EMG show, for a few seconds, activity that resembles the state of wakefulness [60]. The alternation, the amount and the quality of NREMS and REMS could be altered by many different experimental variables. Among them are sleep debt because of previous sleep deprivation, experimental manipulation, or even genetic, circadian, metabolic, pharmacological, environmental, developmental and immune related factors [61, 62].

Spontaneous transitions between sleep stages and wakefulness are frequent, reversible, and relatively fast, and different factors contribute to these state switches. The first scientific model that aimed at explaining transitions between behavioral states was proposed by Alexander Borbély, called the “two processes model” [63, 64]. In this model, two processes are taking place: process C and process S. The body's internal biological clock, known as the circadian system (process C), regulates the sleep-wake cycle at the physiological level. This circadian rhythm is controlled by the expression of different clock genes and their interaction in a feedback loop system that occurs in every cell of the body. The anatomical site of this mechanism, however, resides in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is referred to as the master circadian clock of the whole organism [65, 66]. Homeostatic sleep drive (process S) can induce sleep because of the accumulation of different somnogenic substances in the brain and body that, over a certain threshold, induces or even forces the animal to fall asleep [67-70]. This model emphasizes the role of sleep pressure and sleep homeostasis in sleep transitions. However, among other models, it has been proposed that sleep could be

controlled by brain neural networks that are capable of dictating vigilance state maintenance and transitions. In these models, the interactions between different brain areas and neuromodulatory systems are involved in controlling sleep-wake cycling patterns [71].

1.2.1. Wake-promoting pathways

In 1949, two neurophysiologists, Giuseppe Moruzzi and Horace W. Magoun found that when stimulating the rostral tegmental part of the pons in the brainstem, animals under experiment suddenly awoke from sleep or anesthesia, and strong cerebral activity and behavioral motion were induced [72]. Conversely, when this area is lesioned or its projections are transected, animals fall irreversibly into a comatose state [73]. They named this area the ascending RAS of the brainstem, which comprises several monoaminergic nuclei. Among them are noradrenergic neurons (NA) of the locus coeruleus (LC), serotonergic neurons (5-HT) of the dorsal and medial raphe (DR) nuclei, and dopaminergic neurons from the ventral tegmental area (VTA) and substantia nigra [7].

Neurons of the RAS send ascending projections to the forebrain along two major pathways (Fig. 1). The first, dorsal, extends throughout the thalamus, and the second, ventral, passes through the hypothalamus and reaches the basal forebrain (BF) and the cortex. The dorsal pathway enables thalamic processing of signals related to sensation, motor responses, and cognition. In contrast, the ventral pathway is mostly important to produce a state of behavioral wakefulness, as demonstrated when experimentally lesioned [2]. The consequences of a lesion to the ventral pathway could result in animals or subjects being unable to stay awake and experiencing hypersomnolence. These nuclei receive reciprocal connections that allow them to act in an orchestrated manner and consequently induce wakefulness because of their simultaneous activation. Thanks to this direct interconnectivity between subcortical and brainstem areas, they

can enhance and regulate RAS overall tone and maintain aroused and awakened states of the organism [11].

Apart from monoaminergic nuclei, the RAS contains cholinergic neurons of the laterodorsal tegmentum (LDT) and pedunculopontine tegmentum (PPT), and glutamatergic cells located in the parabrachial nucleus and pre-coeruleus nucleus, which are fast-acting neurons that are readily able to promote wakefulness because of their direct projections to the forebrain areas. Important to mention, these nuclei are also known to actively participate in REMS generation and maintenance [11]. Another wake-promoting area is the tuberomammillary nucleus (TMN), located in the posterior hypothalamus, which releases histamine and can promote wakefulness and arousal [74].

At the cortical level, wakefulness is defined as an overall fast and desynchronized low-amplitude activity at the EEG level, with a predominant presence of theta, alpha, beta, and gamma rhythms [4, 30]. Various regions of the cerebral cortex are involved in maintaining wakefulness. These include the prefrontal cortex, which is crucial for executive functions and decision-making. Parietal, temporal and occipital areas instead, which are involved in sensory processing and perception, are important for perceptual awareness [75]. The basal forebrain is another structure that also contributes to cortical activity during wakefulness. It contains cholinergic neurons that release acetylcholine, a neurotransmitter that plays an important role in attention and wakefulness, and these neurons have wide projections to the cortex and contribute to cognitive processes [76].

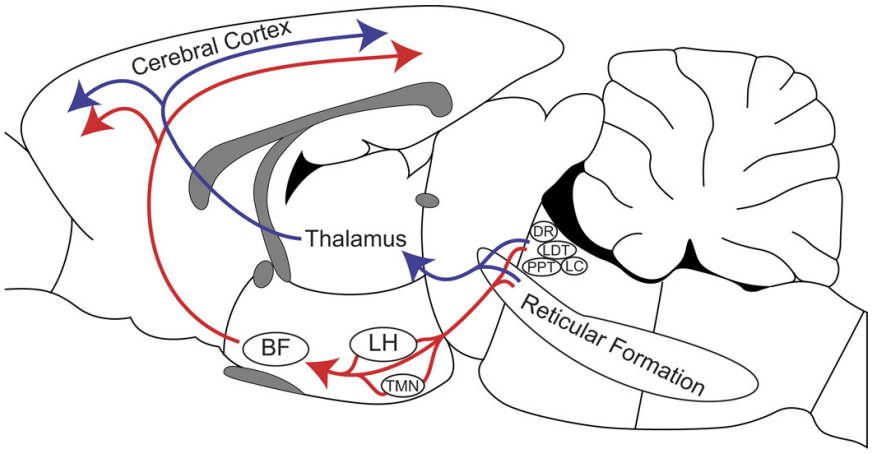


Fig. 1. Dorsal and ventral pathways of the reticular ascending system (RAS). The dorsal pathway is mostly implicated in bottom-up sensory processing, while the ventral pathway is important for generating arousal tone. Noradrenergic, serotonergic and cholinergic neural projections form these pathways. DR, dorsal raphe; LDT, laterodorsal tegmentum; PPT peduculopontine tegmentum; LC, locus coeruleus; LH lateral hypothalamus; TMN, tuberomammillary nucleus; BF, basal forebrain. From Brown et al. [2].

1.2.2. Sleep-generating pathways

The most important sleep-generating areas of the brain are the VLPO and the median preoptic nucleus (MnPO) of the hypothalamus (Fig. 2) [77]. VLPO is located at the base of the brain, lateral to the optic chiasma and could be distinguished into two components. The first component is called the VLPO cluster, usually referred to as VLPO, and the other is the extended VLPO (eVLPO) (Fig. 3) [78]. Firing rate of VLPO neurons is almost doubled during sleep compared to waking and is even higher during recovery sleep after sleep deprivation [79]. Other experiments in which lesions of this area were performed have shown the importance of the VLPO in orchestrating sleep. Loss of VLPO cluster neurons produced a strong reduction in NREMS, but did not correlate with loss of REMS [80]. Instead, lesions of the eVLPO correlated with decreased total REMS but not with a lower amount of NREMS [80]. In line with these results, when rats were exposed to a period of darkness during the day, a condition that doubled the total REMS time, a concomitant increase in the activity of the eVLPO was observed [78]. These observations suggest that the VLPO contains specific subregions that can promote REMS or NREMS distinctively, namely the eVLPO and the VLPO cluster.

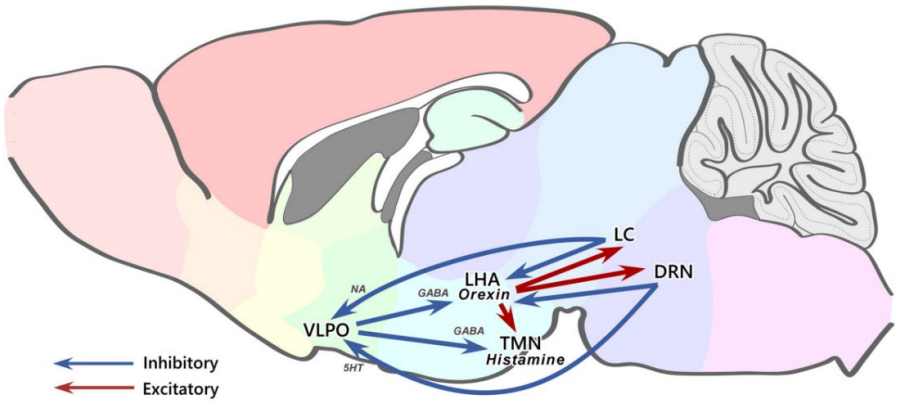


Fig. 2. Inhibitory and excitatory connections between wake-promoting and sleep-generating areas. The inter-play of these neurons can produce a state of wakefulness or generate sleep. Interconnectivity is usually composed of excitatory-inhibitory feedback loops. (VLPO, ventrolateral preoptic neurons; LHA; lateral hypothalamus; TMN, tuberomammillary nucleus; DRN, dorsal raphe nucleus; LC locus coeruleus). From Saper et al. [81].

The VLPO and MnPO have sleep-active GABAergic neurons, which also produce the neuropeptide galanin. The VLPO typically exhibits an increase in activity at spontaneous wake to sleep transitions and has a progressive enhancement of firing activity from light to deep NREMS. In MnPO neurons, an increase in the marker gene for neuronal activation, c-Fos, has been documented in GABAergic neurons after a period of sleep deprivation, suggesting that this area can accumulate sleep pressure and then dissipate it during the sleep rebound [82]. VLPO neurons are excited by adenosine, which accumulates extracellularly during sustained wakefulness [83]. Adenosine has been defined as a somnogenic substance and is therefore intimately linked with sleep pressure. The VLPO and MnPO innervate strongly and inhibit arousal-promoting brain regions such as LC and DR monoaminergic cells, BF cholinergic neurons, and LH hypocretinergic cells [84]. Conversely, they receive feedback inputs from the same projecting areas, which in turn can inhibit VLPO and MnPO [85]. Thus, VLPO and MnPO sleep-active neurons inhibit arousal systems, and *vice versa*.

Of importance, since no hypocretin receptors are reported in sleep-active VLPO galaninergic neurons, it seems that hypocretin cells can modulate neural activity in this area of the hypothalamus indirectly through projections on neighboring VLPO GABAergic cells.

Therefore, VLPO GABAergic neurons are excited by hypocretin afferents, which in turn inhibit VLPO galaninergic neurons, which are sleep-generating [86]. Lastly, in the LH, intermingled with hypocretin neurons, is another class of neurons expressing the peptide melanin-concentrating hormone (MCH) [87, 88]. The majority of MCH neurons also produce the inhibitory neurotransmitter GABA. A role for MCH neurons in orchestrating REMS is proposed. Optogenetic activation of MCH neurons during NREMS was found to promote transitions to REMS, and stimulation during REMS prolonged REMS episode duration [89]. Instead, optogenetic silencing of MCH neurons had no effects on REMS duration, thus indicating that activity of MCH neurons is sufficient but not necessary to promote REMS [89].

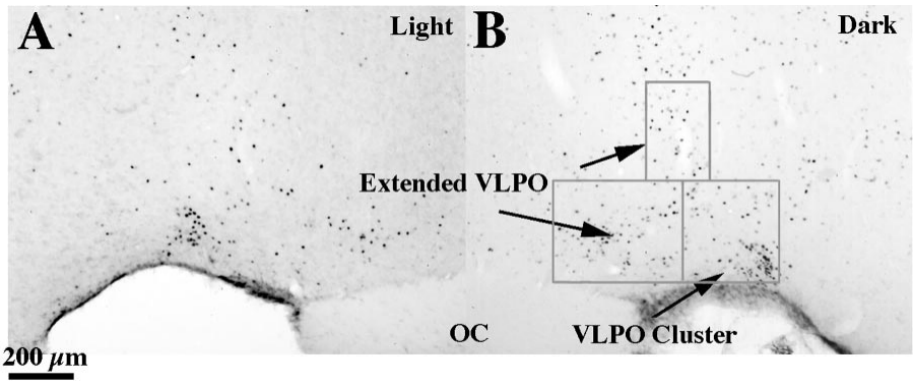


Fig. 3. Extended and cluster VLPO. eVLPO resides more medial and more dorsal to the cVLPO. (VLPO, ventrolateral preoptic area). From Lu et al. [78].

1.2.3. The flip-flop switch model of sleep

Among the different models proposed to explain transitions between and permanence within vigilance states, the flip-flop model was introduced a few years after the discovery of hypocretin neurons by the Boston group of Prof. Clifford Saper and colleagues (Fig. 4) [81, 90, 91]. This model aims at explaining the presence of a particular vigilance state and the non-coexistence of any other state at the same time [81, 92, 93]. In this model, mutual inhibition between sleep-generating and wake-promoting pathways can lead the organism into one of these two states. Dysfunction within these pathways could give rise to sleep and/or wake abnormalities and potentially uncontrolled transitions.

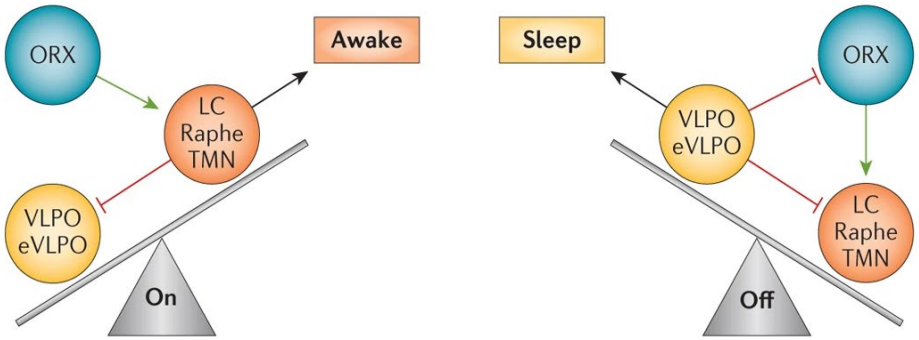


Fig. 4. Flip-flop switch model with the interplay of its wake-promoting and sleep-generating nuclei. Orexin neurons are external to the two antagonist sides and their role is to help maintaining the system in the awake state. (TMN, tuberomammillary nucleus; LC, locus coeruleus; VLPO, ventrolateral preoptic area; eVLPO, extended ventrolateral preoptic area; ORX, hypocretin neurons). From Morin et al. [94].

The name flip-flop circuit is taken from electronic engineering vocabulary. In the original terminology, these circuits have two stable states where they can function and process information. Switches between states are rapid and complete once triggered. In sleep research, the flip-flop model is composed of the interactions between reciprocal neuronal networks in which wake-promoting and sleep-generating pathways inhibit each other when active, and their interplay enables vigilance state shifts, thus when one of the two components prevails over the other, stable wakefulness or sleep is achieved.

In the sleep flip-flop network, the side of the sleep-generating components is represented by the VLPO and the MnPO. The other side, mutually in opposition with the first, is composed of wake-promoting areas such as LC, DR and TMN. Axons from the VLPO intensely project to cell bodies of the TMN, DR and LC, and, as already mentioned, the majority of VLPO neurons are GABAergic or galaninergic, therefore they are inhibitory to these nuclei. The relationship between the VLPO and the major monoaminergic groups of the brainstem appears to be reciprocal. In fact, the VLPO is innervated by noradrenergic terminals from the LC and receives serotonergic fibers from the DR, and it was demonstrated in hypothalamic slices that NA and 5-HT can inhibit VLPO neurons [84]. Furthermore, the VLPO is also contacted by the wake-promoting histaminergic axons that also inhibit its firing activity [95].

A fundamental aspect of the flip-flop switch is that if one of the components is mildly weakened, there is a less tendency for the network to remain on the side to which the weakened component belongs. Furthermore, this condition could determine a quick, frequent, and not requested shift between states when the animal should instead be firmly in one of them. To prevent this flickering situation of quick and unwanted switches, other components that secure and stabilize the circuit should be included. Hypocretin neurons are a perfect candidate for this role. Since hypocretin neurons send projections and increases the firing rate of LC, DR and TMN, they make wakefulness more stable and are not affected by the improvised drop in tonic activity of the RAS. Furthermore, hypocretin cells receive inhibitory projections from the VLPO and conversely, hypocretin neurons can silence VLPO neuronal firing by exciting a local inhibitory microcircuit that in turn suppresses sleep-generating VLPO galaninergic cells [86]. Therefore, hypocretin neurons are considered the stabilizing element of the flip-flop circuits, which can prevent abrupt transitions, especially from the wakefulness state.

To summarize, the two halves of the network (wake-promoting and sleep-generation nuclei) are strongly inhibiting each other, therefore creating a feedback loop that is bi-stable, with only two steady positions and a tendency to avoid intermediate conditions because of the stabilizing activity of the third external component: the hypocretin system.

1.3. The hypocretin system

The posterior lateral and perifornical hypothalamus contain a class of neurons that produce the hypocretins (orexins) neuropeptides (Fig. 5). These neurons are crucial for the maintenance of wakefulness, sleep/wake regulation, and vigilance state switching [10, 96, 97]. There are approximately 5,000 hypocretin neurons in the rodent brain and 50,000 in the human brain, and their existence was discovered by two independent groups more than 25 years ago. The group of Prof. Yanagisawa, using a G-protein cell receptor (GPCR) surface assay on cultured cell

lines to screen for orphan receptors, found two novel GPCRs and their correspondent binding neuropeptides [98]. They named the new peptides orexin-A and orexin-B as they appeared to promote feeding (from Greek *orexis,* appetite). Prof. Sutcliffe and his colleagues used a PCR subtractive hybridization method to construct a hypothalamic cDNA library of the rat brain and found the same neuropeptides, but named them hypocretin-1 and hypocretin-2 (Hcrt1, Hcrt2) because of their hypothalamic location and structural similarities with the secretin peptide family [99]. Hypocretin neurons are glutamatergic and also co-express a number of other neuropeptides, including dynorphin, neurotensin and proenkephalin [100].

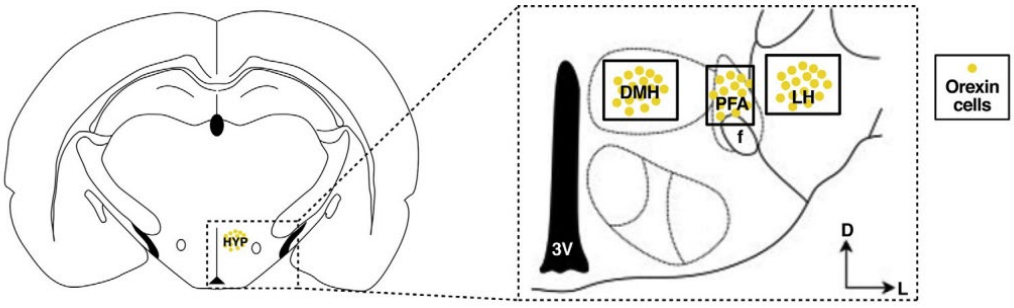


Fig. 5. Anatomical mediolateral subdivision in the hypothalamus of the hypocretin neurons. (DMH, dorsomedial hypothalamus; PFA, perifornical hypothalamus; LH, lateral hypothalamus; f, fornix; 3V, third ventricle). From Harris et al. [101].

There are two hypocretin GPCRs named HcrtR1 and HcrtR2 (or OX1R and OX2R). HcrtR1 has a higher affinity for Hcrt1 over Hcrt2 by an order of magnitude, whereas HcrtR2 displays equal affinity for both neuropeptides [102]. These receptors are expressed at different levels across the brain, for example, HcrtR2 is abundantly expressed in the histaminergic neurons of the TMN, whereas HcrtR1 is highly expressed in noradrenergic cells of the LC (LC^{NA}) [103]. The DR and VTA contain both receptor types [104].

Right after the discovery of the hypocretin/orexin neurons, Yanagisawa and colleagues produced a mouse model deficient in the hypocretin gene ($Hcrt^{KO/KO}$) [105]. They investigated behavioral and polysomnographic phenotypes in hypocretin knockout mice and discovered that this line suffered intermittent attacks of atonia during wakefulness. At the EEG level, during

the attacks, they displayed consistent wake-like activity, therefore it was suggested that these mice have cataplexy episodes. The same year, the Stanford group found that mutations in hypocretin receptor 2 cause canine narcolepsy [106]. Accumulating evidence led the scientific community to consider the absence of hypocretin and/or failure in signaling of the hypocretinergic system as the main cause of human narcolepsy [107].

Hypocretin neurons project widely and target many arousal-regulating regions (Fig. 6). Several of these regions also send reciprocal projections back to hypocretin neurons, potentially participating in feedback loops. Hence, the hypocretinergic system projects densely to the monoaminergic nuclei of the RAS, and these projections are important to increase the firing activity of the RAS and to maintain wakefulness [108]. Outside the brainstem, principal targets of hypocretin fibers are the thalamus, basal forebrain and cortical areas [109]. Hypocretin peptides excite through GPCR other arousal-promoting neurons (noradrenaline, histamine, serotonin, and acetylcholine neurons) either by activating mixed-cation conductance or by inhibiting potassium conductance [110]. Conversely, hypocretin neurons can be inhibited by wake-promoting monoamines such as serotonin and noradrenaline [111].

Pharmacological studies in animal models have established a wake-promoting function for hypocretin neurons. Central infusions of hypocretin increase wakefulness and decrease NREMS and REMS [112]. Administration of hypocretin receptor antagonists decreases wakefulness and increases both NREMS and REMS [113, 114]. Acute optogenetic stimulation of hypocretin neurons evokes wakefulness from both NREMS and REMS [115]. Therefore, hypocretin activity is also related to vigilance state transitions, in particular sleep to wake.

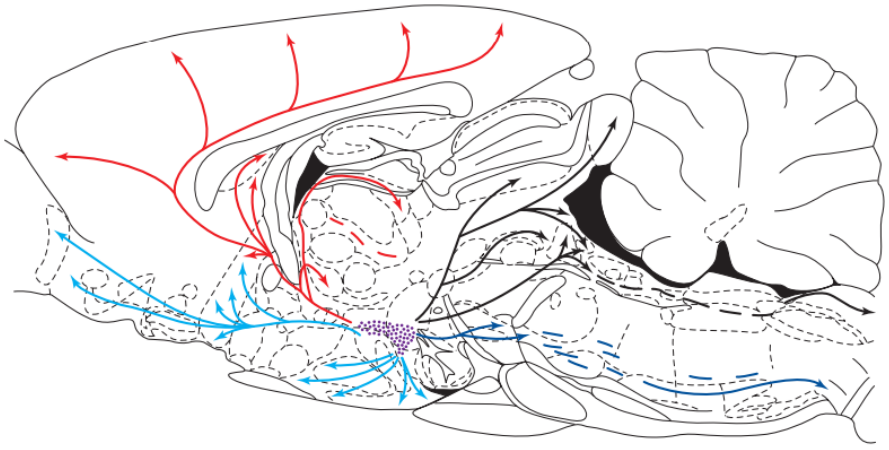


Fig. 6. Hypocretinergic system projections. Hypocretin neurons project extensively throughout the brain and mediate different energetic, arousal, motor and autonomic functions, plus several feeding, emotional and reward related behaviors. (Projections in blue towards the basal forebrain; light-blue to other hypothalamic nuclei; dark-blue to the spinal cord; black to the RAS and the brainstem nuclei; red towards the thalamus, hippocampus and cortex). From Kilduff & Peyron [116].

The firing of hypocretin neurons is generally higher during wakefulness, especially active wake, and lower during sleep [117]. *In vivo* recordings of hypocretin neurons confirmed that these cells are predominantly active when the animal is behaviorally engaged and are usually silent during inactive waking [118]. In the course of sleep, they drastically reduce their activity, and a large body of evidence indicates that they are not directly contributing to this physiological state as opposed to wakefulness. Nevertheless, hypocretin neurons were found to be transiently active during NREMS [119] and during certain phases of REMS [120]. Apart from arousal, hypocretin neurons are also important in several autonomic functions and motivated and social behaviors [121-127]. They also have a significant role in energy balance and feeding [118, 128-130]. It is argued that hypocretin neurons are a heterogeneous population of cells that have a functional dichotomy between the medial and lateral localized subpopulations of the hypothalamus. Cells in the dorsomedial hypothalamus (DMH) and the perifornical area (PeF) are considered to have a role more linked to arousal and sleep/wake regulation (Fig. 7) [101, 131, 132], while those in the LH area are more associated with fear, motivation and reward/addiction [101, 133].

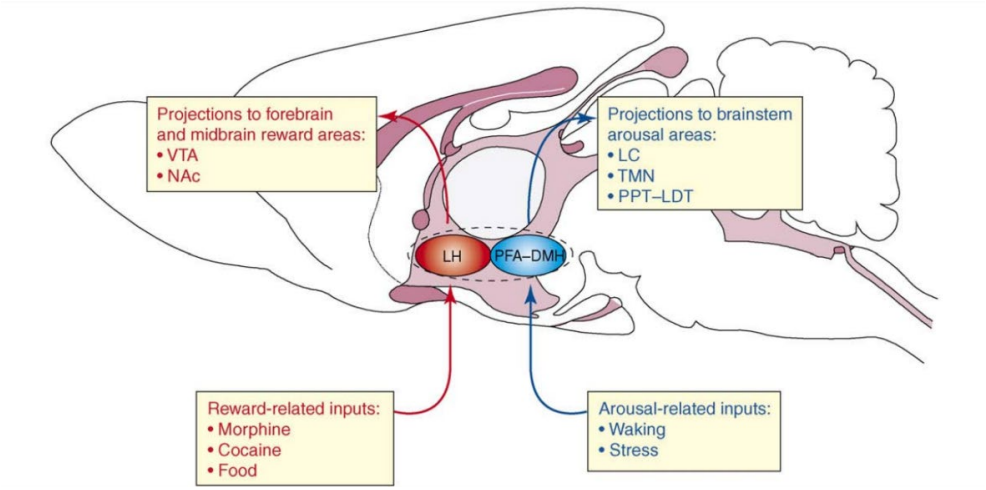


Fig. 7. Functional subdivision of the hypocretin neurons in the hypothalamus, with relevant behaviors or functions highlighted (bottom boxes) and preferred areas contacted by their axons (top boxes). (DMH, dorsomedial hypothalamus; PFA, perifornical hypothalamus; LH, lateral hypothalamus). From Harris et al. [101].

1.3.1. Hypocretin and its clinical implications

Hypocretin neurons are part of a complex neural network that regulates sleep and wakefulness, energy balance, and various physiological functions [96, 134, 135]. They are also implicated in stress responses, emotional regulation, and motivated behaviors [136]. This integration of functions helps coordinate sleep-wake cycles with energetic and physiological needs.

A deficiency or loss of hypocretin neurons is associated with narcolepsy, a neurological disorder characterized by excessive daytime sleepiness, sleep attacks, cataplexy, and disturbances in both NREMS and REMS [137-143]. Narcolepsy leads to fragmented sleep, where both NREMS and REMS episodes occur in a broken pattern, which results in multiple short sleep periods, affecting the overall architecture and quality of sleep [138, 140]. Another important feature of narcolepsy is cataplexy. Cataplexy consists of a sudden loss of muscle tone, usually elicited by an emotional reaction, where consciousness is preserved [216].

Although not exclusive to narcolepsy, some subjects may also manifest REMS behavior disorder (RBD), a particular medical condition in which the patient is unable to produce the characteristic muscle atonia that accompanies REMS, with the dangerous possibility of

forcefully moving during sleep and acting out ongoing dreams and consequently risking injuries because of unawareness and uncoordinated movements during sleep [144-146]. People with narcolepsy often experience REMS abnormalities related to the timing of REMS onset. Normally, a REMS episode occurs after a period of NREMS in the sleep cycle. In narcolepsy, REMS can occur more rapidly after falling asleep, even at sleep onset. These phenomena are known as "sleep-onset REM periods" (SOREMPs) [147, 148].

1.4. Locus coeruleus noradrenergic neurons

There are seven noradrenergic nuclei in the central nervous system (CNS), which were first discovered in rats and labeled from A1 to A7 [149]. The largest noradrenergic nucleus, which contains almost half of all noradrenergic neurons in the CNS, is the A6. This nucleus resides in the dorsal tegmentum of the rostral pons of the brainstem and is commonly indicated with the name “Locus Coeruleus” [150]. The LC was identified in the human brain at the beginning of the XIX century by the German physiologist Johann Christian Reil [151]. At the same epoch, the brothers Joseph and Karl Wenzel observed the identical structure and named it in Latin *locus coeruleus* (“blue area”) [152]. This zone of the brain has a dark-colored appearance because of the presence of neuromelanin, a substance that is necessary for the production of dopamine and noradrenaline, and for the same identical reason, the dopaminergic area of substantia nigra also has a peculiar dark appearance.

The fiber projections from the LC give rise to three pathways: the ascending pathway (dorsal noradrenergic bundle), the cerebellar pathway, and the descending pathway towards the spinal cord [153]. The ascending pathway makes sparse and vast synaptic contacts in sleep related areas located in the hypothalamus, basal forebrain, diverse thalamic nuclei, and multiple cortical regions [154]. Noradrenergic neurons arborize their axons profusely to contact multiple cells at the same time. The terminal fibers contain enlargements named “varicosities”, which

have the structure and function of presynaptic terminals and have been identified as sites of release of NA [155]. Although some of the noradrenergic axons form direct synapses with target neurons, many of the varicosities are located in extrasynaptic space without any identifiable synaptic contacts. Therefore, it has been proposed that NA release is not only capable of mediating direct neurotransmission between neurons but also of neuromodulating the whole brain activity as a diffusible factor [156, 157].

There are three different types of adrenergic receptors in the CNS, which are subdivided in three groups: the α1-adrenoreceptors (α1-AR), α2-adrenoreceptors (α2-AR) and the β-adrenoreceptors (β-AR). The α1-AR and β-AR subtypes mediate excitatory effects, while α2-AR subtype has inhibitory activity on neuronal firing [153]. The α2-AR can also be found on the presynaptic terminals or soma of adrenergic cells, acting as auto-receptors and inhibiting the release of NA itself [158]. LC is considered a major wake-promoting nucleus, and there is a close parallelism between LC activity and level of arousal. Furthermore, LC can inhibit, through α2-AR sleep-generating areas such as the VLPO. Indeed, noradrenaline, released from LC nerve terminals, inhibits VLPO GABAergic neurons via the stimulation of α2-AR and thus promoting wakefulness [159]. The same adrenergic receptors are also present on hypocretin neurons of the hypothalamus and thus NA has an inhibitory effect on this class of cells [160, 161]. Interestingly, hypocretin α2-AR seems to be functional only in sleep-deprived animals, therefore the expression of this receptor is subject to regulation by sleep pressure [162]. Also, hypocretin neurons send projections to the LC and these are strongly excitatory, so that the reciprocal connection of the hypocretinergic and noradrenergic systems constitutes a negative feedback loop involved in preventing excessive activity of the LC arousal pathway during periods of wakefulness [163, 164].

LC^{NA} activity during sleep is linked to state transitions, in particular awakenings. When the basal noradrenergic level is high during NREMS, there are higher chances of stimulus-mediated

NREMS to wake transitions [165]. However, if the basal level of activity is low, there are fewer possibilities of awakenings, and conversely, it is more probable to have a NREMS to REMS transition [166]. Recent studies using the fiber photometry recordings reported that LC activity fluctuates during NREMS [166, 167]. Indeed, LC^{NA} neurons exhibit phasic or burst-like activity during NREMS at precise and coordinated moments (Fig. 8). These fluctuations of neuronal firing that are denominated "LC^{NA} reactivation during NREMS" are thought to be associated with transitions between NREMS and wakefulness, maintenance of a physiological sleep architecture, or memory consolidation. Furthermore, every LC^{NA} reactivation is a time window of sleep fragility because microarousal and complete awakenings are strongly correlated with the bursting activity of noradrenergic cells [166-169]. The LC^{NA} reactivations during NREMS are in the scale of the infra-slow oscillations and are expressed every circa 50 seconds (0.02 Hz), and this LC^{NA} activity is anticorrelated with thalamo-cortical spindles, which occur in between LC^{NA} reactivations when the noradrenergic activity is low. Therefore, high levels of NA during NREMS are inhibiting sleep-spindle generation [167]. Apart from the sleep/wake network, the LC also mediates other functions and is a component of other systems such as the autonomic, the fear/anxiety, the memory and the pain modulation network [154].

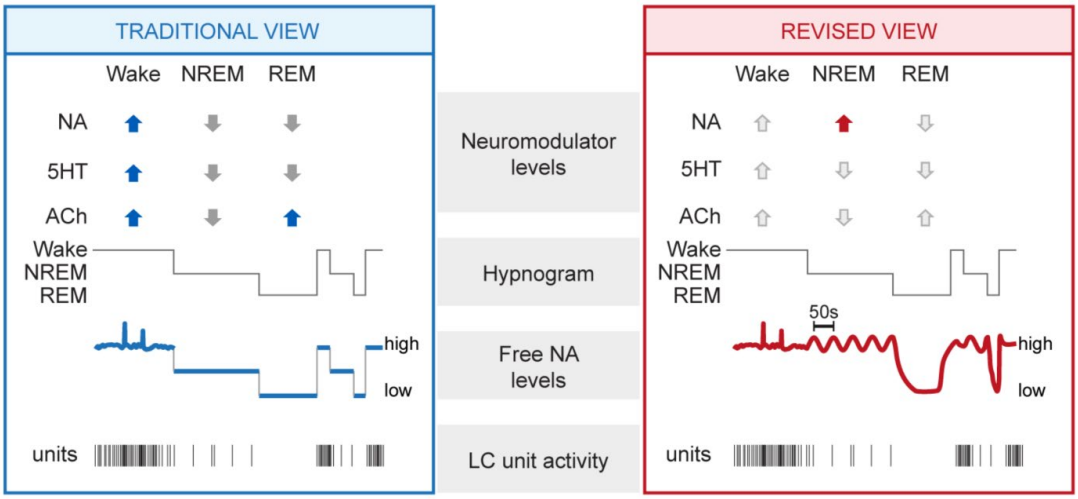


Fig. 8. Locus coeruleus activation across vigilance states, novel insights from literature. Refined techniques are nowadays proving that sleep neuromodulation is important to stabilize its architecture and mediate its functions. From Osorio-Forero et al. [170].

1.5. Thalamic control of sleep-wake cycle

The thalamus resides in the midbrain below the cortex and is structured in two symmetrical halves composed of different nuclei, which are subdivided into different categories depending on their specific afferents [13]. Many of these nuclei receive information from peripheral sensory organs, and the main outputs of these primary thalamic nuclei are directed mainly to the primary cortical areas. Reverberating activity between cortex and thalamus is important not only for sensory processing, but thalamocortical (TC) connections can also promote sleep or wakefulness [171-173]. For instance, thalamic tonic firing can induce wakefulness, while bursting activity can lead to sleep [173, 174]. The TC reverberation model derives from studies conducted in the early 1980s, and it is a complementary model to the flip-flop switch hypothesis [175-179]. This model claims the importance of thalamic activity in generating awareness at the cortical level, and thalamic neurons are considered not just relay-projecting units but important players in controlling global brain states [180].

An important mechanism for sleep maintenance is sensory disconnection from the external environment [181, 182]. Since the ability to perceive, elaborate on, and react to stimuli is maximal during wakefulness, while sleep is usually accompanied by low reactivity to external stimuli, this mechanism of sensory disconnection prevents the animal from being easily awakened, and only intense stimulation can overcome the sleep threshold to arouse the animal. The thalamic sensory nuclei that project to primary areas in the cortex are active during wakefulness, allowing sensory input to reach the cortical mantle and promote sensory processing and awareness. In contrast, these nuclei become less active during sleep, leading to reduced alertness and the avoidance of frequent sleep-wake transitions due to sensory stimulation [12, 183].

The midline nuclei of the thalamus, also indicated as the "high order thalamus", are involved in a general role of diffuse activation of the cortex [184, 185]. The mammalian midline thalamus

consists of five nuclei of paraventricular (PVT), centromedial (CMT), intermediodorsal, rhomboid and reuniens, which receive extensive inputs from the brainstem including from the RAS adrenergic, cholinergic, and serotonergic neurons. They also receive dense innervation from the hypothalamic hypocretinergic cells [186, 187]. The major output of the midline thalamic nuclei is the prefrontal cortex, and tracing studies have shown that the dorsal region of the midline nuclei is strongly connected, mostly with limbic-related parts of the striatum and the prefrontal cortex [188]. Seminal studies demonstrated that low-frequency electrical stimulation of the midline thalamus resulted in the development of cortical slow waves and spindle bursts, which were associated with inattention, drowsiness, and sleep. By contrast, high-frequency stimulation led to desynchronization of the cortical EEG with a concomitant buildup of arousal [189, 190]. Therefore, midline thalamic nuclei are associated with physiological mechanisms that control arousal, attention and consciousness [191].

Recent studies highlighted that some of these nuclei are in phase advance to state transitions, in particular CMT is actively firing before NREMS to wake transitions and also precedes cortical UP states [173]. Conversely, CMT deactivation precedes a reduction in cortical arousal concomitant with sleep onset or even with an induced anesthesia state [192]. Among the midline thalamic nuclei, the densest projections from hypocretin neurons are sent to the PVT [187]. Excitatory PVT neurons can induce awakenings from sleep or anesthesia, and these transitions seem to be strongly regulated by the hypocretinergic inputs they receive [193]. Therefore, midline thalamic nuclei are considered important components of the arousal network and are mainly involved in promoting wakefulness and anticipating cortical state transitions.

Timing of cortical state transition is heterogeneous and some regions could anticipate or have a lag compared to the others to complete the switch [173, 192]. There is also some evidence for different transition times across laminae of the same cortical region [194]. The prevailing theory about state transitions at the cortical level is based on the activity of inhibitory cells, especially

interneurons that are able to mediate cortico-cortical connectivity and regulate firings across distant regions of the cortex [195, 196]. A large body of evidence also indicates that sleep can even occur locally [197, 198]. Indeed, at the cortical level, the fundamental properties of sleep can also be seen in isolated neuronal ensembles such as cortical columns or restricted microcircuits [199-202]. In this perspective, sleep is defined as a local neuronal activity that is self-generated and self-sustained and that could be disconnected from the whole brain activity.

1.6. Aims of the thesis

In the present thesis project, I will develop a series of arguments that sleep is a naturally occurring dynamic behavior controlled by elaborate and precise neural mechanisms and that the activity of a sleep-controlling brain neural network is sufficient *per se* to induce and maintain vigilance states. Moreover, I propose that transitions within vigilance states are controlled by the communication between the components of this broad neural network, composed of sleep-generating and wake-promoting areas. Particular attention will be given to the hypocretin system, and by using a mouse line lacking a functional hypocretinergic system to better understand why sleep is fragmented yet still regulated. Failure in communication or dysregulation in connectivity between these circuits leads to arousal disorders and disruptions of sleep architecture, abnormal physiological transitions between vigilance states or even sleep-related disorders such as insomnia, narcolepsy, or REMS behavior disorder [203]. For these clinical conditions, the research conducted in the present thesis is relevant for both the scientific community and the public at large.

2. Results

To achieve the aims of this thesis, we studied several neural circuits involved in the regulation of vigilance state transitions, localized in the hypothalamus, brainstem, thalamus, and cortex. Considering several studied neurocircuits with different aims and using different techniques, we organized the findings of this PhD thesis into three separate projects:

a. The first project investigated the role of hypothalamic hypocretin neurons and LC noradrenergic cells in vigilance state transitions using the fiber photometry technique. The results of this project, which are the major scientific contribution of this thesis, are provided as a preprint attached to this thesis. A summary of the project is also provided in the section 2.1. of this chapter.

b. The second project studied the activity of extended VLPO GABAergic cells across vigilance states and their transitions using the fiber photometry technique. The findings of this project are provided in the section 2.2. of this chapter.

c. The 3rd project concerns the generalization pathways of sleep-wake patterns across different thalamic and cortical sites. Using multisite local field potential (LFP) and unit recordings, up to 5 regions simultaneously, we aimed to find how sleep or wake states arise and then propagate in thalamic and cortical sites. Although I have collected extensive data, and in different thalamo-cortical configurations, for this project, the detailed analysis is still ongoing and requires additional time. Thus, only a summary of the collected data and preliminarily results of this project are provided in the section 2.3. of this chapter.

2.1. A mediatory role for hypocretin neurons during sleep

Preprint title: Hypothalamic control of noradrenergic neurons stabilizes sleep

Authors: Gianandrea Broglia, Giorgio Corsi, Pierre-Hugues Prouvot Bouvier, Mehdi Tafti, and Mojtaba Bandarabadi

Hypocretin is a key player in sleep-wake regulation and is part of a broader neural network that controls arousal and vigilance state transitions. Lack of hypocretin leads to the debilitating condition of narcolepsy, and in its absence sleep architecture and vigilance state are highly compromised. We took advantage of Cre expressing mouse lines to record Ca^{2+} activity of wake-promoting nuclei in the hypothalamus and brainstem. Selected regions and neural populations for this investigation were posterior hypothalamus hypocretinergic cells and LC^{NA} neurons. To this end, we employed the *Hcrt-IRES-Cre* and *Dbh-Cre* lines. To gain insight on the LC^{NA} dynamics in the absence of hypocretin, we generated a novel strain of narcolepsy mouse model by crossing *Dbh-Cre* with $Hcrt^{KO/KO}$ mice. We also characterized this novel mutant line for its sleep behavior and electrophysiological phenotypes using coupled EEG/EMG recordings. The importance of this work is to expand recent findings on noradrenergic activity during NREMS [191, 92], and to foster knowledge relative to the flip-flop network model of sleep and arousal generation [53]. We report several outstanding findings related to $Hcrt^{KO/KO}$ mice, overall regarding sleep behavior, activity of hypocretin cells during REMS, LC^{NA} reactivations during NREMS, EEG theta activity during REMS, and abnormal sleep architecture. A preprint of this study can be found in the thesis Annex.

2.2. Role of the extended VLPO in regulating vigilance states

The role of inhibitory neuronal populations of the VLPO in sleep is extensively studied by different groups [80, 84, 204-214]. The core area of the VLPO (cVLPO) is principally active during NREMS and is particularly important for the generation and maintenance of NREMS [77]. The dorsal and more medial sides of the VLPO are referred to as the extended VLPO (eVLPO) and seminal references highlight the importance of this area for the regulation of REMS [78, 80]. However, no study has reported the *in vivo* activity of eVLPO GABAergic (eVLPO^{GABA}) subpopulations across vigilance states, and whether they have different dynamics from previously reported cVLPO activity. Thus, we investigated the dynamics of eVLPO^{GABA} neurons using *in vivo* calcium imaging in *Vgat-Cre* mice. Additionally, as the absence of hypocretin dysregulates REMS architecture and components, we generated homozygous hypocretin-knockout *Hcrt$^{KO/KO}$xVgat-Cre* mice, by crossing *Vgat-Cre* mice with *Hcrt$^{KO/KO}$* mice, to investigate eVLPO^{GABA} activity in the absence of hypocretin.

To record neuronal activity, a calcium indicator (ssAAV-9/2-hSyn1-chI-dlox-GCaMP6m(rev)-dlox-WPRE-SV40p(A), titer 4.2x10E12 vg/ml) was delivered in the eVLPO (AP: 0.000; ML: 0.500; DV: 5.500) of transgenic mice. Afterward, optic fibers (200 μm diameter) were implanted and placed upon the medial region of the VLPO, which corresponds to the eVLPO and data were collected using a fiber photometry setup.

Scoring of the vigilance states and analysis of photometry signals were performed as described in the “Methods” part of the previous section (attached preprint). Implantation sites of fibers and injected areas were confirmed with post-mortem brain fixation in 4% parafolmadehyde and with 60 μm thick coronal sections using a cryostat. Sections were visualized and images acquired under a bright-field microscope or confocal microscope in the case of IHC using antibodies against VGAT, CRE and DAPI.

Our photometry recordings from freely behaving *Vgat-cre* mice revealed that eVLPO^{GABA} neurons are mainly active during REMS but also show some activity during wakefulness (Fig. 9). Instead, the same population of cells shows sparse activity during NREMS (Fig. 9a,c). We then investigated eVLPO^{GABA} neuronal activity across transitions in vigilance states and observed a low bump of signal right after awakenings from NREMS (Fig. 9b). Activity of eVLPO^{GABA} neurons at transitions towards NREMS did not show clear changes and decreased after transitions to NREMS, suggesting that eVLPO^{GABA} neurons might not play a role in promoting NREMS as opposed to cVLPO cell populations.

Compared to the dynamics of NREMS transitions (onset and termination), we found a strong calcium signal at transitions to REMS and during REMS. Indeed, eVLPO^{GABA} neuronal activity increased slowly and constantly s few seconds prior to REMS onset and reached a high level after the state switch. The calcium signal is constantly high across REMS episodes and drops right after REMS to wake transitions (Fig. 9b). The average calcium signals across vigilance states show that REMS episodes have the highest eVLPO^{GABA} neuronal activity, but wakefulness also shows moderate levels of calcium signal.

We also compared eVLPO^{GABA} neuronal activity between control (*Vgat-Cre*) and hypocretin knockout (*Hcrt$^{KO/KO}$xVgat-Cre*) mice. We found similar trends and dynamics of neuronal activity across vigilance states and transitions between two genotypes (Fig. 9a,b). However, the average $\Delta F/F$ analysis revealed a higher activity during REMS and lower activity during wake in the absence of hypocretin compared to controls (Fig. 9c). These results suggest differential effects of hypocretin neurons on eVLPO^{GABA} neuronal activity during wakefulness and REMS, with excitatory and inhibitory effects during wakefulness and REMS, respectively. One possible explanation is the heterogenicity of hypocretinergic neuronal activity across wakefulness and REMS, which we reported in the results of the first project.

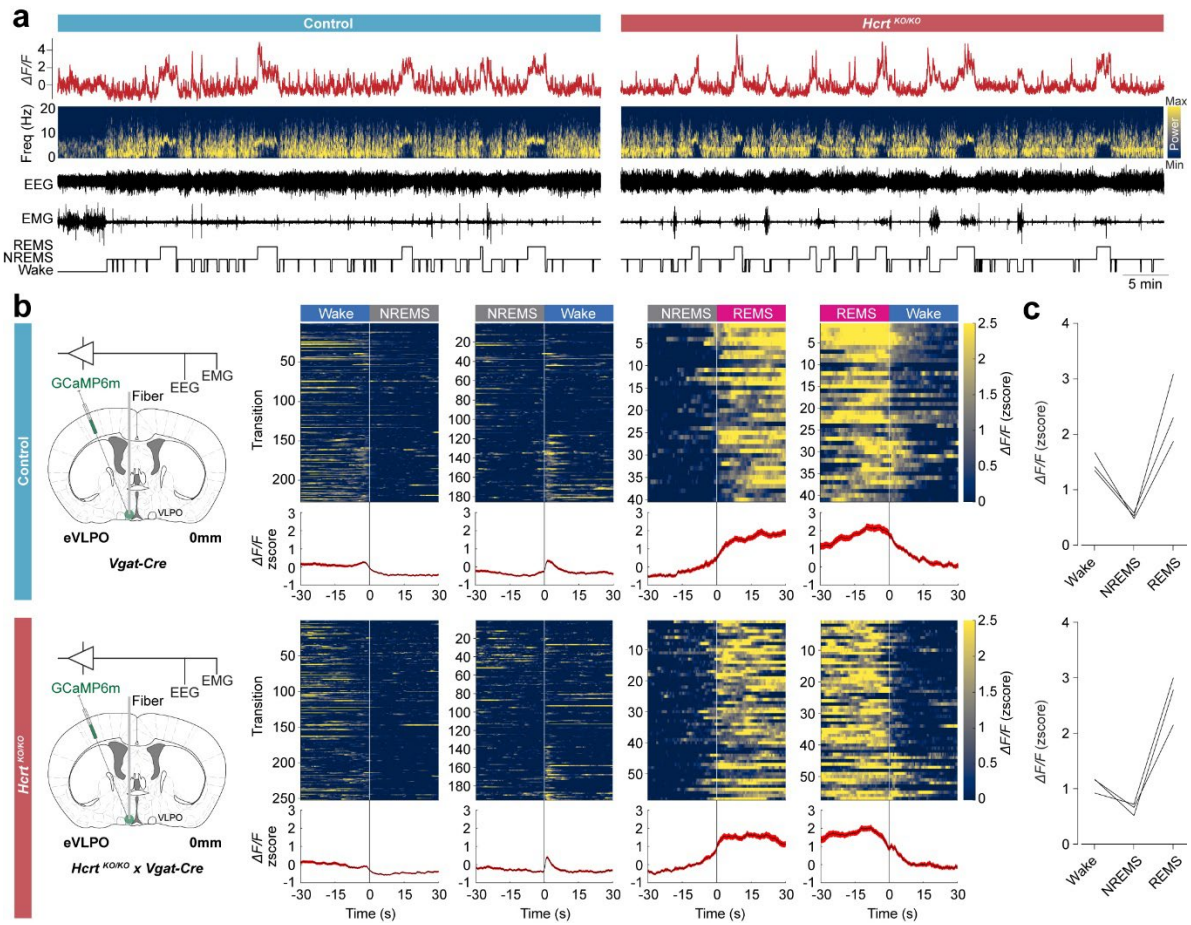


Fig. 9. Activity of eVLPO GABAergic neurons across vigilance states.

a) Representative fiber photometry calcium recordings of eVLPO GABAergic neurons, time-frequency representations of EEG signals, and raw EEG/EMG signals, from *Vgat-Cre* (left) and *Hcrt*$^{KO/KO}$*xVgat-Cre* (right). Hypnogram is depicted below.

b) Transition panels of vigilance states in control (top) and narcoleptic (bottom) mice injected with GCaMP6m. Normalized color-coded *ΔF/F* for every transition (top) and averaged signal for the 30 sec before and after the transition (bottom). Data are from 3 mice for *Vgat-Cre* and 3 mice for *Hcrt*$^{KO/KO}$*xVgat-Cre*.

c) Average *ΔF/F* of calcium signal across each vigilance state during the whole recording session (ZT4-7). Data are from 3 mice for *Vgat-Cre* (top) and 3 mice for *Hcrt*$^{KO/KO}$*xVgat-Cre* (bottom).

As the release of the most monoamine neurotransmitters, e.g., norepinephrine, serotonin, and histamine, ceases during REMS [166, 167, 215-217], we hypothesized that eVLPO^{GABA} neurons might be responsible for their inhibition. To test this, we simultaneously recorded the activity of eVLPO^{GABA} neurons and the LC noradrenergic system, which is the best-characterized monoaminergic system involved in the regulation of vigilance states (Fig. 10). We first injected GCaMP6m in the eVLPO and GRAB$_{NE}$ in the LC of *Vgat-Cre* mice and then implanted optic fibers above these two regions. Dual-site fiber photometry recordings revealed a strong anti-correlation between the activity of eVLPO^{GABA} cells and local norepinephrine release within the LC during REMS (Fig. 10b). In fact, LC^{NA} is silent during REMS and displays a constant decay signal (Fig. 10b). Instead, as already mentioned above, eVLPO^{GABA} neurons show very high and sustained activity during REMS.

Another important finding is that at transitions from NREMS to REMS, LC^{NA} activity is continuously decreasing, while eVLPO activity concomitantly increases and reaches a peak once REMS is consolidated (Fig. 10c). The dynamics of the two areas are also evidently anti-correlated at the awakenings from REMS. Among vigilance states, LC^{NA} has the lowest level of recorded $\Delta F/F$ signal during REMS, but abruptly increases its activity at REMS to wake transitions (Fig. 10c). Conversely, activity of eVLPO is highest during REMS and has a sudden decrease at REMS termination (Fig. 10c). Furthermore, during NREMS small signal fluctuations of the eVLPO are anti-correlated with LC^{NA} reactivations.

In summary, our findings indicate that eVLPO and LC^{NA} are strongly anti-correlated in their activity across all vigilance states, suggesting functionally direct connections between the two areas that are involved in the regulation of the sleep-wake cycle [77]. Further experiments, including retrograde tracing and optogenetic manipulations, are planned to first confirm the connectivity of the two nuclei and, second, investigate causality and role of eVLPO^{GABA} neurons in the induction or maintenance of REMS.

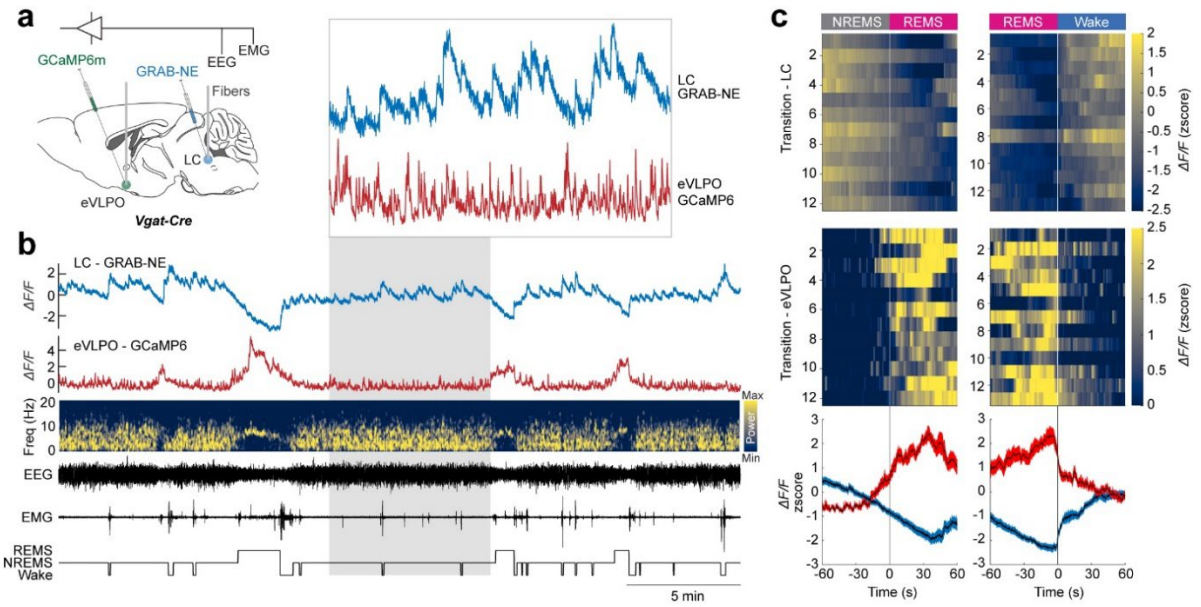


Fig. 10. Activity of the LC noradrenergic system is anti-correlated with eVLPO GABAergic neurons during REMS.

a) Schematic coronal sections of fiber implantation.

b) Example of photometry recordings using $GRAB_{NE}$ in the LC and GCaMP6m in the eVLPO across vigilance states from a single animal. From top to bottom, LC noradrenergic signal, eVLPO GABAergic calcium signal, time-frequency representation of EEG signals, and raw EEG/EMG signals. Hypnogram is depicted below.

c) Normalized color-coded *ΔF/F* photometry activity of regional norepinephrine release in the LC (top) and calcium signal from eVLPO GABAergic neurons (middle) for NREMS to REMS and REMS to wake transitions. Averaged signal for the 60 sec before and after the transition (blue LC; red eVLPO) (bottom).

2.3. From local to global sleep

The main aims of this project focused on (1) how sleep and wake states are generated locally and then generalized across the thalamo-cortical structures, and (2) how different inputs to the cortex are responsible for local cortical and then global cortical sleep. To investigate these questions, I performed multisite LFP/unit recordings from different thalamic nuclei and cortical sites using tetrodes, combined with EEG/EMG recordings, in spontaneously sleeping freely behaving mice.

Methods

Animals. Adult C57BL/6J mice were used for tetrode implantations. Animals were group-housed with *ad libitum* access to standard food pellets and water at a constant temperature (23±1 °C) and humidity (30-40%), and a 12h/12h light/dark cycle with lights on from 8:00 a.m. to 8:00 p.m. Animals were 12-18 weeks old at the time of the experiments. All procedures were carried out in accordance with the Swiss federal laws and approved by the veterinary service of the State of Vaud, Switzerland (license VD3524).

Multisite tetrode implantation. Tetrodes were made manually by twisting four stands of tungsten wires (12.5 µm, California Fine Wire, USA) with the help of a twisting machine (Thorlabs). Tetrodes were connected to an electrode interface board (Neuralynx, USA) using gold pins and final end of tetrodes were incapsulated in glass capillaries of 100 µm. Extremities protruding from glass capillaries were cut with designed scissor to have each tetrode exposing 100 µm circa from its capillary. Tetrode tips were electroplated in gold non-cyanide solution (Neuralynx, USA) using the nanoZ device (White Matter LLC, USA), and their impedance was measured. If the impedances of all wires were not between 500 KΩ and 800 KΩ, tetrode tips were cut and gold-plated again until reaching the criterion.

Adult male mice (12-14 weeks) underwent stereotaxic surgery (Kopf Instruments, USA) for implantation of tetrodes and EEG/EMG electrodes. Anesthesia was achieved with the intraperitoneal injection of ketamine/xylazine (100/20 mg/kg) diluted in 0.9% saline. Isoflurane at 1-3% was given through a face mask to maintain animal sedated. To record multisite LFP and single-unit activity, tetrodes were inserted in the midline thalamus and different cortical sites, combined with EEG/EMG electrodes. The PVT (AP -1.45; ML -0.75; DV -2.7; 15° tilt towards midline), CMT (AP -1.35; ML -0.75; DV -3.7; 15° tilt towards midline), CING (AP 1.8; ML -0.3; DV -1.8), somatosensory barrel cortex (BARR: AP -1.5; ML -2.5; DV -1.250), motor cortex (MOT: AP 1.2; ML -1.75; DV -2), premotor cortex (pMOT: AP +2.2; ML -1; DV -1.3) and the insular cortex (INS: AP -1; ML -3.75; DV -3.9) were targeted. Three golden screws were placed on the right hemisphere at coordinates (AP +1.8; ML +1.8) for frontal EEG, (AP -2; ML +2.5) for parietal EEG, and (AP -6; ML +1.8) for the ground electrode. Two plastic-covered gold wires were inserted into the neck muscles and served as EMG electrodes. All electrodes and wires were fixed using the dental resin (Relyx 3M). The EEG/ground screws and EMG wires were soldered to a digital interface board (Neuralynx, USA), and the whole implant was covered by dental cement.

Multisite LFP/unit recordings. Mice were allowed to recover from the surgical implantation for one week, and then connected to a tethered 32-channel digital headstage (RHD2132, Intan Technologies). Animals were habituated to the recording cable in their open-top home cages for 3 days and kept tethered for the duration of the experiments. All recordings were performed between 16-18 weeks of age. All channels were sampled at 20 KHz using the Intan RHD USB interface board and the Evaluation System Software (RHD2000 evaluation software, Intan Technologies, USA). The baseline of natural sleep from 12:00 to 18:00 (ZT4 to ZT10) were recorded for at least two days. Then animals underwent sleep deprivation followed by a sleep recovery session recording. For four hours, starting at 08:00 (ZT0), gentle disturbance with the

help of a stick was performed if animals were stationary to avoid sleep. At 12:00 (ZT4), animals were left undisturbed, and a new recording session started. Sleep recovery recordings lasted 6 hours for tetrode experiments.

Histological confirmation of the implanted sites. At the end of recordings, a current was applied to make lesions in the implanted sites for histological confirmation. Subsequently, mice were deeply anesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with PBS, followed by 4% paraformaldehyde. For cryostat (CM3050S, Leica, Germany) sectioning, brains were removed, fixed in 4% paraformaldehyde and kept in 30% sucrose/PBS at 4 °C overnight, and snap-frozen in isopentane. The frozen sliced using a cryostat in 30 µm coronal sections for histological confirmation of the implanted sites. Images were acquired with the LSM 900 confocal microscope (Carl Zeiss, Germany) and analyzed using the Zen software 2012 (Carl Zeiss, Germany). Microscopy investigation was also performed to locate the tip of implanted tetrodes. Data were excluded if the locations were not confirmed.

Scoring and quantification of vigilance states. Scoring of vigilance states was performed manually using visual inspection of EEG/EMG recordings as described previously [172, 218]. A custom toolbox written in MATLAB was used to visualize time and frequency characteristics of EEG/EMG traces. To allow precise scoring of microarousals and transition times, a 1-second epoch scoring was used. Wake was defined as periods of either theta band EEG activity accompanied by EMG bursts of movement-related activity, or periods that mice were immobile including feeding and grooming behaviors. NREMS was scored as periods with a relatively high amplitude low frequency delta band EEG activity accompanied by reduced muscle tone relative to wakefulness and associated with behavioral quiescence. REMS episodes were scored as sustained periods of theta band EEG activity and behavioral quiescence associated with muscle atonia, except for brief phasic muscle twitches. Transition to wake was defined as the first epoch with a rapid increase in muscle tone concurrent with low-amplitude fast-frequency

EEG activity. Wake to NREMS transition was defined as the first epoch containing high-amplitude delta band activity appearing after EMG silencing. REMS onset was defined as an epoch with the absence of EMG tone concomitant with recursive synchronized theta rhythm. Number, duration, and state fragmentation of wakefulness, NREMS, and REMS were quantified using MATLAB scripts.

Single-unit analysis. To obtain single-unit activity, the multi-unit activity was first extracted from bandpass filtered recordings (600-4000 Hz, 4th-order elliptic filter, 0.1 dB passband ripple, -40 dB stopband attenuation), and by applying a detection threshold equal to 7.5 times the median of the absolute value of the filtered signal. Detected multiunit activity was automatically sorted using the WaveClus toolbox to obtain single-unit activity [219]. Automatically sorted spikes were visually inspected for noise clusters and clusters with a completely symmetric shape were excluded from further analysis. State-specific average spike rates were calculated as total number of action potentials during each state divided by total time spent in that state, and dynamics of single-unit activity were assessed across each state and during transitions using overlapped moving average windows with different time-resolutions to uncover short and long-term trends of unit activity.

Preliminarily results

I performed multisite recordings in four different configurations with the PVT, CMT, and CING as the fixed recording sites, and a variable 4th recording site in the BARR (6 mice), MOT (6 mice), pMOT (5 mice) or INS (5 mice) (Fig. 11). These configurations allow us to explore the sequencing of vigilance state transitions between thalamic nuclei and cortical sites with diverse functions. Implanted animals were recorded for two baseline days, and a gentle sleep deprivation of 4 h (8:00-12:00am) was performed on the third day.

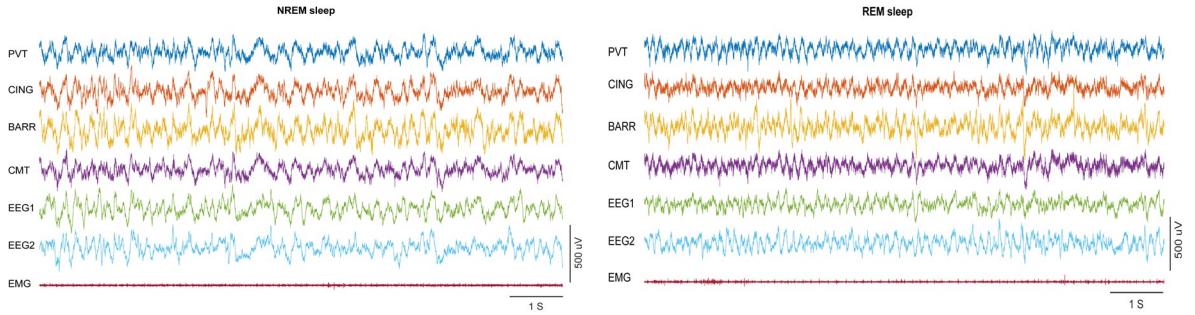


Fig. 11. Multisite thalamic and cortical LFP recordings.

Representative multisite thalamic and cortical LFP recordings, as well as frontal/parietal EEGs and EMG signals, during NREMS and REMS. The PVT, CMT, and CING sites are preserved in our different configuration recordings.

Our preliminary results indicate that the generation of vigilance states in thalamo-cortical sites can occur both sequentially and in cascade; e.g., the motor cortex (M1) shows a delayed transition to wakefulness compared to the midline thalamic nuclei (sequential transition), while the cingulate cortex wakes up in parallel with the midline thalamic nuclei (cascade transition, Fig. 12a). Our preliminary results also show that the activity of non-sensory thalamic cells switches to NREMS earlier than that of cortical neurons (Fig. 12b). We are further investigating the order of transitions using high-temporal resolution LFP analysis, e.g., wavelet-based time-frequency analysis, to uncover the local and global patterns of sleep-wake generalization. For targeting the neocortex, we implant tetrodes in layer VI of cortical sites, as this layer receives the hypocretinergic innervations and cortico-cortical projections in layer 4 spread electrical signals to the whole cortex.

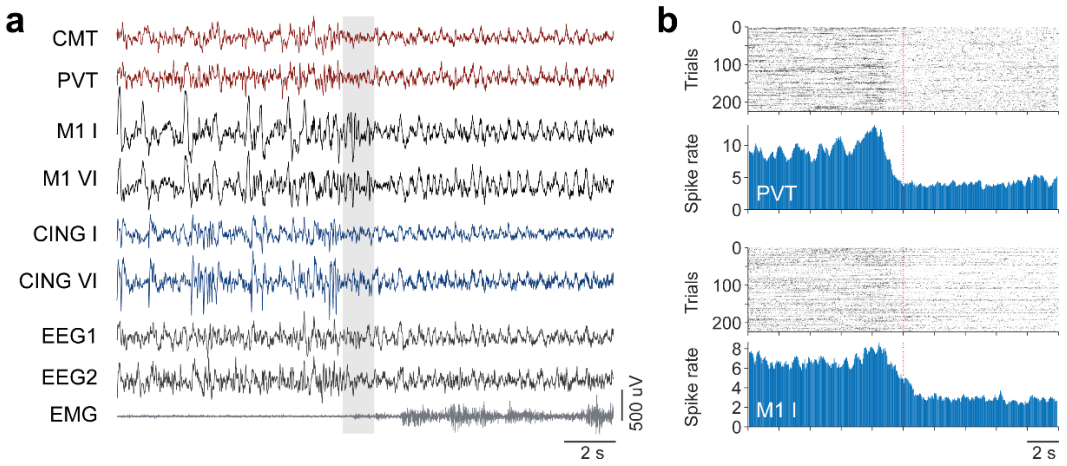


Fig. 12. Sequential and cascade generation of vigilance states in thalamo-cortical sites.
a) Representative multisite LFP recordings from six thalamo-cortical sites, as well as EEGs and EMG signals, during a transition from NREMS to wakefulness. The highlighted part in gray shows the transition event. The motor cortex (M1) shows a delayed transition to wakefulness, a sequential transition, compared to the midline thalamic nuclei (CMT and PVT) and the cingulate cortex (CING). The CING cortex wakes up in parallel with midline thalamic nuclei, a cascade transition.
b) Representative activity of PVT and M1 neurons during transitions from wakefulness to NREMS indicates that PVT cells switch to the sleep mode earlier than motor cortical neurons. Each trial is a wake-to-NREMS transition.

The typical activity of a thalamic/cortical unit is to increase firing upon sleep to wake transition and decrease it in the opposite case (Fig. 13). This is expected for most neurons since it is known that spiking activity correlates with the elaboration of neural information, and during sleep this process is reduced [30, 220]. We termed these neurons “ON-OFF” cells, as they show increased or decreased firing after the transition.

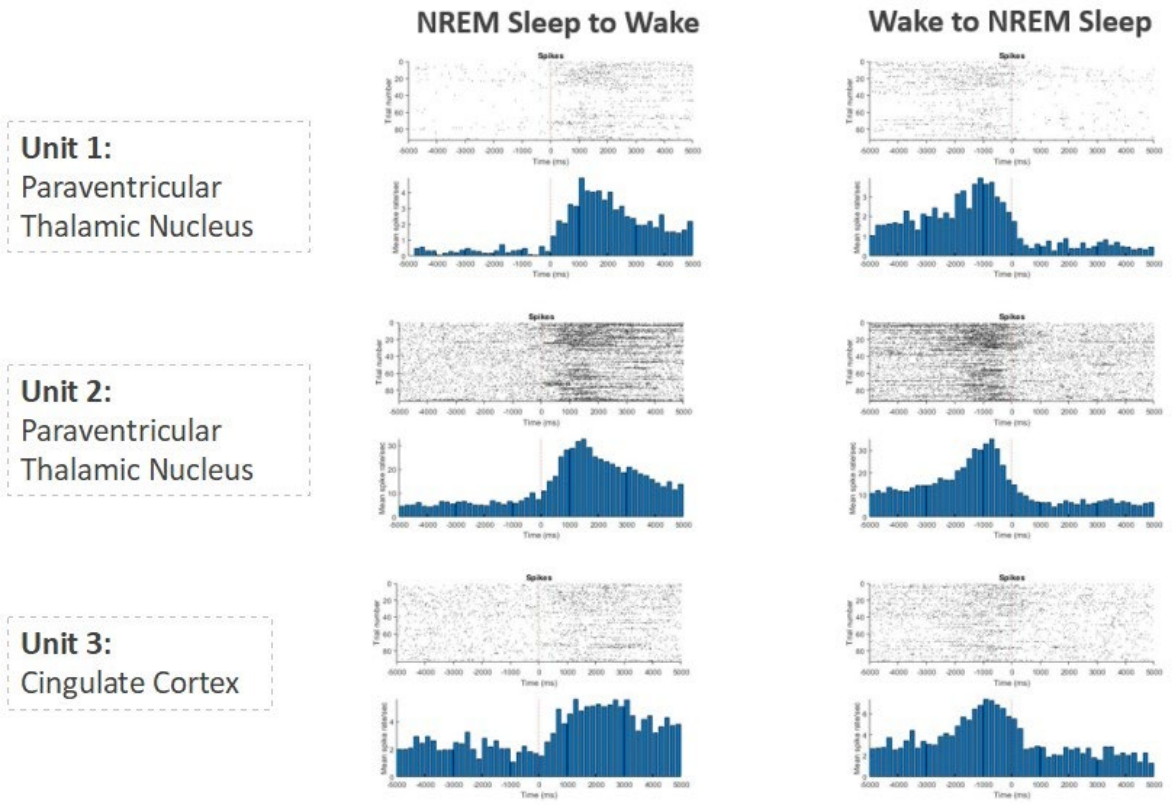


Fig. 13. The concept of "ON-OFF" cells.

Representative thalamic and cortical spike activity across transitions. Raster plots of thalamic and cortical units and their averages during vigilance state transitions. Dashed red lines indicate NREMS-to-wake or wake-to-NREMS transition times.

We could identify another class of neurons that seems to anticipate transitions and show increased/decreased firing only during the switch (Fig. 14). Activity of these identified cells is symmetric and distributed in a window of ~2 s, having a peak/through at the transition time. We provisionally named them "transition cells", hypothesizing that they are high-order hierarchy neurons in their local microcircuits that can activate/inhibit neighboring cells by receiving inputs from subcortical or subthalamic downstream.

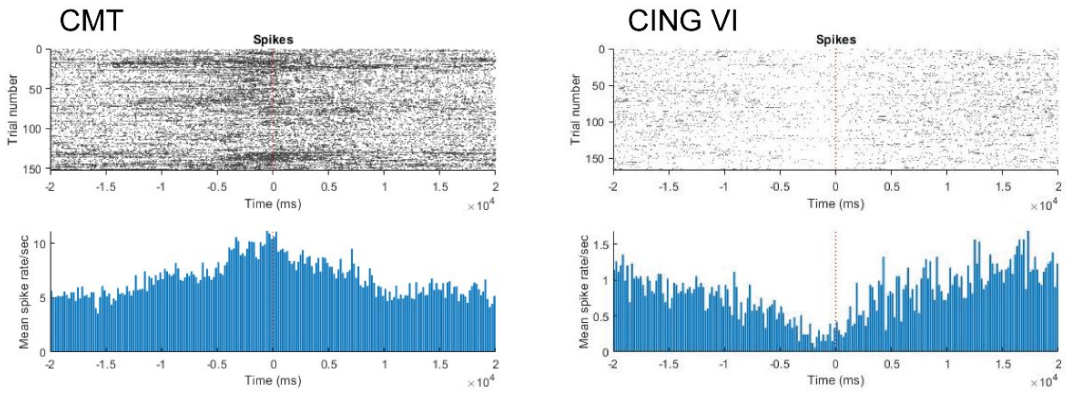


Fig. 14. The concept of "transition cells".

Transition cells show increased or decreased firing only during the transitions. Representative spike activity of two single units from the centromedial thalamus (CMT) and cingulate cortex during wake to NREMS transitions.

2.4. Personal contribution

To carry out the projects described above, I first established the multichannel fiber photometry and multisite electrophysiology techniques in the lab, and synchronized recordings of different setups, e.g., EEG/EMG with fiber photometry recordings. I performed all surgeries on mice, including craniotomies, viral injections, and fiber/tetrode with EEG/EMG implantations. I carried out all *in vivo* recordings on mice and then sacrificed animals with transcardial perfusion to obtain brain tissues for analysis. I made cryostat or vibratome sectioning of collected brains for histological confirmation of the injection and fiber implantation sites, and imaged sections with a bright-field microscope. I scored all electrophysiological recordings and significantly contributed to the data analysis. I also contributed to the designing of the experiments together with my supervisors, Dr. Mojtaba Bandarabadi and Prof. Mehdi Tafti. Immunohistochemistry and confocal microscopy were run in collaboration with Dr. Giorgio Corsi and Dr. Pierre-Hugues Prouvot-Bouvier. I also contributed to the writing/editing of the manuscript and the preparation of the figures.

3. General discussion

In the first part of this thesis, we investigated the activity of specific neural populations across different vigilance states and their transitions, using fiber photometry technique with AAVs carrying GECI or neurotransmitter reporters in several Cre lines [46-51, 91, 105, 115, 221, 222]. We recorded Ca^{2+} activity from hypocretin cells of the hypothalamus in the *Hcrt-IRES-Cre* line, LC noradrenergic cells in the *Dbh-Cre* line, and $eVLPO^{GABA}$ cells in the *Vgat-Cre* line. Moreover, we also used the $GRAB_{NE}$ sensor to screen NA release in the midline thalamus and prefrontal cortex [50]. Central points in our research were hypothalamic hypocretin and LC^{NA} cells because of their importance in current scientific knowledge about the mechanisms of sleep-wake transitions and their maintenance [11, 93, 223]. We also crossed these Cre lines with $Hcrt^{KO/KO}$ mice to obtain novel mutants that are Cre positive on our gene of interest while lacking a functional hypocretinergic system, therefore constituting an experimental model of narcolepsy [105]. Our aim was to investigate the neural activity of LC^{NA} and $eVLPO^{GABA}$ cells and study their differences between $Hcrt^{KO/KO}$ and control mice during vigilance states.

We made several unexpected and scientifically relevant findings that should be debated and potentially included in theoretical and experimental models that are used for understanding the generation of arousal states and their physiological or disease-related mechanisms of transition. We first identified a subpopulation of hypocretin neurons located in the PeF hypothalamus that are active during REMS and wakefulness, compared to the classical population of LH^{HCRT} cells that are mainly active during wakefulness [9, 224]. Moreover, we recorded a low activity of hypocretin cells during NREMS, as recently reported [119]. We found that the hypocretin system actively contributes to the regulation of NREMS and REMS and can neuromodulate LC^{NA} cells, directly or indirectly, especially during NREMS. In fact, the activity of LC^{NA} during NREMS is severely compromised and altered in the absence of hypocretin neurons, considering NREMS LC^{NA} reactivations. Sleep architecture, sleep oscillations, and the amount and duration

of sleep episodes are also evidently affected in animals without a functional hypocretinergic system. As in narcolepsy patients, our animals express less stable NREMS and REMS [140, 218, 225].

All together, these findings open a novel scenario for the comprehension of the hypocretin neurocircuitry and its involvement in sleep. Nevertheless, although these findings need to be replicated with different approaches, they already help to revisit notions related to the function of sleep pathways and partially explain the causes of an altered structure of sleep architecture in narcolepsy. Future investigations are also needed to better understand the active role of hypocretin during sleep and its ability to mediate LC^{NA} neurons.

3.1. Role of hypocretin neurons during REMS

We unexpectedly found that PeF^{HCRT} neurons are highly active during REMS. Currently, there are only a few studies that indicated or hypothesized that hypocretin neurons are active during REMS [116, 226-228]. Some studies suggest that hypocretin neurons are active during phasic REMS [120], and that extracellular levels of hypocretin rise during REMS episodes [229]. However, these findings have never been considered conclusive due to technical limitations and scarce reproducibility. Using Ca^{2+} imaging, recent studies reported that LH^{HCRT} neurons are active during NREMS and REMS [119, 228]. However, these studies did not record PeF^{HCRT} cells. Our results are the first to describe that PeF^{HCRT} neurons are mainly REMS-active using an *in vivo* cell-population recording technique. We also recorded LH^{HCRT} and found similar results to the recent papers [119, 228]. However, we also observed a dichotomy between the LH^{HCRT} cell population and the more medial PeF^{HCRT} neurons. The former was found principally wake-active, and we also found a low activity during REMS and a scarce signal during NREMS. The latter instead, had similar low fluctuations during NREMS but a strong and evident signal during REMS and wake episodes.

Narcoleptic mice show a higher percentage of REMS, with shorter bout durations, compared to controls, indicating a more fragmented REMS. We conclude that sleep fragmentation is another result indicating the importance of hypocretin neurocircuitry in maintaining a physiological sleep architecture and stabilizing REMS. Supporting our findings, a recent work showed that hypocretin neurons can accumulate REMS pressure, and if mice do not dissipate this need in the first part of the day, they could express REMS abnormalities and unstructured sleep later during the same day [228]. In fact, in this study optogenetic inhibition of hypocretin cells during ZT3-8 increased total REMS during the subsequent ZT8-12 [228].

3.1.1. Hypocretin connectivity with the brainstem REMS circuits

A complex broad neurocircuits, composed of several REM-on nuclei, regulates REMS generation and its propagation throughout the brain [59]. Prof. Michel Jouvet demonstrated that the feline nucleus pontis oralis (NPO) is an important structure for REMS onset, and focal lesions to the NPO are sufficient to devoid the animals of REMS [230]. In addition, with his "pontine cat" preparation, which comprises all neurons caudal to the pons, Jouvet also demonstrated that REMS generation takes place at the brainstem level [231].

A series of experiments conducted on cats demonstrated that micro-injections of hypocretin-1 peptide in the NPO can induce REMS if the animal is in NREMS, while consolidate wakefulness if the peptide is injected during wakefulness [232-234]. The NPO plays a key role in generating REMS through its connections with the other brainstem cholinergic nuclei. Two main cholinergic nuclei of the brainstem, the LDT and the PPT, are implicated in both arousal and REMS generation [11]. Interestingly, immunoreactivity for hypocretin receptors, especially HcrtR1, is reported on both LDT and PPT [187], and hypocretin has an excitatory effect on the LDT/PPT cholinergic neurons that can potentiate the firing of NPO neurons [232, 235, 236]. Conversely, the NPO sends projections to glycinergic neurons of the ventral gigantocellular

reticular nucleus and the ventromedial medulla, which are inhibitory nuclei able to induce muscle atonia during REMS and cataplexy, because of their direct projections to spinal motoneurons [237, 238]. Therefore, when cholinergic neurons of the LDT/PPT are tonically active, there is an increase in the firing amplitude and frequency of NPO neurons and thus a conceivable facilitation for the induction of REMS.

Another important area for REMS generation is the dorsal part of the NPO, which is called the sublaterodorsal tegmental nucleus (SLD) in mice [239]. The SLD is mainly composed of REM-on glutamatergic neurons which express HcrtR1 and receives hypocretin efferents [109, 187]. Iontophoretic injection of kainic acid (a glutamate agonist) in the SLD excites local neurons and induces REMS-like state [240, 241]. Also, hypocretin can depolarize and increase the firing activity of SLD neurons, as shown *in vitro* [242]. A recent study provided strong evidence that hypocretin projections to the SLD can prolong REMS [242]. Using optogenetics, they excited hypocretin terminals in the SLD and extended REMS episodes. Nonetheless, they performed REMS deprivation and found an increased Fos expression in SLD-projecting hypocretin neurons after REMS [242]. Furthermore, optogenetic or chemogenetic silencing of hypocretin axons in the SLD destabilizes and shortens REMS bouts. Altogether, their findings provide strong evidence that hypocretin neurons have a role in REMS generation and stabilization.

3.1.2. Dichotomy of hypocretin neurons

Several research groups distinguished hypocretin neurons into two functional clusters, PeF/DMH and LH neurons. The principal functions of PeF/DMH hypocretin neurons are related to sleep-wake control, whereas LH^{HCRT} neurons participate in reward, feeding, and addiction processes [101, 243, 244]. Indeed, LH^{HCRT} neurons project strongly to the dopaminergic reward-associated areas, such as the VTA and the nucleus accumbens, or emotional processing structures, such as the central amygdala [245, 246]. In contrast, PeF^{HCRT} neurons project heavily

to the arousal-related areas, such as the LC and PPT/LDT [101, 104, 247]. Moreover, Estabrooke et al. reported that activation of Fos in PeF/DMH hypocretin neurons shows diurnal changes, consistent with a role in the production or maintenance of wakefulness, while LH^{HCRT} neurons do not show such diurnal variation [131]. Another study, using a two-chamber conditioned place preference experiment, showed that Fos activation in LH^{HCRT} neurons is highly correlated with the repeated preference for the drug-paired or food-paired environment, while no correlation was found in PeF/DMH hypocretin neurons [248].

Of note, although there is a general dichotomy between PeF/DMH and LH hypocretin neurons in terms of their functions, these distinctions are not always absolute, and there is still ongoing research to understand the complexity of hypocretin neurons and their functional distribution within the hypothalamus. In conclusion, since in the scientific field there is a general tendency to record hypocretin neurons located in the LH, there is a lack of studies on the sleep function of the PeF^{HCRT}. To our knowledge, our study is the first to report REMS-active PeF^{HCRT} neurons and provides evidence for the existence of an LH versus PeF/DMH hypocretin functional dichotomy.

3.2. A mediatory role for hypocretin neurons during NREMS

The LC is a key player in promoting wakefulness and maintaining arousal. Release of noradrenaline (or norepinephrine) from LC neurons enhances alertness, attention, and the ability to stay awake [11, 249]. Noradrenergic neurons in the LC send projections throughout the brain, including the cerebral cortex, thalamus, and wake-promoting areas [2]. The LC is involved in the transitions between different sleep-wake states [250]. Its activation leads to transitions from NREMS or REMS to wakefulness. LC neurons are less active during NREMS than during wakefulness, but they do not become entirely silent, instead continue to fire at a lower rate [170]. LC is a REM-off area and is completely silent during REMS [167].

3.2.1. Hypocretinergic control of NREMS LC reactivation

While the LC is typically more active during wakefulness and at transitions from sleep to wake, it also exhibits a unique pattern of activity during NREMS, which is referred to as NREMS LC^{NA} reactivations [166-169]. This infra-slow oscillation is important for the modulation of natural or sensory-related arousability and is hypothesized to contribute to memory consolidation processes during sleep [165, 168]. NREMS LC^{NA} reactivations can cluster thalamo-cortical spindles in time frames between LC reactivations as well as during LC silent periods before NREMS to REMS transitions [167, 172]. Every reactivation provides a window of sleep fragility and a potential awakening [166, 167, 251].

This phenomenon, NREMS LC^{NA} reactivation, is also found in our recordings from $Hcrt^{KO/KO}$ mice, indicating that hypocretin cells are not necessary for their occurrence. However, we found a higher rate of NREMS LC reactivations in $Hcrt^{KO/KO}$*xDbh-Cre* mice, where the time intervals between reactivations are almost halved. Thus, hypocretin presence could be important for the timing of NREMS LC^{NA} reactivations. Indeed, the hypothalamic hypocretin signal is correlated but is in advance of the noradrenaline fluctuations during NREMS, possibly having a role in causing or leading these fluctuations. The other less probable hypothesis is that hypocretin cells can inhibit LC^{NA} neurons. However, this is not confirmed by our data, as we found a correlation between the two signals.

In summary, less robustly organized and faster cycles of noradrenergic activity occur during NREMS in the absence of a functional hypocretin system. $Hcrt^{KO/KO}$ and control mice show similar amounts of NREMS but a higher number of bouts with shorter durations, indicating that NREMS architecture is highly affected. The shorter NREMS bouts are possibly a direct consequence of the higher NREMS LC^{NA} reactivations that are causing more frequent microarousals and awakenings.

3.2.2. Altered noradrenergic activity during REMS transitions

At transitions from REMS to wake, we found that LC^{NA} neurons are activated earlier in $Hcrt^{KO/KO}$ mice than controls, indicating that hypocretin neurons can organize the firing of noradrenergic cells prior to REMS to wake transitions [238, 252]. We hypothesize two mechanisms for the role of hypocretin at this transition. First, REMS-active hypocretin neurons have an inhibitory role on the LC^{NA} system during REMS, which helps to maintain REMS, and their absence causes fluctuations in LC^{NA} neurons and thus premature termination of REMS. Second, wake-active hypocretin neurons can modulate LC^{NA} neurons to activate them concomitantly at the REMS to wake transition, so that the transition is induced rapidly, considering that precise ensemble activation of LC neurons is crucial for quick and physiological transitions in vigilance states [159, 165].

At transitions from NREMS to REMS, we found that LC^{NA} neurons behave differently in $Hcrt^{KO/KO}$ mice compared to controls. The inactive periods of LC^{NA} neurons prior to REMS transitions are significantly shorter in $Hcrt^{KO/KO}$ mice compared to controls. The incapacity to shut down LC^{NA} neurons prior to REMS onset in narcoleptic mice could be explained by structural or functional deficits in communication between the different components of the sleep/arousal networks. Consequently, an unstable and disproportioned NREMS LC^{NA} reactivation can occur, and this prevents the brain from easily entering REMS. Another possibility is that hypocretin neurons can entrain NREMS LC^{NA} reactivations and dictate a slower pace of their occurrence, allowing for longer periods of LC inactivation and higher possibilities of NREMS to REMS transitions. Altogether, these findings suggest that hypocretin neurons can control the activity of LC^{NA} neurons during REMS transitions, where LC^{NA} activity is less coordinated, and potentially unstructured, in $Hcrt^{KO/KO}$ mice, therefore REMS architecture is detrimentally affected.

3.2.3. Functional connectivity of hypocretin and noradrenergic systems

LC is a wake-promoting structure able to produce awakenings when experimentally stimulated [163, 249]. Several studies indicated that hypocretin cells can control LC^{NA} spiking activity during wakefulness [81, 92, 93], and also mediate arousal from sleep through this LC^{NA} activation [164, 249]. In state transition analysis, we found a correlation between noradrenergic and hypocretinergic activity in wake-NREMS and NREMS-wake transitions, where activity of both decreases and increases, respectively. In addition, an increase in LH^{HCRT} neuronal activity also correlated with the LC^{NA} signal at the REMS to wake transitions. Hypocretin signal is always in advance of LC^{NA} activity during these transitions. However, we could not establish a direct relationship between the hypocretin and the noradrenergic systems. Therefore, further experiments with the help of optogenetics or chemogenetics are needed to test this possibility.

It is peculiar that we found REMS-active hypocretin neurons in the perifornical hypothalamus, but lack of activity in the LC^{NA} neurons that could be directly elicited by the former [111]. We hypothesize that sleep-generating and stabilizing mechanisms are strongly inhibiting RAS during REMS, while PeF^{HCRT} neurons are active. Another possibility is the existence of different subpopulations of hypocretin neurons that project to distinct REM-on and REM-off brain nuclei. Indeed, a previous study in rats showed that there is a specific and non-overlapping distribution of two hypocretin subpopulations projecting to the SLD and the LC [242]. The distribution of these two subpopulations was sparse through all the mediolateral axis of the posterior hypothalamus, therefore no clear distinction emerged between PeF versus LH localization of these two subpopulations. The existence of distinct hypocretin subpopulations projecting to REM-on SLD and REM-off LC nuclei can explain why hypocretin activity during REMS is not exciting the wake-promoting LC region. We hypothesize that LC-projecting hypocretin neurons are not active during REMS, but SLD-projecting hypocretin neurons are.

3.3. Hypocretinergic modulation of sleep oscillations

The slow oscillation, delta waves, and spindles are the dominant EEG activities during NREMS. We found a shift in the peak frequency of NREMS delta waves in *Hcrt*$^{KO/KO}$ mice compared to controls, suggesting that hyperactivated LC^{NA} neurons modulate thalamo-cortical networks, which are responsible for the generation of these waves. Furthermore, we quantified the number of spindles and found a significant decrease in *Hcrt*$^{KO/KO}$ mice, which can be explained due to a higher rate of NREMS LC^{NA} reactivations. Considering that these fluctuations are preventing spindle generation, higher LC activity would be detrimental for thalamo-cortical networks to develop spindles [167]. Spindle characteristics, including duration, central frequency, and number of spindle cycles also significantly decreased in *Hcrt*$^{KO/KO}$ mice. Alternations of sleep spindle structure are associated with memory and cognitive impairment, schizophrenia, and mental retardation [253-258], suggesting that they might be involved in sleep-dependent neuropsychological and memory impairments reported in narcolepsy patients [259-263].

REMS theta oscillations contribute to sleep-dependent memory consolidation, and interference in theta generation causes memory impairments [264-267]. Moreover, modulation of gamma power by the theta phase is linked to working and short-term memory [267]. We found a higher theta power and theta-gamma coupling during REMS in *Hcrt*$^{KO/KO}$ mice compared to controls. Lack of hypocretin increases theta power during cataplexy and REMS [140, 228, 268], while it decreases theta and theta-dominated periods during wakefulness [140], suggesting a differential role of hypocretin neurons in modulation of theta oscillations during REMS and wakefulness. Hypocretin neurons project to GABAergic and cholinergic neurons of the medial septum (MS), which in turn project to the hippocampus and can modulate the generation of theta rhythms [76, 269-271]. Specific lesions of MS cholinergic neurons decrease theta power, while specific lesions of GABAergic hypocretin-receiving neurons impede the generation of theta rhythms [269, 272, 273]. As both cholinergic and GABAergic neurons of the MS express dense HcrtR2

[271], hypocretin projections to the MS can control the generation of theta oscillations [76, 271]. Therefore, it is possible that REMS-active hypocretin neurons, presumably PeF^{HCRT} neurons, play an inhibitory role in the theta generation pathways during REMS, while wake-active hypocretin neurons, presumably LH^{HCRT} neurons, play an excitatory role.

3.4. Extended VLPO as a potential regulator of REMS

The eVLPO is an important area for sleep and is composed of GABAergic neurons principally active during REMS, as shown using c-Fos expression [78]. Using *in vivo* calcium imaging, we confirmed that $eVLPO^{GABA}$ neurons are highly active during REMS, and this activity is anti-correlated with the noradrenergic system. To our knowledge, this is the first *in vivo* study reporting $eVLPO^{GABA}$ activity during REMS in spontaneously behaving mice. In contrast, there is no evidence for the cVLPO activity during REMS, which has been shown using Ca^{2+} imaging, electrophysiology, and c-Fos experiments [77]. As recorded activity of $eVLPO^{GABA}$ neurons during NREMS is lower compared to other states, this suggests little contamination of recordings from the cVLPO region. We did not observe any difference in the calcium activity at transitions of vigilance states between controls and $Hcrt^{KO/KO}$ mice. However, the average of recorded calcium signals across vigilance states shows slight differences between the two genotypes, with a higher value for REMS and a lower one for wakefulness in $Hcrt^{KO/KO}$ mice compared to controls, suggesting state-specific modulation of $eVLPO^{GABA}$ neurons by the hypocretin system. We hypothesize that the heterogenicity in the activity of hypocretin neurons (PeF vs. LH) across REMS and wakefulness might be responsible for this finding.

The *Slc32* gene is the promoter for the expression of CRE protein in the *Vgat-Cre* line and allows possible recombination of a Cre-lox site with consequent translation and amplification of GECI virus-delivered GCaMP6m. *Slc32* is important for the transport of GABA-filled nano-vesicles from the soma of the neurons to the presynaptic area, where they are released [274].

There are at least two classes of neurons expressing *Slc32* in the VLPO, one produces the galanin neuropeptide along with the GABA neurotransmitter (hereafter VLPO^{GAL}), and the second one only expresses GABA and not galanin (hereafter VLPO^{GABA}) [86]. VLPO^{GAL} neurons, which project widely to wake-active areas, are sleep-active neurons and important for the generation or maintenance of sleep [77, 159], while VLPO^{GABA} neurons can control the excitability of regional micro-circuitries and are important for inhibiting VLPO^{GAL} neurons during wakefulness [86]. Calcium recordings of VLPO GABAergic neurons, using a miniscope or fiber photometry in the *Vgat-Cre* line, showed that these neurons are active across all vigilance states with different subgroups of neurons that fire in one or more states [275, 276]. However, these reports were based on the activity of cVLPO alone or both eVLPO and cVLPO together. In our recordings, which are centered solely on the eVLPO, there is no evident NREMS activity, but the signal is only present during REMS and wakefulness. Since local inhibitory microcircuits of GABAergic neurons can repress activity of NREM-active cVLPO galaninergic cells during wakefulness [86], we hypothesize that a similar microcircuit exists in the eVLPO, and we possibly recorded a strong activity of presumably eVLPO^{GAL} neurons during REMS and eVLPO^{GABA} cells during wakefulness. Thus, we suggest that the eVLPO^{GAL} neuronal activity is important for the generation and consolidation of REMS, while eVLPO^{GABA} cells for inhibiting REM-promoting eVLPO^{GAL} neurons during wakefulness to avoid entering REMS at inappropriate times.

In double implantation experiments, we found a strong anti-correlation between the eVLPO^{GABA} and the LC^{NA} neurons during REMS and at the NREMS-REMS or REMS-wake transitions. Their signal dynamics were mirrored to the other and evolved with similar intensity, suggesting a functional and direct connectivity between two regions, as reported by *ex vivo* tracing experiment [78]. To expand this notion *in vivo*, we recommend recording and manipulating eVLPO^{GAL} neurons in the Galanin-Cre line. A limitation of our investigation is

the possible contamination with cVLPO neuronal activity. Therefore, the weak calcium signal during NREMS could reflect proximal cVLPO activity. To rule out this possibility, we suggest the use of a high spatial resolution Ca^{2+} recording centered on the eVLPO.

3.5. Thalamo-cortical state switch

In our preliminary investigations of multisite LFP/unit recordings in thalamo-cortical sites, we found different classes of neurons at vigilance state transitions and could divide them into two main categories of "ON-OFF" and "transition" cells. Firing pattern of neurons in the first class changes abruptly at the transition moment, while the firing frequency of cells in the second class changes in a circa 2-3 seconds before the switch and shows a peak or a through at the transition point. After the transition is completed, they return to the similar firing frequency as before the state switch. Considering firing frequency, both fast-spiking (presumably interneurons) and slow-active (potentially excitatory) neurons were found in both of the categories. Since "transition" cells anticipate changes in the activity of "ON-OFF" neurons, we hypothesize that they could lead transitions at the regional microcircuit level and regulate local states.

Local sleep is an established concept in the field [197, 198, 277-282], and the opposite concept also exists and local desynchronized activity is important to allocate resources and enhance spiking activity in cortical areas that are sensory-stimulated or necessary for processing behavior-relevant information [283, 284]. State-transitions are emergent properties determined by the interaction of microcircuits and of sparse subcortical projections, thus some cortical areas can be in advance to other ones during transitions. For instance, we found that the motor cortex is in delay during NREMS-wake transitions, compared to the midline thalamus and other recorded cortical sites, suggesting that midline thalamic neurons do not directly activate this region at the transition time. Another possibility is that local cortical factors, such as

intracellular Cl^- or extracellular K^+ concentrations, determine the difference in timing of regional switching [285, 286].

In summary, our preliminary evidence points out the existence of at least two different categories of cells at state transitions. Their subtypes should be investigated and possibly manipulated to confirm our hypothesis regarding their role in controlling state transitions. In addition, why the motor cortex is in delay with respect to the other recorded regions is another finding that should be explored by exciting or inhibiting midline thalamic nuclei while recording this region. Moreover, we suggest using back-propagating tracing techniques to visualize the extent of their TC connectivity.

3.6. Conclusion

In the present thesis, I reported the results of research carried out using fiber photometry recording on the activity of neurons from the hypothalamus and brainstem. For this purpose, I used different genetic mouse lines coupled with AAV vectors to target specific populations of neurons. The focus of the experiments was on the neural activity during vigilance states and transitions between them.

We discovered a subpopulation of hypocretin neurons located in the PeF hypothalamus that are active during REMS and wakefulness. Furthermore, this subpopulation displays a low-level activation during NREMS. Consequently, the activity of the PeF hypocretin neurons is correlated with the LC^{NA} neurons during NREMS and wakefulness, but not during REMS since the LC is a REMS-off area. This is a very interesting novel result since there are only a few reports indicating that the hypocretin neurons are active during sleep, and to our knowledge there is no report on the PeF hypocretin neurons that are active during REMS. Indeed, the flip-flop model proposes that the hypocretin neurons are active and contribute only to the maintenance of the wakefulness state. Instead, we found active hypocretin cells both during

NREMS and REMS, and there are alterations in both sleep states in the absence of the functional hypocretin system.

The activity of LC during NREMS was recently reported. Here we found that the lack of hypocretin would lead to alternations in the rhythmicity of NREMS LC reactivations. In *Hcrt*$^{KO/KO}$ mice, the time between each NREMS LC reactivation is almost halved and therefore the number of reactivations is almost doubled. The NREMS LC reactivations represent a "window of fragility" for the NREMS and this is reflected in a higher number of NREMS bouts with a shorter duration. The main hypothesis proposed in this thesis is that more frequent NREMS LC reactivations lead to a less stable and more fragile NREMS. Although this result was not tested for its behavioral effects, it is possible to speculate that memory formation could also be detrimentally affected and/or that the restorative function of sleep could not be completely achieved. Therefore, it is necessary to better explore the connection between the NREMS LC reactivations in the absence of the functional hypocretin system and the consequences on the behavioral performance using different memory-related tasks.

The claim resulting from our investigations is that there is a time-pacing function for the hypocretin system in organizing NREMS LC reactivations and the ability to control the vigilance states maintenance and transitions. This role of the hypocretin cells is reflected in consolidated NREMS in the presence of a functional hypocretin system. In contrast, the hypocretin-deleted mice cannot express physiological NREMS and experience a fragmented sleep/wake cycle. Effects due to the lack of hypocretin were also seen at the EEG level. We did find that NREMS spindles occurred with different electrophysiological characteristics and at a lower rate in *Hcrt*$^{KO/KO}$ mice, since their occurrence is indirectly inhibited by the hyperactivity of the LC during NREMS.

In *Hcrt*$^{KO/KO}$ mice, REMS bouts are more frequent and of shorter duration, and the LC silent period necessary to switch from NREMS to REMS is also shorter compared to controls. The

total amount of REMS is also higher in the absence of hypocretin neuropeptides. In addition, in this phase of sleep, we found noticeable effects at the EEG level, with higher theta power and higher theta-gamma coupling. The unexpected ability of hypocretin neurons to stabilize REMS could foster scientific knowledge on clinical conditions such as SOREMP, RBD, and narcolepsy with cataplexy, and explain the presence of fragmented and intrusive REMS or REMS-like events in patients where the hypocretin system is absent or impaired.

Additionally, I recorded the activity of inhibitory GABAergic neurons in the eVLPO, where the seminal papers have proposed the involvement of this area in REMS regulation. I found that eVLPO GABAergic neurons are highly active during REMS, and in particular, their activity increases before the REMS onset and has sustained activity during the whole REMS episode. However, since we did not address the use of optogenetics we could not assess if the activity of eVLPO is sufficient or complementary for the generation and maintenance of REMS.

The findings reported in this thesis on hypocretin neurons active during both REMS and NREMS represent an important step forward in understanding the mechanisms of vigilance states control. However, they deserve further investigations to reconsider the functions of hypocretin neuropeptides in promoting and stabilizing not only wakefulness, but also sleep. The present results should lead us to consider hypocretin as an active component in sleep neuromodulation. The current models of sleep-wake regulation, e.g., the flip-flop model, should be revisited, and more research should be performed in the future on this intriguing question.

References

1. Luppi, P.H. and P. Fort, *Neuroanatomical and Neurochemical Bases of Vigilance States.* Handb Exp Pharmacol, 2019. **253**: p. 35-58.
2. Brown, R.E., et al., *Control of sleep and wakefulness.* Physiol Rev, 2012. **92**(3): p. 1087-187.
3. Holst, S.C. and H.P. Landolt, *Sleep-Wake Neurochemistry.* Sleep Med Clin, 2022. **17**(2): p. 151-160.
4. Adamantidis, A.R., C. Gutierrez Herrera, and T.C. Gent, *Oscillating circuitries in the sleeping brain.* Nat Rev Neurosci, 2019. **20**(12): p. 746-762.
5. Eban-Rothschild, A., L. Appelbaum, and L. de Lecea, *Neuronal Mechanisms for Sleep/Wake Regulation and Modulatory Drive.* Neuropsychopharmacology, 2018. **43**(5): p. 937-952.
6. Steriade, M., *Arousal: revisiting the reticular activating system.* Science, 1996. **272**(5259): p. 225-6.
7. Jones, B.E., *Arousal and sleep circuits.* Neuropsychopharmacology, 2020. **45**(1): p. 6-20.
8. Li, S.B. and L. de Lecea, *The hypocretin (orexin) system: from a neural circuitry perspective.* Neuropharmacology, 2020. **167**: p. 107993.
9. Li, S.B., W.J. Giardino, and L. de Lecea, *Hypocretins and Arousal.* Curr Top Behav Neurosci, 2017. **33**: p. 93-104.
10. Nishino, S., *Hypothalamus, hypocretins/orexin, and vigilance control.* Handb Clin Neurol, 2011. **99**: p. 765-82.
11. Jones, B.E., *Arousal systems.* Front Biosci, 2003. **8**: p. s438-51.
12. Steriade, M., *The corticothalamic system in sleep.* Front Biosci, 2003. **8**: p. d878-99.
13. Halassa, M.M., *The Thalamus*. 2022, Cambridge: Cambridge University Press.
14. Halassa, M.M., *Thalamocortical dynamics of sleep: roles of purinergic neuromodulation.* Semin Cell Dev Biol, 2011. **22**(2): p. 245-51.
15. da Mota Gomes, M., *Encephalitis lethargica epidemic milestones in early sleep neurobiology researches.* Sleep Med, 2020. **74**: p. 349-356.
16. Sak, J. and A. Grzybowski, *Brain and aviation: on the 80th anniversary of Constantin von Economo's (1876-1931) death.* Neurol Sci, 2013. **34**(3): p. 387-91.
17. Berger, H., *Über das Elektrenkephalogramm des Menschen.* Archiv für Psychiatrie und Nervenkrankheiten, 1929. **87**(1): p. 527-570.
18. Aserinsky, E. and N. Kleitman, *Regularly occurring periods of eye motility, and concomitant phenomena, during sleep.* Science, 1953. **118**(3062): p. 273-4.
19. Li, J., M.V. Vitiello, and N.S. Gooneratne, *Sleep in Normal Aging.* Sleep Med Clin, 2018. **13**(1): p. 1-11.
20. Tsai, S.C., *Excessive sleepiness.* Clin Chest Med, 2010. **31**(2): p. 341-51.
21. Yaremchuk, K., *Sleep Disorders in the Elderly.* Clin Geriatr Med, 2018. **34**(2): p. 205-216.
22. Thorpy, M.J., *Classification of sleep disorders.* Neurotherapeutics, 2012. **9**(4): p. 687-701.
23. Ohayon, M.M., et al., *How age and daytime activities are related to insomnia in the general population: consequences for older people.* J Am Geriatr Soc, 2001. **49**(4): p. 360-6.
24. Ellenbogen, J.M., *Sleepiness.* Semin Neurol, 2016. **36**(5): p. 449-455.
25. Gandhi, K.D., et al., *Excessive Daytime Sleepiness: A Clinical Review.* Mayo Clin Proc, 2021. **96**(5): p. 1288-1301.

26. Schneider, L., *Neurobiology and Neuroprotective Benefits of Sleep.* Continuum (Minneap Minn), 2020. **26**(4): p. 848-870.
27. Krueger, J.M., et al., *Sleep function: Toward elucidating an enigma.* Sleep Med Rev, 2016. **28**: p. 46-54.
28. Zielinski, M.R. and J.M. Krueger, *Sleep and innate immunity.* Front Biosci (Schol Ed), 2011. **3**(2): p. 632-42.
29. Yankova, G., O. Bogomyakova, and A. Tulupov, *The glymphatic system and meningeal lymphatics of the brain: new understanding of brain clearance.* Rev Neurosci, 2021. **32**(7): p. 693-705.
30. Vyazovskiy, V.V. and K.D. Harris, *Sleep and the single neuron: the role of global slow oscillations in individual cell rest.* Nat Rev Neurosci, 2013. **14**(6): p. 443-51.
31. Rasmussen, M.K., H. Mestre, and M. Nedergaard, *The glymphatic pathway in neurological disorders.* Lancet Neurol, 2018. **17**(11): p. 1016-1024.
32. Xie, L., et al., *Sleep drives metabolite clearance from the adult brain.* Science, 2013. **342**(6156): p. 373-7.
33. Jessen, N.A., et al., *The Glymphatic System: A Beginner's Guide.* Neurochem Res, 2015. **40**(12): p. 2583-99.
34. Siegel, J.M., *Clues to the functions of mammalian sleep.* Nature, 2005. **437**(7063): p. 1264-71.
35. Nath, R.D., et al., *The Jellyfish Cassiopea Exhibits a Sleep-like State.* Curr Biol, 2017. **27**(19): p. 2984-2990 e3.
36. Allada, R. and J.M. Siegel, *Unearthing the phylogenetic roots of sleep.* Curr Biol, 2008. **18**(15): p. R670-R679.
37. Miyazaki, S., C.Y. Liu, and Y. Hayashi, *Sleep in vertebrate and invertebrate animals, and insights into the function and evolution of sleep.* Neurosci Res, 2017. **118**: p. 3-12.
38. Rechtschaffen, A., et al., *Physiological correlates of prolonged sleep deprivation in rats.* Science, 1983. **221**(4606): p. 182-4.
39. Shaw, P.J., et al., *Stress response genes protect against lethal effects of sleep deprivation in Drosophila.* Nature, 2002. **417**(6886): p. 287-91.
40. Siegel, J.M., *Sleep function: an evolutionary perspective.* Lancet Neurol, 2022. **21**(10): p. 937-946.
41. Tononi, G. and C. Cirelli, *Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration.* Neuron, 2014. **81**(1): p. 12-34.
42. Girardeau, G. and V. Lopes-Dos-Santos, *Brain neural patterns and the memory function of sleep.* Science, 2021. **374**(6567): p. 560-564.
43. Tononi, G. and C. Cirelli, *Sleep and synaptic down-selection.* Eur J Neurosci, 2020. **51**(1): p. 413-421.
44. Lokhandwala, S. and R.M.C. Spencer, *Relations between sleep patterns early in life and brain development: A review.* Dev Cogn Neurosci, 2022. **56**: p. 101130.
45. Kelly, J.M. and M.T. Bianchi, *Mammalian sleep genetics.* Neurogenetics, 2012. **13**(4): p. 287-326.
46. Kos, C.H., *Cre/loxP system for generating tissue-specific knockout mouse models.* Nutr Rev, 2004. **62**(6 Pt 1): p. 243-6.
47. Bouabe, H. and K. Okkenhaug, *Gene targeting in mice: a review.* Methods Mol Biol, 2013. **1064**: p. 315-36.
48. Qian, T., et al., *Current and emerging methods for probing neuropeptide transmission.* Curr Opin Neurobiol, 2023. **81**: p. 102751.
49. Oh, J., C. Lee, and B.K. Kaang, *Imaging and analysis of genetically encoded calcium indicators linking neural circuits and behaviors.* Korean J Physiol Pharmacol, 2019. **23**(4): p. 237-249.

50. Feng, J., et al., *A Genetically Encoded Fluorescent Sensor for Rapid and Specific In Vivo Detection of Norepinephrine.* Neuron, 2019. **102**(4): p. 745-761 e8.
51. Duffet, L., et al., *A genetically encoded sensor for in vivo imaging of orexin neuropeptides.* Nat Methods, 2022. **19**(2): p. 231-241.
52. Merten, J.E., et al., *The use of rodent models to better characterize the relationship among epilepsy, sleep, and memory.* Epilepsia, 2022. **63**(3): p. 525-536.
53. Le Bon, O., et al., *Ultradian cycles in mice: definitions and links with REMS and NREMS.* J Comp Physiol A Neuroethol Sens Neural Behav Physiol, 2007. **193**(10): p. 1021-32.
54. Davis, C.J., et al., *Delta wave power: an independent sleep phenotype or epiphenomenon?* J Clin Sleep Med, 2011. **7**(5 Suppl): p. S16-8.
55. Vyazovskiy, V.V., et al., *Cortical firing and sleep homeostasis.* Neuron, 2009. **63**(6): p. 865-78.
56. Luthi, A., *Sleep Spindles: Where They Come From, What They Do.* Neuroscientist, 2014. **20**(3): p. 243-56.
57. Fernandez, L.M.J. and A. Luthi, *Sleep Spindles: Mechanisms and Functions.* Physiol Rev, 2020. **100**(2): p. 805-868.
58. Luppi, P.H., O. Clement, and P. Fort, *Paradoxical (REM) sleep genesis by the brainstem is under hypothalamic control.* Curr Opin Neurobiol, 2013. **23**(5): p. 786-92.
59. Luppi, P.H. and P. Fort, *Sleep-wake physiology.* Handb Clin Neurol, 2019. **160**: p. 359-370.
60. Cardis, R., et al., *Cortico-autonomic local arousals and heightened somatosensory arousability during NREMS of mice in neuropathic pain.* Elife, 2021. **10**.
61. Vassalli, A. and D.J. Dijk, *Sleep function: current questions and new approaches.* Eur J Neurosci, 2009. **29**(9): p. 1830-41.
62. Kryger, M.H., et al., *Principles and practice of sleep medicine*. Seventh edition. ed. 2022, Philadelphia, PA: Elsevier.
63. Borbely, A.A., *A two process model of sleep regulation.* Hum Neurobiol, 1982. **1**(3): p. 195-204.
64. Borbely, A.A., *Sleep regulation. Introduction.* Hum Neurobiol, 1982. **1**(3): p. 161-2.
65. Welsh, D.K., J.S. Takahashi, and S.A. Kay, *Suprachiasmatic nucleus: cell autonomy and network properties.* Annu Rev Physiol, 2010. **72**: p. 551-77.
66. Hastings, M.H., E.S. Maywood, and M. Brancaccio, *Generation of circadian rhythms in the suprachiasmatic nucleus.* Nat Rev Neurosci, 2018. **19**(8): p. 453-469.
67. Gamaldo, C.E., A.K. Shaikh, and J.C. McArthur, *The sleep-immunity relationship.* Neurol Clin, 2012. **30**(4): p. 1313-43.
68. Krueger, J.M., *The role of cytokines in sleep regulation.* Curr Pharm Des, 2008. **14**(32): p. 3408-16.
69. Maret, S., et al., *Homer1a is a core brain molecular correlate of sleep loss.* Proc Natl Acad Sci U S A, 2007. **104**(50): p. 20090-5.
70. Franken, P., D. Chollet, and M. Tafti, *The homeostatic regulation of sleep need is under genetic control.* J Neurosci, 2001. **21**(8): p. 2610-21.
71. Scammell, T.E., E. Arrigoni, and J.O. Lipton, *Neural Circuitry of Wakefulness and Sleep.* Neuron, 2017. **93**(4): p. 747-765.
72. Moruzzi, G. and H.W. Magoun, *Brain stem reticular formation and activation of the EEG.* Electroencephalogr Clin Neurophysiol, 1949. **1**(4): p. 455-73.
73. Kovalzon, V.M., *Ascending Reticular Activating System of the Brain.* Translational Neuroscience and Clinics, 2016. **2**(4): p. 275-285.
74. Venner, A., et al., *Reassessing the Role of Histaminergic Tuberomammillary Neurons in Arousal Control.* J Neurosci, 2019. **39**(45): p. 8929-8939.

75. Bear, M.F., B.W. Connors, and M.A. Paradiso, *Neuroscience: Exploring the Brain*. 2016: Wolters Kluwer.
76. Arrigoni, E., T. Mochizuki, and T.E. Scammell, *Activation of the basal forebrain by the orexin/hypocretin neurones.* Acta Physiol (Oxf), 2010. **198**(3): p. 223-35.
77. Arrigoni, E. and P.M. Fuller, *The Sleep-Promoting Ventrolateral Preoptic Nucleus: What Have We Learned over the Past 25 Years?* Int J Mol Sci, 2022. **23**(6).
78. Lu, J., et al., *Selective activation of the extended ventrolateral preoptic nucleus during rapid eye movement sleep.* J Neurosci, 2002. **22**(11): p. 4568-76.
79. Alam, M.A., et al., *Neuronal activity in the preoptic hypothalamus during sleep deprivation and recovery sleep.* J Neurophysiol, 2014. **111**(2): p. 287-99.
80. Lu, J., et al., *Effect of lesions of the ventrolateral preoptic nucleus on NREM and REM sleep.* J Neurosci, 2000. **20**(10): p. 3830-42.
81. Saper, C.B., T.C. Chou, and T.E. Scammell, *The sleep switch: hypothalamic control of sleep and wakefulness.* Trends Neurosci, 2001. **24**(12): p. 726-31.
82. Gong, H., et al., *Activation of c-fos in GABAergic neurones in the preoptic area during sleep and in response to sleep deprivation.* J Physiol, 2004. **556**(Pt 3): p. 935-46.
83. Choi, I.S., et al., *Astrocyte-derived adenosine excites sleep-promoting neurons in the ventrolateral preoptic nucleus: Astrocyte-neuron interactions in the regulation of sleep.* Glia, 2022. **70**(10): p. 1864-1885.
84. Gallopin, T., et al., *Identification of sleep-promoting neurons in vitro.* Nature, 2000. **404**(6781): p. 992-5.
85. Saito, Y.C., et al., *Monoamines Inhibit GABAergic Neurons in Ventrolateral Preoptic Area That Make Direct Synaptic Connections to Hypothalamic Arousal Neurons.* J Neurosci, 2018. **38**(28): p. 6366-6378.
86. De Luca, R., et al., *Orexin neurons inhibit sleep to promote arousal.* Nat Commun, 2022. **13**(1): p. 4163.
87. Luppi, P.H., C. Peyron, and P. Fort, *Role of MCH neurons in paradoxical (REM) sleep control.* Sleep, 2013. **36**(12): p. 1775-6.
88. Verret, L., et al., *A role of melanin-concentrating hormone producing neurons in the central regulation of paradoxical sleep.* BMC Neurosci, 2003. **4**: p. 19.
89. Jego, S., et al., *Optogenetic identification of a rapid eye movement sleep modulatory circuit in the hypothalamus.* Nat Neurosci, 2013. **16**(11): p. 1637-43.
90. Fuller, P.M., J.J. Gooley, and C.B. Saper, *Neurobiology of the sleep-wake cycle: sleep architecture, circadian regulation, and regulatory feedback.* J Biol Rhythms, 2006. **21**(6): p. 482-93.
91. Saper, C.B., et al., *Sleep state switching.* Neuron, 2010. **68**(6): p. 1023-42.
92. Saper, C.B., T.E. Scammell, and J. Lu, *Hypothalamic regulation of sleep and circadian rhythms.* Nature, 2005. **437**(7063): p. 1257-63.
93. Lu, J., et al., *A putative flip-flop switch for control of REM sleep.* Nature, 2006. **441**(7093): p. 589-94.
94. Morin, C.M., et al., *Insomnia disorder.* Nat Rev Dis Primers, 2015. **1**(1): p. 15026.
95. Liu, Y.W., J. Li, and J.H. Ye, *Histamine regulates activities of neurons in the ventrolateral preoptic nucleus.* J Physiol, 2010. **588**(Pt 21): p. 4103-16.
96. Tyree, S.M., J.C. Borniger, and L. de Lecea, *Hypocretin as a Hub for Arousal and Motivation.* Front Neurol, 2018. **9**: p. 413.
97. Sakurai, T., *Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis.* Sleep Med Rev, 2005. **9**(4): p. 231-41.
98. Sakurai, T., et al., *Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior.* Cell, 1998. **92**(4): p. 573-85.

99. de Lecea, L., et al., *The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity.* Proc Natl Acad Sci U S A, 1998. **95**(1): p. 322-7.
100. Nishino, S. and T. Sakurai, *The Orexin/Hypocretin System: Physiology and Pathophysiology*. 1. Aufl. ed. 2005, Totowa, NJ: Humana Press. 397-397.
101. Harris, G.C. and G. Aston-Jones, *Arousal and reward: a dichotomy in orexin function.* Trends Neurosci, 2006. **29**(10): p. 571-7.
102. Thompson, M.D., et al., *OX1 and OX2 orexin/hypocretin receptor pharmacogenetics.* Front Neurosci, 2014. **8**: p. 57.
103. Bourgin, P., et al., *Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons.* J Neurosci, 2000. **20**(20): p. 7760-5.
104. Greco, M.A. and P.J. Shiromani, *Hypocretin receptor protein and mRNA expression in the dorsolateral pons of rats.* Brain Res Mol Brain Res, 2001. **88**(1-2): p. 176-82.
105. Chemelli, R.M., et al., *Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation.* Cell, 1999. **98**(4): p. 437-51.
106. Lin, L., et al., *The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene.* Cell, 1999. **98**(3): p. 365-76.
107. Mignot, E.J., *History of narcolepsy at Stanford University.* Immunol Res, 2014. **58**(2-3): p. 315-39.
108. Kohlmeier, K.A., et al., *Differential actions of orexin receptors in brainstem cholinergic and monoaminergic neurons revealed by receptor knockouts: implications for orexinergic signaling in arousal and narcolepsy.* Front Neurosci, 2013. **7**: p. 246.
109. Peyron, C., et al., *Neurons containing hypocretin (orexin) project to multiple neuronal systems.* J Neurosci, 1998. **18**(23): p. 9996-10015.
110. Kukkonen, J.P. and C.S. Leonard, *Orexin/hypocretin receptor signalling cascades.* Br J Pharmacol, 2014. **171**(2): p. 314-31.
111. Sakurai, T., Y.C. Saito, and M. Yanagisawa, *Interaction between Orexin Neurons and Monoaminergic Systems.* Front Neurol Neurosci, 2021. **45**: p. 11-21.
112. Espana, R.A., et al., *Wake-promoting and sleep-suppressing actions of hypocretin (orexin): basal forebrain sites of action.* Neuroscience, 2001. **106**(4): p. 699-715.
113. Sun, Y., R.K. Tisdale, and T.S. Kilduff, *Hypocretin/Orexin Receptor Pharmacology and Sleep Phases.* Front Neurol Neurosci, 2021. **45**: p. 22-37.
114. Mahoney, C.E., T. Mochizuki, and T.E. Scammell, *Dual orexin receptor antagonists increase sleep and cataplexy in wild type mice.* Sleep, 2020. **43**(6).
115. Li, S.B., et al., *Optical probing of orexin/hypocretin receptor antagonists.* Sleep, 2018. **41**(10).
116. Kilduff, T.S. and C. Peyron, *The hypocretin/orexin ligand-receptor system: implications for sleep and sleep disorders.* Trends Neurosci, 2000. **23**(8): p. 359-65.
117. Guillaumin, M.C.C. and D. Burdakov, *Neuropeptides as Primary Mediators of Brain Circuit Connectivity.* Front Neurosci, 2021. **15**: p. 644313.
118. Gonzalez, J.A., et al., *Inhibitory Interplay between Orexin Neurons and Eating.* Curr Biol, 2016. **26**(18): p. 2486-2491.
119. Li, S.B., et al., *Hyperexcitable arousal circuits drive sleep instability during aging.* Science, 2022. **375**(6583): p. eabh3021.
120. Lee, M.G., O.K. Hassani, and B.E. Jones, *Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle.* J Neurosci, 2005. **25**(28): p. 6716-20.
121. Peleg-Raibstein, D., P. Viskaitis, and D. Burdakov, *Eat, seek, rest? An orexin/hypocretin perspective.* J Neuroendocrinol, 2023. **35**(9): p. e13259.
122. Williams, R.H. and D. Burdakov, *Hypothalamic orexins/hypocretins as regulators of breathing.* Expert Rev Mol Med, 2008. **10**: p. e28.

123. Grujic, N., et al., *Control and coding of pupil size by hypothalamic orexin neurons.* Nat Neurosci, 2023. **26**(7): p. 1160-1164.
124. Sakurai, T., *The role of orexin in motivated behaviours.* Nat Rev Neurosci, 2014. **15**(11): p. 719-31.
125. Borgland, S.L., et al., *Orexin A/hypocretin-1 selectively promotes motivation for positive reinforcers.* J Neurosci, 2009. **29**(36): p. 11215-25.
126. Dawson, M., et al., *Hypocretin/orexin neurons encode social discrimination and exhibit a sex-dependent necessity for social interaction.* Cell Rep, 2023. **42**(7): p. 112815.
127. Eban-Rothschild, A., et al., *VTA dopaminergic neurons regulate ethologically relevant sleep-wake behaviors.* Nat Neurosci, 2016. **19**(10): p. 1356-66.
128. Gonzalez, J.A., et al., *Metabolism-independent sugar sensing in central orexin neurons.* Diabetes, 2008. **57**(10): p. 2569-76.
129. Williams, R.H., et al., *Adaptive sugar sensors in hypothalamic feeding circuits.* Proc Natl Acad Sci U S A, 2008. **105**(33): p. 11975-80.
130. Willie, J.T., et al., *To eat or to sleep? Orexin in the regulation of feeding and wakefulness.* Annu Rev Neurosci, 2001. **24**: p. 429-58.
131. Estabrooke, I.V., et al., *Fos expression in orexin neurons varies with behavioral state.* J Neurosci, 2001. **21**(5): p. 1656-62.
132. Freeman, L.R. and G. Aston-Jones, *Activation of medial hypothalamic orexin neurons during a Go/No-Go task.* Brain Res, 2020. **1731**: p. 145928.
133. James, M.H., et al., *Increased Number and Activity of a Lateral Subpopulation of Hypothalamic Orexin/Hypocretin Neurons Underlies the Expression of an Addicted State in Rats.* Biol Psychiatry, 2019. **85**(11): p. 925-935.
134. Latifi, B., et al., *Sleep-Wake Cycling and Energy Conservation: Role of Hypocretin and the Lateral Hypothalamus in Dynamic State-Dependent Resource Optimization.* Front Neurol, 2018. **9**: p. 790.
135. Peleg-Raibstein, D. and D. Burdakov, *Do orexin/hypocretin neurons signal stress or reward?* Peptides, 2021. **145**: p. 170629.
136. Giardino, W.J. and L. de Lecea, *Hypocretin (orexin) neuromodulation of stress and reward pathways.* Curr Opin Neurobiol, 2014. **29**: p. 103-8.
137. Barateau, L., et al., *Narcolepsy.* J Sleep Res, 2022. **31**(4): p. e13631.
138. Mignot, E., et al., *Sleep Problems in Narcolepsy and the Role of Hypocretin/Orexin Deficiency.* Front Neurol Neurosci, 2021. **45**: p. 103-116.
139. Liblau, R.S., et al., *Hypocretin (orexin) biology and the pathophysiology of narcolepsy with cataplexy.* Lancet Neurol, 2015. **14**(3): p. 318-28.
140. Vassalli, A., et al., *Electroencephalogram paroxysmal theta characterizes cataplexy in mice and children.* Brain, 2013. **136**(Pt 5): p. 1592-608.
141. Bassetti, C.L.A., et al., *Narcolepsy - clinical spectrum, aetiopathophysiology, diagnosis and treatment.* Nat Rev Neurol, 2019. **15**(9): p. 519-539.
142. Dauvilliers, Y., et al., *CSF hypocretin-1 levels in narcolepsy, Kleine-Levin syndrome, and other hypersomnias and neurological conditions.* J Neurol Neurosurg Psychiatry, 2003. **74**(12): p. 1667-73.
143. Seifinejad, A., et al., *Epigenetic silencing of selected hypothalamic neuropeptides in narcolepsy with cataplexy.* Proc Natl Acad Sci U S A, 2023. **120**(19): p. e2220911120.
144. Antelmi, E., et al., *REM sleep behavior disorder in narcolepsy: A secondary form or an intrinsic feature?* Sleep Med Rev, 2020. **50**: p. 101254.
145. Peever, J., P.H. Luppi, and J. Montplaisir, *Breakdown in REM sleep circuitry underlies REM sleep behavior disorder.* Trends Neurosci, 2014. **37**(5): p. 279-88.
146. Dauvilliers, Y., et al., *REM sleep behaviour disorder.* Nat Rev Dis Primers, 2018. **4**(1): p. 19.

147. Tafti, M., et al., *Sleep onset rapid-eye-movement episodes in narcolepsy: REM sleep pressure or nonREM-REM sleep dysregulation?* J Sleep Res, 1992. **1**(4): p. 245-250.
148. Mignot, E., et al., *Correlates of sleep-onset REM periods during the Multiple Sleep Latency Test in community adults.* Brain, 2006. **129**(Pt 6): p. 1609-23.
149. Dahlstrom, A. and K. Fuxe, *Localization of monoamines in the lower brain stem.* Experientia, 1964. **20**(7): p. 398-9.
150. Chandler, D.J., et al., *Redefining Noradrenergic Neuromodulation of Behavior: Impacts of a Modular Locus Coeruleus Architecture.* J Neurosci, 2019. **39**(42): p. 8239-8249.
151. Maeda, T., *The locus coeruleus: history.* J Chem Neuroanat, 2000. **18**(1-2): p. 57-64.
152. Wenzel, J. and C. Wenzel, *De penitiori structura cerebri.* Tubing, 1812. **18**(2): p. 168.
153. Szabadi, E., *Functional neuroanatomy of the central noradrenergic system.* J Psychopharmacol, 2013. **27**(8): p. 659-93.
154. Samuels, E.R. and E. Szabadi, *Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part I: principles of functional organisation.* Curr Neuropharmacol, 2008. **6**(3): p. 235-53.
155. Livett, B.G., *Histochemical visualization of peripheral and central adrenergic neurones.* Br Med Bull, 1973. **29**(2): p. 93-9.
156. Descarries, L. and N. Mechawar, *Ultrastructural evidence for diffuse transmission by monoamine and acetylcholine neurons of the central nervous system.* Prog Brain Res, 2000. **125**: p. 27-47.
157. Wahis, J. and M.G. Holt, *Astrocytes, Noradrenaline, alpha1-Adrenoreceptors, and Neuromodulation: Evidence and Unanswered Questions.* Front Cell Neurosci, 2021. **15**: p. 645691.
158. Huang, H.P., et al., *Physiology of quantal norepinephrine release from somatodendritic sites of neurons in locus coeruleus.* Front Mol Neurosci, 2012. **5**: p. 29.
159. Liang, Y., et al., *The NAergic locus coeruleus-ventrolateral preoptic area neural circuit mediates rapid arousal from sleep.* Curr Biol, 2021. **31**(17): p. 3729-3742 e5.
160. Yamanaka, A., et al., *Hypothalamic orexin neurons regulate arousal according to energy balance in mice.* Neuron, 2003. **38**(5): p. 701-13.
161. Li, Y. and A.N. van den Pol, *Direct and indirect inhibition by catecholamines of hypocretin/orexin neurons.* J Neurosci, 2005. **25**(1): p. 173-83.
162. Uschakov, A., et al., *Sleep-deprivation regulates alpha-2 adrenergic responses of rat hypocretin/orexin neurons.* PLoS One, 2011. **6**(2): p. e16672.
163. Carter, M.E., L. de Lecea, and A. Adamantidis, *Functional wiring of hypocretin and LC-NE neurons: implications for arousal.* Front Behav Neurosci, 2013. **7**: p. 43.
164. Carter, M.E., et al., *Mechanism for Hypocretin-mediated sleep-to-wake transitions.* Proc Natl Acad Sci U S A, 2012. **109**(39): p. E2635-44.
165. Hayat, H., et al., *Locus coeruleus norepinephrine activity mediates sensory-evoked awakenings from sleep.* Sci Adv, 2020. **6**(15): p. eaaz4232.
166. Kjaerby, C., et al., *Memory-enhancing properties of sleep depend on the oscillatory amplitude of norepinephrine.* Nat Neurosci, 2022. **25**(8): p. 1059-1070.
167. Osorio-Forero, A., et al., *Noradrenergic circuit control of non-REM sleep substates.* Curr Biol, 2021. **31**(22): p. 5009-5023 e7.
168. Swift, K.M., et al., *Abnormal Locus Coeruleus Sleep Activity Alters Sleep Signatures of Memory Consolidation and Impairs Place Cell Stability and Spatial Memory.* Curr Biol, 2018. **28**(22): p. 3599-3609 e4.
169. Antila, H., et al., *A noradrenergic-hypothalamic neural substrate for stress-induced sleep disturbances.* Proc Natl Acad Sci U S A, 2022. **119**(45): p. e2123528119.
170. Osorio-Forero, A., et al., *When the Locus Coeruleus Speaks Up in Sleep: Recent Insights, Emerging Perspectives.* Int J Mol Sci, 2022. **23**(9).

171. Krone, L.B., et al., *A role for the cortex in sleep-wake regulation.* Nat Neurosci, 2021. **24**(9): p. 1210-1215.
172. Bandarabadi, M., et al., *A role for spindles in the onset of rapid eye movement sleep.* Nat Commun, 2020. **11**(1): p. 5247.
173. Gent, T.C., et al., *Thalamic dual control of sleep and wakefulness.* Nat Neurosci, 2018. **21**(7): p. 974-984.
174. Llinas, R.R. and M. Steriade, *Bursting of thalamic neurons and states of vigilance.* J Neurophysiol, 2006. **95**(6): p. 3297-308.
175. Deschenes, M., J.P. Roy, and M. Steriade, *Thalamic bursting mechanism: an inward slow current revealed by membrane hyperpolarization.* Brain Res, 1982. **239**(1): p. 289-93.
176. Steriade, M. and M. Deschenes, *The thalamus as a neuronal oscillator.* Brain Res, 1984. **320**(1): p. 1-63.
177. Deschenes, M., et al., *Electrophysiology of neurons of lateral thalamic nuclei in cat: resting properties and burst discharges.* J Neurophysiol, 1984. **51**(6): p. 1196-219.
178. Jahnsen, H. and R. Llinas, *Ionic basis for the electro-responsiveness and oscillatory properties of guinea-pig thalamic neurones in vitro.* J Physiol, 1984. **349**: p. 227-47.
179. Llinas, R. and H. Jahnsen, *Electrophysiology of mammalian thalamic neurones in vitro.* Nature, 1982. **297**(5865): p. 406-8.
180. Steriade, M. and R.R. Llinas, *The functional states of the thalamus and the associated neuronal interplay.* Physiol Rev, 1988. **68**(3): p. 649-742.
181. Sharon, O. and Y. Nir, *Attenuated Fast Steady-State Visual Evoked Potentials During Human Sleep.* Cereb Cortex, 2018. **28**(4): p. 1297-1311.
182. Nir, Y., et al., *Auditory responses and stimulus-specific adaptation in rat auditory cortex are preserved across NREM and REM sleep.* Cereb Cortex, 2015. **25**(5): p. 1362-78.
183. Steriade, M., *Ascending control of thalamic and cortical responsiveness.* Int Rev Neurobiol, 1970. **12**: p. 87-144.
184. Colavito, V., et al., *Limbic thalamus and state-dependent behavior: The paraventricular nucleus of the thalamic midline as a node in circadian timing and sleep/wake-regulatory networks.* Neurosci Biobehav Rev, 2015. **54**: p. 3-17.
185. Vertes, R.P., S.B. Linley, and A.K.P. Rojas, *Structural and functional organization of the midline and intralaminar nuclei of the thalamus.* Front Behav Neurosci, 2022. **16**: p. 964644.
186. Krout, K.E., R.E. Belzer, and A.D. Loewy, *Brainstem projections to midline and intralaminar thalamic nuclei of the rat.* J Comp Neurol, 2002. **448**(1): p. 53-101.
187. Marcus, J.N., et al., *Differential expression of orexin receptors 1 and 2 in the rat brain.* J Comp Neurol, 2001. **435**(1): p. 6-25.
188. Vertes, R.P., *Interactions among the medial prefrontal cortex, hippocampus and midline thalamus in emotional and cognitive processing in the rat.* Neuroscience, 2006. **142**(1): p. 1-20.
189. Dempsey, E.W. and R.S. Morison, *The Production of Rhythmically Recurrent Cortical Potentials after Localized Thalamic Stimulation.* American Journal of Physiology-Legacy Content, 1941. **135**(2): p. 293-300.
190. Morison, R.S. and E.W. Dempsey, *Mechanism of thalamocortical augmentation and repetition.* American Journal of Physiology, 1943. **138**(2): p. 0297-0308.
191. Groenewegen, H.J. and H.W. Berendse, *The specificity of the 'nonspecific' midline and intralaminar thalamic nuclei.* Trends Neurosci, 1994. **17**(2): p. 52-7.

192. Baker, R., et al., *Altered activity in the central medial thalamus precedes changes in the neocortex during transitions into both sleep and propofol anesthesia.* J Neurosci, 2014. **34**(40): p. 13326-35.
193. Ren, S., et al., *The paraventricular thalamus is a critical thalamic area for wakefulness.* Science, 2018. **362**(6413): p. 429-434.
194. Blackwood, E.B., B.P. Shortal, and A. Proekt, *Weakly Correlated Local Cortical State Switches under Anesthesia Lead to Strongly Correlated Global States.* J Neurosci, 2022. **42**(48): p. 8980-8996.
195. Bosman, C. and N. Aldunate, *The state of cortical microcircuits during wakefulness and sleep.* Arch Ital Biol, 2018. **156**(3): p. 127-136.
196. Zucca, S., et al., *An inhibitory gate for state transition in cortex.* Elife, 2017. **6**.
197. Siclari, F. and G. Tononi, *Local aspects of sleep and wakefulness.* Curr Opin Neurobiol, 2017. **44**: p. 222-227.
198. Vyazovskiy, V.V., et al., *Local sleep in awake rats.* Nature, 2011. **472**(7344): p. 443-7.
199. Bandarabadi, M., A. Vassalli, and M. Tafti, *Sleep as a default state of cortical and subcortical networks.* Current Opinion in Physiology, 2020. **15**: p. 60-67.
200. Hinard, V., et al., *Key electrophysiological, molecular, and metabolic signatures of sleep and wakefulness revealed in primary cortical cultures.* J Neurosci, 2012. **32**(36): p. 12506-17.
201. Jewett, K.A., et al., *Tumor necrosis factor enhances the sleep-like state and electrical stimulation induces a wake-like state in co-cultures of neurons and glia.* Eur J Neurosci, 2015. **42**(4): p. 2078-90.
202. Lemieux, M., et al., *The impact of cortical deafferentation on the neocortical slow oscillation.* J Neurosci, 2014. **34**(16): p. 5689-703.
203. M, K.P. and V. Latreille, *Sleep Disorders.* Am J Med, 2019. **132**(3): p. 292-299.
204. Sherin, J.E., et al., *Activation of ventrolateral preoptic neurons during sleep.* Science, 1996. **271**(5246): p. 216-9.
205. Chung, S., et al., *Identification of preoptic sleep neurons using retrograde labelling and gene profiling.* Nature, 2017. **545**(7655): p. 477-481.
206. Moffitt, J.R., et al., *Molecular, spatial, and functional single-cell profiling of the hypothalamic preoptic region.* Science, 2018. **362**(6416).
207. Gallopin, T., et al., *The endogenous somnogen adenosine excites a subset of sleep-promoting neurons via A2A receptors in the ventrolateral preoptic nucleus.* Neuroscience, 2005. **134**(4): p. 1377-90.
208. Sangare, A., et al., *Serotonin differentially modulates excitatory and inhibitory synaptic inputs to putative sleep-promoting neurons of the ventrolateral preoptic nucleus.* Neuropharmacology, 2016. **109**: p. 29-40.
209. Vanini, G., et al., *Activation of Preoptic GABAergic or Glutamatergic Neurons Modulates Sleep-Wake Architecture, but Not Anesthetic State Transitions.* Curr Biol, 2020. **30**(5): p. 779-787 e4.
210. Kroeger, D., et al., *Galanin neurons in the ventrolateral preoptic area promote sleep and heat loss in mice.* Nat Commun, 2018. **9**(1): p. 4129.
211. Chamberlin, N.L., et al., *Effects of adenosine on gabaergic synaptic inputs to identified ventrolateral preoptic neurons.* Neuroscience, 2003. **119**(4): p. 913-8.
212. Venner, A., et al., *An Inhibitory Lateral Hypothalamic-Preoptic Circuit Mediates Rapid Arousals from Sleep.* Curr Biol, 2019. **29**(24): p. 4155-4168 e5.
213. Luthi, A., *Sleep: The Very Long Posited (VLPO) Synaptic Pathways of Arousal.* Curr Biol, 2019. **29**(24): p. R1310-R1312.
214. Kim, J.H., et al., *Astrocytes in the Ventrolateral Preoptic Area Promote Sleep.* J Neurosci, 2020. **40**(47): p. 8994-9011.

215. Dong, H., et al., *Genetically encoded sensors for measuring histamine release both in vitro and in vivo.* Neuron, 2023. **111**(10): p. 1564-1576 e6.
216. Kato, T., et al., *Oscillatory population-level activity of dorsal raphe serotonergic neurons is inscribed in sleep structure.* J Neurosci, 2022. **42**(38): p. 7244-55.
217. Wan, J., et al., *A genetically encoded sensor for measuring serotonin dynamics.* Nat Neurosci, 2021. **24**(5): p. 746-752.
218. Seifinejad, A., et al., *Hypocretinergic interactions with the serotonergic system regulate REM sleep and cataplexy.* Nat Commun, 2020. **11**(1): p. 6034.
219. Quiroga, R.Q., Z. Nadasdy, and Y. Ben-Shaul, *Unsupervised spike detection and sorting with wavelets and superparamagnetic clustering.* Neural Comput, 2004. **16**(8): p. 1661-87.
220. Kepecs, A., X.J. Wang, and J. Lisman, *Bursting neurons signal input slope.* J Neurosci, 2002. **22**(20): p. 9053-62.
221. Guo, Q., et al., *Multi-channel fiber photometry for population neuronal activity recording.* Biomed Opt Express, 2015. **6**(10): p. 3919-31.
222. Pisano, F., et al., *Depth-resolved fiber photometry with a single tapered optical fiber implant.* Nat Methods, 2019. **16**(11): p. 1185-1192.
223. Saper, C.B., *The neurobiology of sleep.* Continuum (Minneap Minn), 2013. **19**(1 Sleep Disorders): p. 19-31.
224. Tyree, S.M. and L. de Lecea, *Optogenetic Investigation of Arousal Circuits.* Int J Mol Sci, 2017. **18**(8).
225. Rolls, A., et al., *Optogenetic disruption of sleep continuity impairs memory consolidation.* Proc Natl Acad Sci U S A, 2011. **108**(32): p. 13305-10.
226. Torterolo, P. and M.H. Chase, *The hypocretins (orexins) mediate the "phasic" components of REM sleep: A new hypothesis.* Sleep Sci, 2014. **7**(1): p. 19-29.
227. Torterolo, P., S. Sampogna, and M.H. Chase, *Hypocretinergic and non-hypocretinergic projections from the hypothalamus to the REM sleep executive area of the pons.* Brain Res, 2013. **1491**: p. 68-77.
228. Ito, H., et al., *Deficiency of orexin signaling during sleep is involved in abnormal REM sleep architecture in narcolepsy.* Proc Natl Acad Sci U S A, 2023. **120**(41): p. e2301951120.
229. Mileykovskiy, B.Y., L.I. Kiyashchenko, and J.M. Siegel, *Behavioral correlates of activity in identified hypocretin/orexin neurons.* Neuron, 2005. **46**(5): p. 787-98.
230. Vanni-Mercier, G., et al., *Carbachol microinjections in the mediodorsal pontine tegmentum are unable to induce paradoxical sleep after caudal pontine and prebulbar transections in the cat.* Neurosci Lett, 1991. **130**(1): p. 41-5.
231. Jouvet, M., *[Research on the neural structures and responsible mechanisms in different phases of physiological sleep].* Arch Ital Biol, 1962. **100**: p. 125-206.
232. Xi, M.C., et al., *Induction of active (REM) sleep and motor inhibition by hypocretin in the nucleus pontis oralis of the cat.* J Neurophysiol, 2002. **87**(6): p. 2880-8.
233. Xi, M. and M.H. Chase, *The injection of hypocretin-1 into the nucleus pontis oralis induces either active sleep or wakefulness depending on the behavioral state when it is administered.* Sleep, 2010. **33**(9): p. 1236-43.
234. Xi, M.C. and M.H. Chase, *Neuronal mechanisms of active (rapid eye movement) sleep induced by microinjections of hypocretin into the nucleus pontis oralis of the cat.* Neuroscience, 2006. **140**(1): p. 335-42.
235. Ishibashi, M., et al., *Orexin Receptor Activation Generates Gamma Band Input to Cholinergic and Serotonergic Arousal System Neurons and Drives an Intrinsic Ca(2+)-Dependent Resonance in LDT and PPT Cholinergic Neurons.* Front Neurol, 2015. **6**: p. 120.

236. Xi, M.C., F.R. Morales, and M.H. Chase, *Interactions between GABAergic and cholinergic processes in the nucleus pontis oralis: neuronal mechanisms controlling active (rapid eye movement) sleep and wakefulness.* J Neurosci, 2004. **24**(47): p. 10670-8.
237. Uchida, S., et al., *A Discrete Glycinergic Neuronal Population in the Ventromedial Medulla That Induces Muscle Atonia during REM Sleep and Cataplexy in Mice.* J Neurosci, 2021. **41**(7): p. 1582-1596.
238. Luppi, P.H., et al., *Paradoxical (REM) sleep genesis: the switch from an aminergic-cholinergic to a GABAergic-glutamatergic hypothesis.* J Physiol Paris, 2006. **100**(5-6): p. 271-83.
239. Sakai, K. and Y. Koyama, *Are there cholinergic and non-cholinergic paradoxical sleep-on neurones in the pons?* Neuroreport, 1996. **7**(15-17): p. 2449-53.
240. Boissard, R., et al., *Localization of the GABAergic and non-GABAergic neurons projecting to the sublaterodorsal nucleus and potentially gating paradoxical sleep onset.* Eur J Neurosci, 2003. **18**(6): p. 1627-39.
241. Boissard, R., et al., *The rat ponto-medullary network responsible for paradoxical sleep onset and maintenance: a combined microinjection and functional neuroanatomical study.* Eur J Neurosci, 2002. **16**(10): p. 1959-73.
242. Feng, H., et al., *Orexin signaling modulates synchronized excitation in the sublaterodorsal tegmental nucleus to stabilize REM sleep.* Nat Commun, 2020. **11**(1): p. 3661.
243. Gonzalez, J.A., et al., *Convergent inputs from electrically and topographically distinct orexin cells to locus coeruleus and ventral tegmental area.* Eur J Neurosci, 2012. **35**(9): p. 1426-32.
244. Harris, G.C. and G. Aston-Jones, *Activation in extended amygdala corresponds to altered hedonic processing during protracted morphine withdrawal.* Behav Brain Res, 2007. **176**(2): p. 251-8.
245. Baimel, C., et al., *Orexin/hypocretin role in reward: implications for opioid and other addictions.* Br J Pharmacol, 2015. **172**(2): p. 334-48.
246. Pan, Y.P., et al., *Involvement of orexin-A in the regulation of neuronal activity and emotional behaviors in central amygdala in rats.* Neuropeptides, 2020. **80**: p. 102019.
247. Khanday, M.A. and B.N. Mallick, *REM sleep modulation by perifornical orexinergic inputs to the pedunculo-pontine tegmental neurons in rats.* Neuroscience, 2015. **308**: p. 125-33.
248. Harris, G.C., M. Wimmer, and G. Aston-Jones, *A role for lateral hypothalamic orexin neurons in reward seeking.* Nature, 2005. **437**(7058): p. 556-9.
249. de Lecea, L., *Optogenetic control of hypocretin (orexin) neurons and arousal circuits.* Curr Top Behav Neurosci, 2015. **25**: p. 367-78.
250. Carter, M.E., et al., *Tuning arousal with optogenetic modulation of locus coeruleus neurons.* Nat Neurosci, 2010. **13**(12): p. 1526-33.
251. Lecci, S., et al., *Coordinated infraslow neural and cardiac oscillations mark fragility and offline periods in mammalian sleep.* Sci Adv, 2017. **3**(2): p. e1602026.
252. Verret, L., et al., *Localization of the neurons active during paradoxical (REM) sleep and projecting to the locus coeruleus noradrenergic neurons in the rat.* J Comp Neurol, 2006. **495**(5): p. 573-86.
253. Fogel, S.M. and C.T. Smith, *The function of the sleep spindle: a physiological index of intelligence and a mechanism for sleep-dependent memory consolidation.* Neurosci Biobehav Rev, 2011. **35**(5): p. 1154-65.

254. Fogel, S., et al., *Sleep spindles: a physiological marker of age-related changes in gray matter in brain regions supporting motor skill memory consolidation.* Neurobiol Aging, 2017. **49**: p. 154-164.
255. Seibt, J., et al., *Cortical dendritic activity correlates with spindle-rich oscillations during sleep in rodents.* Nat Commun, 2017. **8**(1): p. 684.
256. Shibagaki, M., S. Kiyono, and K. Watanabe, *Spindle evolution in normal and mentally retarded children: a review.* Sleep, 1982. **5**(1): p. 47-57.
257. Mayeli, A., et al., *Sleep spindle alterations relate to working memory deficits in individuals at clinical high-risk for psychosis.* Sleep, 2022. **45**(11).
258. Ferrarelli, F. and G. Tononi, *What Are Sleep Spindle Deficits Telling Us About Schizophrenia?* Biol Psychiatry, 2016. **80**(8): p. 577-8.
259. Asp, A., et al., *Impaired procedural memory in narcolepsy type 1.* Acta Neurol Scand, 2022. **146**(2): p. 186-193.
260. Medrano-Martinez, P., A. Gomez-Sacristan, and R. Peraita-Adrados, *Is memory impaired in narcolepsy type 1?* J Sleep Res, 2022. **31**(5): p. e13593.
261. Rogers, A.E. and R.S. Rosenberg, *Tests of memory in narcoleptics.* Sleep, 1990. **13**(1): p. 42-52.
262. Mazzetti, M., et al., *Sleep-dependent consolidation of motor skills in patients with narcolepsy-cataplexy.* Arch Ital Biol, 2012. **150**(2-3): p. 185-93.
263. Gagnon, K., et al., *Sleep Stage Transitions and Sleep-Dependent Memory Consolidation in Children with Narcolepsy–Cataplexy.* Children, 2023. **10**(10): p. 1702.
264. Boyce, R., et al., *Causal evidence for the role of REM sleep theta rhythm in contextual memory consolidation.* Science, 2016. **352**(6287): p. 812-6.
265. Boyce, R., S. Williams, and A. Adamantidis, *REM sleep and memory.* Curr Opin Neurobiol, 2017. **44**: p. 167-177.
266. Sartor, G.C. and G.S. Aston-Jones, *A septal-hypothalamic pathway drives orexin neurons, which is necessary for conditioned cocaine preference.* J Neurosci, 2012. **32**(13): p. 4623-31.
267. Bandarabadi, M., et al., *Dynamic modulation of theta-gamma coupling during rapid eye movement sleep.* Sleep, 2019. **42**(12): p. 1-11.
268. Vassalli, A. and P. Franken, *Hypocretin (orexin) is critical in sustaining theta/gamma-rich waking behaviors that drive sleep need.* Proc Natl Acad Sci U S A, 2017. **114**(27): p. E5464-E5473.
269. Smith, H.R. and K.C. Pang, *Orexin-saporin lesions of the medial septum impair spatial memory.* Neuroscience, 2005. **132**(2): p. 261-71.
270. Colgin, L.L., *Mechanisms and functions of theta rhythms.* Annu Rev Neurosci, 2013. **36**: p. 295-312.
271. Wu, M., et al., *Hypocretin/orexin innervation and excitation of identified septohippocampal cholinergic neurons.* J Neurosci, 2004. **24**(14): p. 3527-36.
272. Gerashchenko, D., R. Salin-Pascual, and P.J. Shiromani, *Effects of hypocretin-saporin injections into the medial septum on sleep and hippocampal theta.* Brain Res, 2001. **913**(1): p. 106-15.
273. Lee, M.G., et al., *Hippocampal theta activity following selective lesion of the septal cholinergic system.* Neuroscience, 1994. **62**(4): p. 1033-47.
274. Omote, H. and Y. Moriyama, *Vesicular neurotransmitter transporters: an approach for studying transporters with purified proteins.* Physiology (Bethesda), 2013. **28**(1): p. 39-50.
275. Luo, M., et al., *Divergent Neural Activity in the VLPO During Anesthesia and Sleep.* Adv Sci (Weinh), 2023. **10**(2): p. e2203395.

276. Kostin, A., et al., *Calcium Dynamics of the Ventrolateral Preoptic GABAergic Neurons during Spontaneous Sleep-Waking and in Response to Homeostatic Sleep Demands.* Int J Mol Sci, 2023. **24**(9).
277. Vyazovskiy, V.V., C. Cirelli, and G. Tononi, *Electrophysiological correlates of sleep homeostasis in freely behaving rats.* Prog Brain Res, 2011. **193**: p. 17-38.
278. Nir, Y., et al., *Regional slow waves and spindles in human sleep.* Neuron, 2011. **70**(1): p. 153-69.
279. Fernandez, L.M., et al., *Thalamic reticular control of local sleep in mouse sensory cortex.* Elife, 2018. **7**: p. e39111.
280. Krueger, J.M., et al., *Local sleep.* Sleep Med Rev, 2019. **43**: p. 14-21.
281. Vantomme, G., et al., *Regulation of Local Sleep by the Thalamic Reticular Nucleus.* Front Neurosci, 2019. **13**(576): p. 576.
282. Sarasso, S., et al., *Local sleep-like cortical reactivity in the awake brain after focal injury.* Brain, 2020. **143**(12): p. 3672-3684.
283. Poulet, J.F.A. and S. Crochet, *The Cortical States of Wakefulness.* Front Syst Neurosci, 2018. **12**: p. 64.
284. Nobili, L., et al., *Dissociated wake-like and sleep-like electro-cortical activity during sleep.* Neuroimage, 2011. **58**(2): p. 612-9.
285. Alfonsa, H., et al., *Intracellular chloride regulation mediates local sleep pressure in the cortex.* Nat Neurosci, 2023. **26**(1): p. 64-78.
286. Rasmussen, R., et al., *Cortex-wide Changes in Extracellular Potassium Ions Parallel Brain State Transitions in Awake Behaving Mice.* Cell Rep, 2019. **28**(5): p. 1182-1194 e4.

Hypothalamic control of noradrenergic neurons stabilizes sleep

Gianandrea Broglia[1], Giorgio Corsi[1], Pierre-Hugues Prouvot Bouvier[1], Mehdi Tafti[1], and Mojtaba Bandarabadi[1,*]

[1]*Department of Biomedical Sciences, University of Lausanne, Lausanne, Switzerland*

[*] *Correspondence to:*
Mojtaba Bandarabadi, PhD.
Department of Biomedical Sciences
University of Lausanne
Rue du Bugnon 7
1005 Lausanne, Switzerland
Tel: +41 (0) 21 692 55 55
mojtaba.bandarabadi@unil.ch

Abstract

Hypocretin/orexin neurons are essential to stabilize sleep, but the underlying mechanisms remain elusive. We report that hypocretin neurons of the perifornical hypothalamus are highly active during rapid eye movement sleep and show state-specific correlation with noradrenergic neurons. Deletion of hypocretin gene significantly increased periodic reactivations of locus coeruleus noradrenergic neurons during sleep and dysregulated their activity across transitions, suggesting a role for hypocretin neurons in mediating neuromodulation to stabilize sleep.

Main text

Spontaneous transitions in vigilance states have deterministic neural substrates allowing the maintenance of stable sleep-wake cycle over time[1]. Complex interconnected wake-promoting and sleep-generating pathways mutually inhibit each other to induce rapid and complete state transitions[2,3]. Hypocretin/orexin (HCRT) neurons are critical for the proper regulation of these transitions[4], in addition to attention, motivation, and appetite[5,6]. Hypocretin neurons are localized in the lateral hypothalamus (LH), perifornical area of hypothalamus (PeF), and a sparse distribution in the dorsomedial hypothalamus (DMH)[7,8]. They project to all wake-promoting monoaminergic and cholinergic nuclei, the midline thalamus, and the cortex[9], and can promote wakefulness and maintain arousal by stimulating these areas. Deficiency in hypocretin neurotransmission causes the sleep disorder narcolepsy, associated with chronic sleepiness, fragmented sleep, abnormal transitions to rapid eye movement sleep (REMS), and sudden loss of muscle tone during wakefulness in both humans and animals[10-12].

Locus coeruleus noradrenergic (LC^{NA}) neurons, which receive the densest projections of hypocretin cells[9], show periodic reactivations during non-rapid eye movement sleep (NREMS) and can gate sleep to wake transitions[13,14]. Optogenetic activation of LH^{HCRT} or LC^{NA} neurons induces rapid awaking from both NREMS and REMS[15,16]. Hypocretin neurons play a crucial role in stabilizing vigilance states in the current theoretical models, e.g. the flip-flop switch model[3,17]. However, LH^{HCRT} neurons are relatively silent during sleep[18], and the current models cannot explain long-term regulation of sleep-wake cycle in a steady manner in the absence of hypocretin[19,20]. Therefore, other hypocretin subpopulations or mechanisms might be involved to maintain consolidated sleep and regulate vigilance state transitions.

First, to test whether PeF^{HCRT} neurons have different temporal dynamics from LH^{HCRT} cells across sleep-wake states and during spontaneous transitions, we performed *in vivo* calcium imaging in the PeF and LH, combined with EEG/EMG recordings in freely behaving *Hcrt-IRES-Cre* mice (Supp. Fig. 1,2). While LH^{HCRT} cells are mainly active during wakefulness, as in previous reports[18,21,22], we unexpectedly found that PeF^{HCRT} neurons are highly active during REMS and wakefulness (Fig. 1a-c). We also recorded a weak calcium signal from LH^{HCRT} neurons during REMS (Fig. 1b,c), consistent with a recent study reporting that a small subpopulation of LH^{HCRT} neurons are active during REMS[22]. Both PeF^{HCRT} and LH^{HCRT} neurons also showed sparse activity during NREMS, with a peak at NREMS to wake transitions (Fig. 1b). To investigate the correlation between activity of PeF^{HCRT} neurons and the noradrenergic system, we simultaneously recorded PeF^{HCRT} calcium signal and regional norepinephrine release in the LC and in its major projecting target of the paraventricular nucleus of the thalamus (PVT). We found that PeF^{HCRT} neuronal activity correlates with norepinephrine release in both LC and PVT during wakefulness and NREMS, while anti-correlated during REMS (Fig. 1d), suggesting different PeF^{HCRT} subpopulations that are active during wake/NREMS and REMS.

Next, to investigate the activity of LC^{NA} neurons in the absence of hypocretin, we generated hypocretin knockout (*Hcrt*$^{KO/KO}$) mice harboring Cre recombinase in noradrenergic neurons by breeding *Hcrt*$^{KO/KO}$ and *Dbh-Cre* mice (*Hcrt*$^{KO/KO}$x*Dbh-Cre*). We then performed *in vivo* calcium imaging of LC^{NA} neurons in freely behaving knockout (*Hcrt*$^{KO/KO}$x*Dbh-Cre*) and control (*Dbh-Cre*) mice, combined with EEG/EMG recordings. We found that LC^{NA} neurons are periodically reactivated during NREMS, as reported recently[13,14], in both genotypes (Fig. 2a). Multisite fiber photometry of the LC and its projections in the PVT and cingulate cortex revealed that the norepinephrine

release during these reactivations reaches both thalamic and cortical sites, similar as during wakefulness (Supp. Fig. 3). LC^{NA} neuronal activity increased prior to NREMS to wake transitions in both knockout and control mice (Fig. 2b), consistent with the role of LC^{NA} reactivations in providing a fragility window for transitions[13,14]. However, we found that LC^{NA} reactivations during NREMS are significantly more frequent in hypocretin knockout mice compared to controls ($p = 0.0005$, unpaired *t*-test), which resulted from a shorter refractory period ($p < 0.0001$, unpaired *t*-test; Fig. 2c). *Hcrt*$^{KO/KO}$ mice showed fragmented NREMS episodes, reflected in higher number of shorter bouts (number: $p = 0.0017$, duration: $p = 0.0015$, unpaired *t*-test; Fig. 2d), but similar total NREMS amount compared to controls (Supp. Fig. 4a,b). These data suggest a mediatory role for the hypocretin system during NREMS, where shorter sleep episodes result from hyperactivated LC^{NA} neurons in the absence of hypocretin signaling.

We next investigated the effects of overactivity of LC^{NA} neurons in *Hcrt*$^{KO/KO}$ mice on the brain oscillations during NREMS. We calculated the power spectral density (PSD) of EEG signal and found a shift in the peak frequency of delta oscillations in *Hcrt*$^{KO/KO}$ mice compared to controls (Fig. 2e). As LC^{NA} activity is detrimental for the generation of thalamocortical sleep spindles[13], we also quantified spindles and found a significant reduction in the NREMS spindle density and alternations in spindle characterizations ($p < 0.0001$, unpaired *t*-test; Fig. 2f and Supp. Fig. 5). These results indicate the contribution of subcortical neuromodulatory pathways in modulation of thalamocortical brain oscillations during NREMS.

Next, we tested how the absence of hypocretin signaling affects REMS architecture and its components. Although the LC^{NA} neurons are periodically reactivated during NREMS, the noradrenergic tone is completely absent tens of seconds prior to REMS onset and during REMS (Fig. 2a and Fig. 3a). However, we found that this silent period

of LC^{NA} neurons prior to transitions to REMS is significantly shorter in $Hcrt^{KO/KO}$ mice compared to controls (p = 0.0279, unpaired t-test; Fig. 3a,b). Furthermore, LC^{NA} neuronal activity is very high immediately before REMS-to-wake transitions, but the lag between the activation of LC^{NA} neurons and transitions is significantly longer in $Hcrt^{KO/KO}$ mice compared to controls (p = 0.0077, unpaired t-test; Fig. 3a,c). $Hcrt^{KO/KO}$ mice also showed fragmented REMS episodes, reflected in higher number of shorter bouts (number: $p < 0.0001$, duration: p = 0.0162, unpaired t-test; Fig. 3d), and higher total REMS amount compared to controls (Supp. Fig. 4c,d). These results suggest that altered REMS architecture of $Hcrt^{KO/KO}$ mice may result from the dysregulated LC^{NA} activity during sleep.

To assess the contribution of hypocretin neurons in the regulation of REMS oscillations, we calculated the PSD of EEG recordings and found an increased theta power in $Hcrt^{KO/KO}$ mice compared to controls (Fig.3e). We also investigated the interaction of theta rhythms with gamma oscillations, where theta phase modulates gamma power, and found significant increase in theta-gamma, but not theta-fast gamma, coupling in $Hcrt^{KO/KO}$ mice compared to controls (p = 0.0078, unpaired t-test; Fig.3f and Supp. Fig. 6a). These results suggest an inhibitory role for REMS-active hypocretin neurons in theta generation and modulation pathways. As previous reports showed that hypocretin neurons discharge during phasic REMS events[23,24], we also quantified these events, but did not find alternations in the amount and characteristics of phasic REMS events between knockout and control animals (Supp. Fig. 6b), indicating only a correlation and not causation, between hypocretin activity and phasic REMS.

In summary, we found a dichotomy between the LH and PeF subpopulations of hypocretin neurons, where LH^{HCRT} neurons are principally wake-active, while PeF^{HCRT}

neurons are mainly REMS-active. Although the role of LH^{HCRT} neurons in the regulation of arousal levels, reward, feeding, and emotional behaviors is well established[5,6,21], the PeF/DMH neurons are hypothesized to regulate vigilance states only[25]. To our knowledge, our study is the first to report *in vivo* activity of PeF^{HCRT} neurons across vigilance states and during sleep-wake transitions and show that these neurons are highly active during sleep. Although we did not investigate the functional role of PeF^{HCRT} neurons directly, considering that these neurons are mainly REMS-active, they could be responsible for altered architecture and components of REMS in $Hcrt^{KO/KO}$ mice, e.g. the shorter epochs and higher theta power.

Deletion of hypocretin gene resulted in more frequent LC^{NA} reactivations during NREMS. As these reactivations are a window of sleep fragility and possible awakenings[13,14], the fragmented NREMS in $Hcrt^{KO/KO}$ mice could be explained by a mediatory role for the hypocretin system that suppresses the LC^{NA} reactivations during sleep. Moreover, a shorter silent period of LC^{NA} neurons required prior to REMS onset can explain more frequent NREMS-REMS transitions in $Hcrt^{KO/KO}$ mice, and a similar mechanism could cause “sleep onset REM period” in human narcolepsy. Additionally, LC^{NA} activation prior to REMS termination appears earlier in $Hcrt^{KO/KO}$ mice compared to controls, suggesting a possible mechanism behind shorter REMS episodes and their premature termination, and indicating again a mediatory role for REMS-active hypocretin neurons in stabilization of REMS. Although the underlying mechanisms that drive periodic activation of the LC^{NA} neurons, and possibly other monoaminergic neurons, during NREMS are unclear, our results indicate that the hypocretin system has a major role in controlling the timing of this infra-slow oscillation. Altogether, our findings provide a mechanistic answer to the fragmented, but still regulated, sleep in the absence of hypocretin, as in human narcolepsy.

Methods

Animals. *Hcrt-IRES-Cre* knock-in (*Hcrt*$^{tm1.1(cre)Ldl}$) heterozygote[26] and *Dbh-Cre* (*Tg(Dbh-icre)1Gsc*) heterozygote[27,28] mice were locally backcrossed into C57BL/6J mice. Homozygous hypocretin-knockout (*Hcrt*$^{KO/KO}$) mice were used as a mouse model of narcolepsy[12]. *Hcrt*$^{KO/KO}$*xDbh-Cre* mice were generated locally by crossing *Dbh-Cre* mice with *Hcrt*$^{KO/KO}$ mice to investigate noradrenergic neuronal activity in the absence of hypocretin. Genotyping of the lines was carried out using PCR. Animals at 12-18 weeks old at the time of experiments were group-housed with *ad libitum* access to standard food pellets and water at the constant temperature (23±1 °C) and humidity (30-40%), and a 12h/12h light/dark cycle with lights on from 8:00 a.m. to 8:00 p.m. All procedures were carried out in accordance with the Swiss federal laws and approved by the veterinary office of the State of Vaud, Switzerland.

Stereotaxic viral injections. Adeno-associated viruses (AAV) carrying the fluorescent reporter were produced by the viral vector facility of University of Zurich, Switzerland (www.vvf.uzh.ch). To record neuronal activity, a Ca2+ sensor (ssAAV-9/2-hSyn1-chI-dlox-GCaMP6m(rev)-dlox-WPRE-SV40p(A), titer 4.2x10E12 vg/ml) was delivered in Cre-expressing areas of interest in the transgenic mice. For screening neurotransmitter release, a GRAB norepinephrine sensor (ssAAV-9/2-hSyn1-GRAB_NE1h-WPRE-hGHp(A), titer 7.2x10E12 vg/ml) was used[29]. Mice (12-13 weeks) underwent stereotaxic surgery (Kopf Instruments, USA) for injections of AAVs carrying genetically encoded calcium indicators or fluorescent sensors. Anesthesia was achieved with the intraperitoneal injection of ketamine/xylazine (100/20 mg/kg) diluted in 0.9% saline. Isoflurane at 1-3% was given through a face mask to maintain anesthesia. Once the skull was exposed and bregma and lambda were aligned, a hand-drill was used to

insert a nano-syringe (World Precision Instruments, USA) to reach coordinates for vector delivery. AAVs were injected in the perifornical area of hypothalamus (PeF: AP -1.4; ML 0.6; DV -5.1), the lateral hypothalamus (LH: AP -1.4; ML 1; DV -5.1), the locus coeruleus (LC: AP -5.4; ML -0.85; DV -3.7), the paraventricular thalamus (PVT: AP -0.3; ML 0; DV -3.7), and the cingulate cortex (CING: AP +1.4; ML -0.3; DV -1.7). Each viral vector was injected at 300 nl with a speed rate of 50 nl/min. Injected mice were group-housed for three weeks to allow the viruses to achieve proper diffusion and expression.

Implantation of optic fibers and EEG/EMG electrodes. Three weeks after injections, mice underwent a second stereotaxic surgery to implant optic fibers and EEG along with EMG electrodes. Optic fibers (200 µm, 0.37 NA, glass and ferrules from Thorlabs) were inserted slowly above the same coordinates as the injected sites and secured to the skull with dental cement. Two gold plated screws were placed on the right hemisphere at coordinates (AP -2; ML +2.5) for EEG and (AP -6; ML +1.8) for the ground electrode. Two plastic-covered gold wires were inserted into the neck muscles and served as EMG electrodes. All fibers, electrodes and wires were fixed using the dental resin (Relyx 3M). The EEG/ground screws and EMG wires were soldered to a digital interface board (Pinnacle Technology INC, USA), and the whole implant was covered by dental cement (3M). Mice were allowed to recover from surgery for one week before the beginning of recordings.

***In vivo* multisite fiber photometry.** Animals were habituated to the recording cable and optical fibers in their open-top home cages for 3 days and kept tethered for the duration of the experiments. All recordings were performed between 16-18 weeks of

age. To monitor *in vivo* changes in neuronal population spiking activity of specific cell types (calcium signals) or neurotransmitters (GRAB sensor signals) in multiple nuclei, a multi-channel fiber photometry system with two excitation wavelengths of 465 nm and 405 nm was used (bundle-imaging fiber photometry system, Doric Lenses). Interleaved excitation lights were sent into low-autofluorescence optic fiber patch cords (200 µm core, 0.37 NA, Doric Lenses), which were connected to the implanted fibers contained in a 1.25 mm diameter ferrule via a bronze sleeve. The GCaMP/GRAB emission fluorescence signals were collected through the same patch cords with the acquisition rate of 20 Hz using the Doric Neuroscience Studio software (Doric Lenses). The power of 465 nm and 405 nm lights at the tip of the optic fibers was set to 30-40 µW. The EEG/EMG signals were collected at the frequency of 2 kHz using the Pinnacle sleep recording systems and Sirenia software (Pinnacle Technology, USA). Recorded photometry traces were synchronized with EEG/EMG signals using the generated TTL pulses by the Doric Neuroscience Studio, which were recorded by the Sirenia software through a BNC cable.

Immunofluorescence and histological confirmation. Mice were deeply anesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with PBS, followed by 4% paraformaldehyde. Brains were removed, fixed in 4% paraformaldehyde and stored in PBS/sodium azide (0.02%) at 4 °C. Brains were later cut in coronal sections of 60 µm with a vibratome (VT1200S, Leica, Germany). Sections containing areas of viral injection were selected for immunofluorescence staining and sequentially incubated with the primary and secondary antibodies. The primary antibodies were mouse anti-orexin-A (1:200; sc-80263, Santa Cruz Biotechnology), rabbit anti-Cre (1:200; 69050, EMD Millipore), and rabbit anti-MCH

(1:200; H-070-47, Phoenix Pharmaceuticals, Inc.). Images were acquired using the LSM 900 confocal microscope (Carl Zeiss, Germany) and analyzed using the Zen Blue software (Carl Zeiss, Germany). Microscopy investigation was also performed to locate the sites of optical fiber implantation. Data were excluded if the locations were not confirmed.

Scoring and quantification of vigilance states. Scoring of vigilance states was performed manually using visual inspection of EEG/EMG recordings in 1-sec epochs, to allow precise scoring of microarousals and transition times, as described previously[30]. A custom toolbox written in MATLAB was used to visualize time and frequency characteristics of EEG/EMG traces. Wake was defined as periods of either theta band EEG activity accompanied by EMG bursts of movement-related activity, or periods that mice were immobile including feeding and grooming behaviors. NREMS was scored as periods with a relatively high amplitude low frequency delta band EEG activity accompanied by reduced muscle tone relative to wakefulness and associated with behavioral quiescence. REMS episodes were scored as sustained periods of theta band EEG activity and behavioral quiescence associated with muscle atonia, except for brief phasic muscle twitches. Transition to wake was defined as the first epoch with a rapid increase in muscle tone concurrent with low-amplitude fast-frequency EEG activity. Wake to NREMS transition was defined as the first epoch containing high-amplitude delta band activity appearing after EMG silencing. REMS onset was defined as an epoch with the absence of EMG tone concomitant with recursive synchronized theta rhythm. Number, duration, and state fragmentation of wakefulness, NREMS, and REMS were quantified using MATLAB scripts.

Analysis of fiber photometry recordings. The output signals of the fiber photometry system were converted to *ΔF/F* = (*F* - *Fmean*) / *Fmean*, after amplification, digitization, and low-pass filtering. Dynamics of calcium signals and neurotransmitter release during vigilance state transitions were calculated by averaging the ΔF/F signal across wake-NREMS, NREMS-wake, NREMS-REMS, and REMS-wake transitions. Transition heatmaps were obtained by aligning the normalized *ΔF/F* signals (z-scored) at the transition points. To detect prominent activity in calcium signals, the *ΔF/F* signal was lowpass filtered using finite impulse response filters (0.2 Hz, 100-th order), and peaks were detected automatically using the MATLAB "findpeaks" function. For proper calculation of the density and interval of LC^{NA} reactivations during NREMS, only epochs with at least 3 reactivations were considered. Correlation between cell-type specific neuronal dynamics and sleep oscillations were measured using concurrent analysis of fiber photometry recordings and time-frequency analysis of EEG signals across NREMS and REMS and during transitions to these states.

Spectral and time-frequency analysis. Raw electrophysiological recordings were down-sampled to 1000 Hz after applying a low pass filter (Chebyshev Type I, order 8, low pass edge frequency of 400 Hz, passband ripple of 0.05 dB). State-specific power spectral densities (PSDs) of EEG signals were calculated using the Welch's method (MATLAB "pwelch" function), with 4-s windows having 50% overlap and 0.5-Hz frequency resolution. To correct for differences in absolute EEG power between animals, PSDs of each vigilance state was normalized to the weighted total PSD power across 1-45 Hz band of all vigilance states for each animal. The weighted total PSD power for each animal was calculated so that each state contributes equally to the total

EEG power as described previously[31]. Time-frequency heatmaps were obtained using the multitaper spectral analysis.

Detection of sleep spindles. NREMS spindles were detected automatically using an optimized wavelet-based method as described previously[30]. Briefly, the power of EEG signals within 9-16 Hz was estimated using the complex B-spline wavelet function, and smoothed using a 200 ms Hanning window, and then a threshold equal to 3 standard deviations above the mean was applied to detect the potential spindle events. Events shorter than 400 ms or longer than 2 s were discarded. Using band pass-filtered EEG signal in the spindle range (9–16 Hz), we automatically counted the number of cycles of each detected event and excluded those with <5 cycles or more than 30 cycles. To discard artefacts, events with a power in the spindle band lower than 6–8.5 Hz or 16.5–20 Hz power bands were discarded.

Phase-amplitude cross-frequency coupling. Correlation between phase of low frequency oscillations and fast frequency bands, i.e. phase-amplitude cross-frequency coupling, were assessed using the Modulation Index[32,33]. EEG/LFP signals were bandpass-filtered into theta (5-12 Hz), gamma (54-90 Hz), and fast-gamma (110-160 Hz) using finite impulse response filters with an order equal to three cycles of the low-cutoff frequency in both forward and reverse directions to eliminate phase distortion. The instantaneous theta phase and the gamma envelope were estimated using the Hilbert transform. Theta phase was discretized into 18 equal bins (N=18, each 20°) and the average value of gamma envelope within each bin was calculated. The resulting phase-amplitude histogram (P) was compared with a uniform distribution (U) using the Kullback-Leibler distance, $D_{KL}(P,U) = \sum_{j=1}^{N} P(j) * log[P(j)/U(j)]$, and

normalized by *log(N)* to obtain the modulation index, $MI = D_{KL} / log(N)$. To explore other possible cross-frequency coupling patterns between different pairs of low and high-frequency bands, the comodulogram analysis was used[34]. Different frequency bands for phase (1-20 Hz, 1 Hz increments, 2 Hz bandwidth) and amplitude (20-200 Hz, 10 Hz increments, 20 Hz bandwidth) were used and MI values were calculated for all these pairs to obtain the comodulogram graph.

Detection of phasic REMS events. Phasic REMS events, which are transient increase in theta power/frequency during REMS, were detected as described previously[35]. Briefly, EEG signal was bandpass-filtered between 4 and 12 Hz using finite impulse response filters with an order equal to three cycles of the low cutoff frequency, and the individual theta peaks were detected from the filtered signal. The interpeak interval time-series were smoothed using an 11-sample moving average window, and the smoothed interpeak intervals shorter than the 10th percentile were selected as candidate phasic REMS events. The candidate events with the following criteria were considered as phasic REMS: (1) minimum event duration of 900 ms; (2) minimum smoothed interpeak interval shorter than 5th percentile; (3) mean amplitude of theta peaks larger than mean amplitude of theta peaks across all REMS.

Statistics. Animals of all experiments were randomly distributed for recordings, and individuals involved in vigilance state scoring were blinded to animals' genotypes. The GraphPad Prism software was used to perform statistical analysis and compare different conditions via one-way or two-way ANOVA testes followed by multiple comparisons test, or two-sided *t*-tests for parametric data. All results are presented as mean ± SEM.

Code availability. Custom-written MATLAB scripts for data analysis used in this study are available from the corresponding author upon request.

Data availability. The datasets acquired for this study are available from the corresponding author upon request.

Acknowledgements. We thank Anne-Catherine Thomas for her help in animal genotyping. This work was supported by the Swiss National Science Foundation (grant 190605 to M.B. and 201235 to M.T.) and the Novartis Foundation for Medical-Biological Research (grant 21B109 to M.B.).

Author Contribution. G.B., G.C. and P.B. performed the experiments; M.B. and G.B. analyzed the data and wrote the manuscript; All authors discussed the results and commented on the manuscript; M.B. and M.T. conceived and supervised the study.

Competing interests. The authors declare no competing interests.

References

1. Scammell, T.E., Arrigoni, E. & Lipton, J.O. Neural Circuitry of Wakefulness and Sleep. *Neuron* **93**, 747-765 (2017).
2. Lu, J., Sherman, D., Devor, M. & Saper, C.B. A putative flip-flop switch for control of REM sleep. *Nature* **441**, 589-594 (2006).
3. Saper, C.B., Fuller, P.M., Pedersen, N.P., Lu, J. & Scammell, T.E. Sleep state switching. *Neuron* **68**, 1023-1042 (2010).
4. Carter, M.E.*, et al.* Mechanism for Hypocretin-mediated sleep-to-wake transitions. *Proc Natl Acad Sci U S A* **109**, E2635-2644 (2012).
5. Mahler, S.V., Moorman, D.E., Smith, R.J., James, M.H. & Aston-Jones, G. Motivational activation: a unifying hypothesis of orexin/hypocretin function. *Nat Neurosci* **17**, 1298-1303 (2014).
6. Sakurai, T. The role of orexin in motivated behaviours. *Nat Rev Neurosci* **15**, 719-731 (2014).
7. de Lecea, L.*, et al.* The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* **95**, 322-327 (1998).
8. Sakurai, T.*, et al.* Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* **92**, 573-585 (1998).
9. Peyron, C.*, et al.* Neurons containing hypocretin (orexin) project to multiple neuronal systems. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**, 9996-10015 (1998).
10. Nishino, S., Ripley, B., Overeem, S., Lammers, G.J. & Mignot, E. Hypocretin (orexin) deficiency in human narcolepsy. *Lancet* **355**, 39-40 (2000).
11. Lin, L.*, et al.* The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* **98**, 365-376 (1999).
12. Chemelli, R.M.*, et al.* Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**, 437-451 (1999).
13. Osorio-Forero, A.*, et al.* Noradrenergic circuit control of non-REM sleep substates. *Curr Biol* **31**, 5009-5023 e5007 (2021).
14. Kjaerby, C.*, et al.* Memory-enhancing properties of sleep depend on the oscillatory amplitude of norepinephrine. *Nat Neurosci* **25**, 1059-1070 (2022).
15. Adamantidis, A.R., Zhang, F., Aravanis, A.M., Deisseroth, K. & de Lecea, L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature* **450**, 420-424 (2007).
16. Carter, M.E.*, et al.* Tuning arousal with optogenetic modulation of locus coeruleus neurons. *Nat Neurosci* **13**, 1526-1533 (2010).
17. Saper, C.B., Scammell, T.E. & Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *Nature* **437**, 1257-1263 (2005).
18. Li, S.B.*, et al.* Hyperexcitable arousal circuits drive sleep instability during aging. *Science* **375**, eabh3021 (2022).
19. Seifinejad, A., Li, S., Possovre, M.L., Vassalli, A. & Tafti, M. Hypocretinergic interactions with the serotonergic system regulate REM sleep and cataplexy. *Nat Commun* **11**, 6034 (2020).
20. Vassalli, A.*, et al.* Electroencephalogram paroxysmal theta characterizes cataplexy in mice and children. *Brain* **136**, 1592-1608 (2013).

21. Grujic, N., Tesmer, A., Bracey, E., Peleg-Raibstein, D. & Burdakov, D. Control and coding of pupil size by hypothalamic orexin neurons. *Nat Neurosci* **26**, 1160-1164 (2023).
22. Ito, H.*, et al.* Deficiency of orexin signaling during sleep is involved in abnormal REM sleep architecture in narcolepsy. *Proc Natl Acad Sci U S A* **120**, e2301951120 (2023).
23. Lee, M.G., Hassani, O.K. & Jones, B.E. Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**, 6716-6720 (2005).
24. Mileykovskiy, B.Y., Kiyashchenko, L.I. & Siegel, J.M. Behavioral correlates of activity in identified hypocretin/orexin neurons. *Neuron* **46**, 787-798 (2005).
25. Harris, G.C. & Aston-Jones, G. Arousal and reward: a dichotomy in orexin function. *Trends Neurosci* **29**, 571-577 (2006).
26. Giardino, W.J.*, et al.* Parallel circuits from the bed nuclei of stria terminalis to the lateral hypothalamus drive opposing emotional states. *Nat Neurosci* **21**, 1084-1095 (2018).
27. Stanke, M.*, et al.* Target-dependent specification of the neurotransmitter phenotype: cholinergic differentiation of sympathetic neurons is mediated in vivo by gp 130 signaling. *Development* **133**, 141-150 (2006).
28. Parlato, R., Otto, C., Begus, Y., Stotz, S. & Schutz, G. Specific ablation of the transcription factor CREB in sympathetic neurons surprisingly protects against developmentally regulated apoptosis. *Development* **134**, 1663-1670 (2007).
29. Feng, J.*, et al.* A Genetically Encoded Fluorescent Sensor for Rapid and Specific In Vivo Detection of Norepinephrine. *Neuron* **102**, 745-761 e748 (2019).
30. Bandarabadi, M.*, et al.* A role for spindles in the onset of rapid eye movement sleep. *Nat Commun* **11**, 5247 (2020).
31. Franken, P., Malafosse, A. & Tafti, M. Genetic variation in EEG activity during sleep in inbred mice. *The American journal of physiology* **275**, R1127-1137 (1998).
32. Tort, A.B., Komorowski, R., Eichenbaum, H. & Kopell, N. Measuring phase-amplitude coupling between neuronal oscillations of different frequencies. *Journal of neurophysiology* **104**, 1195-1210 (2010).
33. Bandarabadi, M.*, et al.* Dynamic modulation of theta-gamma coupling during rapid eye movement sleep. *Sleep* **42**, 1-11 (2019).
34. Tort, A.B.*, et al.* Dynamic cross-frequency couplings of local field potential oscillations in rat striatum and hippocampus during performance of a T-maze task. *Proc Natl Acad Sci U S A* **105**, 20517-20522 (2008).
35. Mizuseki, K., Diba, K., Pastalkova, E. & Buzsaki, G. Hippocampal CA1 pyramidal cells form functionally distinct sublayers. *Nat Neurosci* **14**, 1174-1181 (2011).

Figures

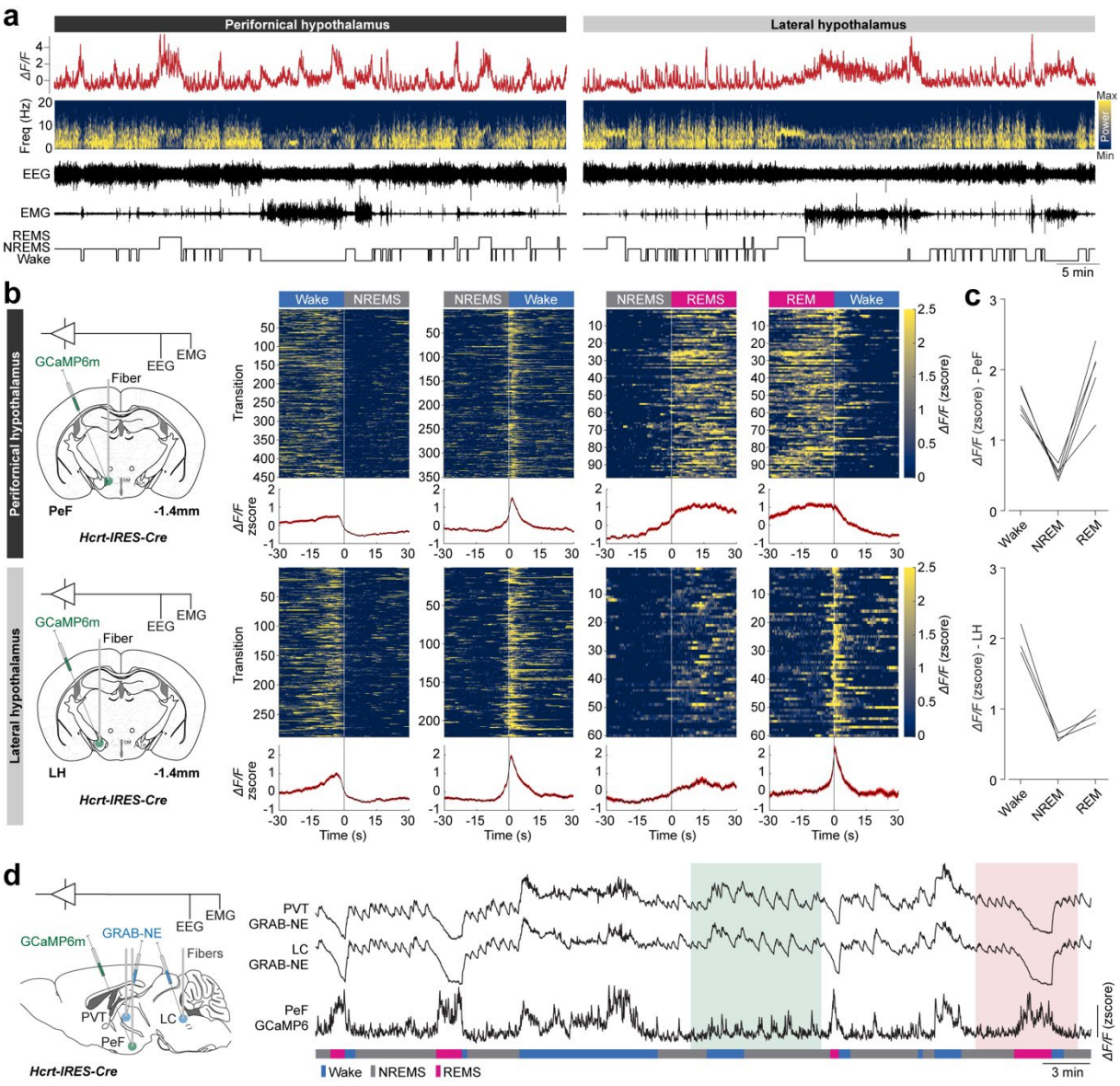


Fig. 1: Hypocretin neurons of the perifornical hypothalamus are REMS-active.

a) Representative fiber photometry calcium activity of hypocretin neurons in the PeF and LH, time-frequency representation of EEG signals, and raw EEG/EMG signals. Hypnogram is depicted below. **b)** Schematic coronal sections with fiber location for each condition, and transition panels of vigilance states for the PeF and the LH hypocretin neurons. Heatmaps show the normalized color-coded *ΔF/F* for each transition (top) and traces represent the averaged signals for the 30 sec before and after the transitions (bottom). Data represent recordings from 5 mice for the PeF and 3 mice for the LH. **c)** Average *ΔF/F* of calcium signal across each vigilance state during the whole recording session (12:00-15:00, ZT4-7; n = 5/3 mice for the PeF/LH). **d)** State-specific correlation between activity of PeF hypocretin neurons

and noradrenergic activity. Representative simultaneous multisite fiber photometry recordings of PeF hypocretin neurons using GCaMP6m and regional norepinephrine release in the LC and PVT using $GRAB_{NE}$. Green box highlights NREMS and wake activity and red one a REMS episode.

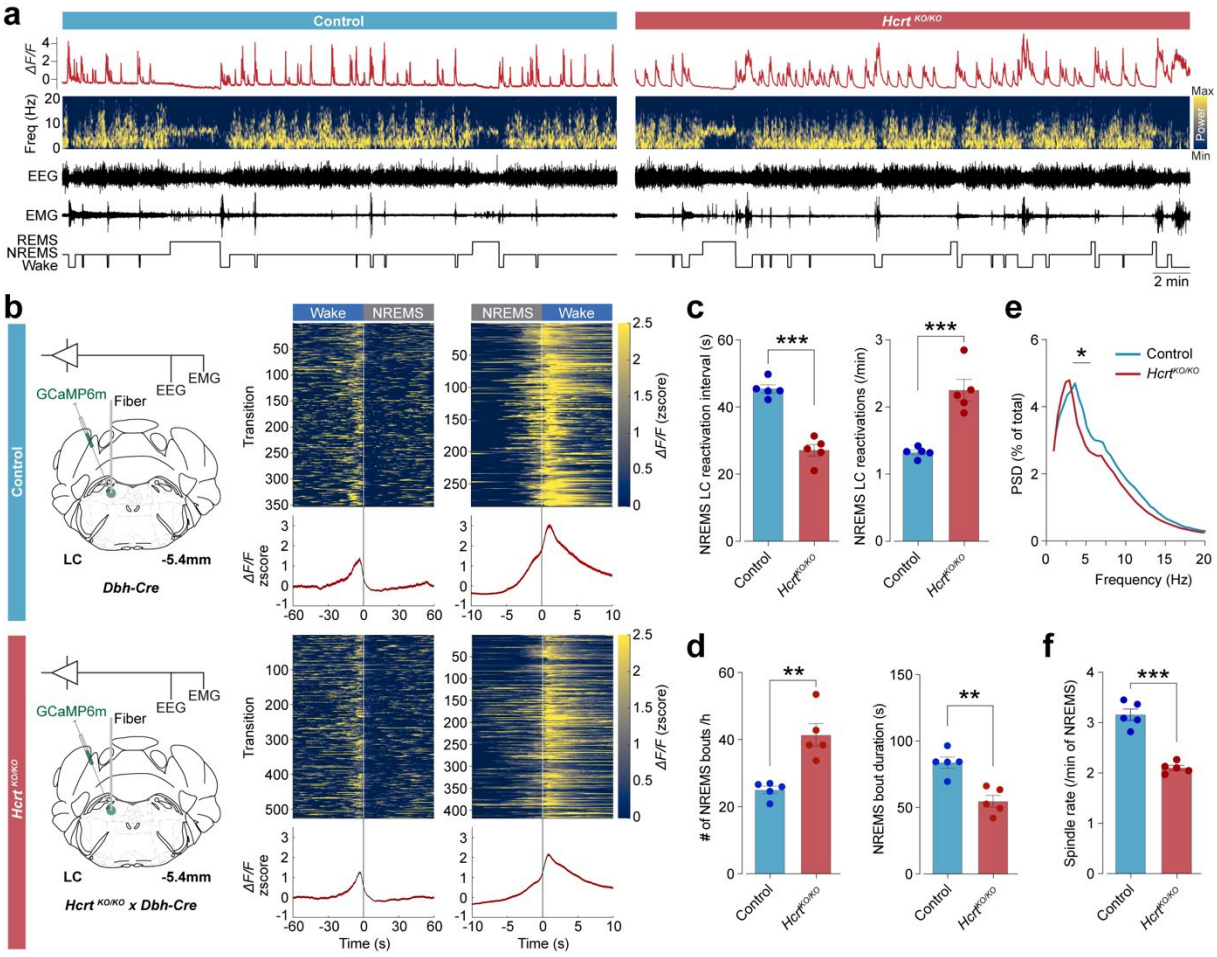


Fig. 2: Hypocretin mediates LC noradrenergic reactivations during NREMS.

a) Representative fiber photometry calcium activity of LC noradrenergic neurons, time-frequency representation of EEG signals, and raw EEG/EMG signals, from control and hypocretin knockout mice. Hypnogram is depicted below. **b)** Heatmaps show the normalized color-coded *ΔF/F* for each transition (top) and traces represent the averaged signals across the transitions (bottom). Data represent recordings from 5 control (*Dbh-Cre*) and 5 hypocretin knockout (*Hcrt*$^{KO/KO}$*xDbh-Cre*) mice. **c)** Quantification of LC noradrenergic reactivations per minute of NREMS ($p = 0.0005$, unpaired *t*-test), and the time interval between LC reactivations during NREMS ($p < 0.0001$, unpaired *t*-test). **d)** Number of NREMS bouts per hour (12:00-15:00; ZT4-7) ($p = 0.0017$, unpaired *t*-test) and mean duration of NREMS bouts in the same recording session ($p = 0.0015$, unpaired *t*-test). **e)** PSD analysis during NREMS (two-way ANOVA, interaction: $p < 0.0001$, followed by Sidak test). **f)** number of spindles per minutes of NREMS ($p < 0.0001$, unpaired *t*-test). n = 5 mice per group.

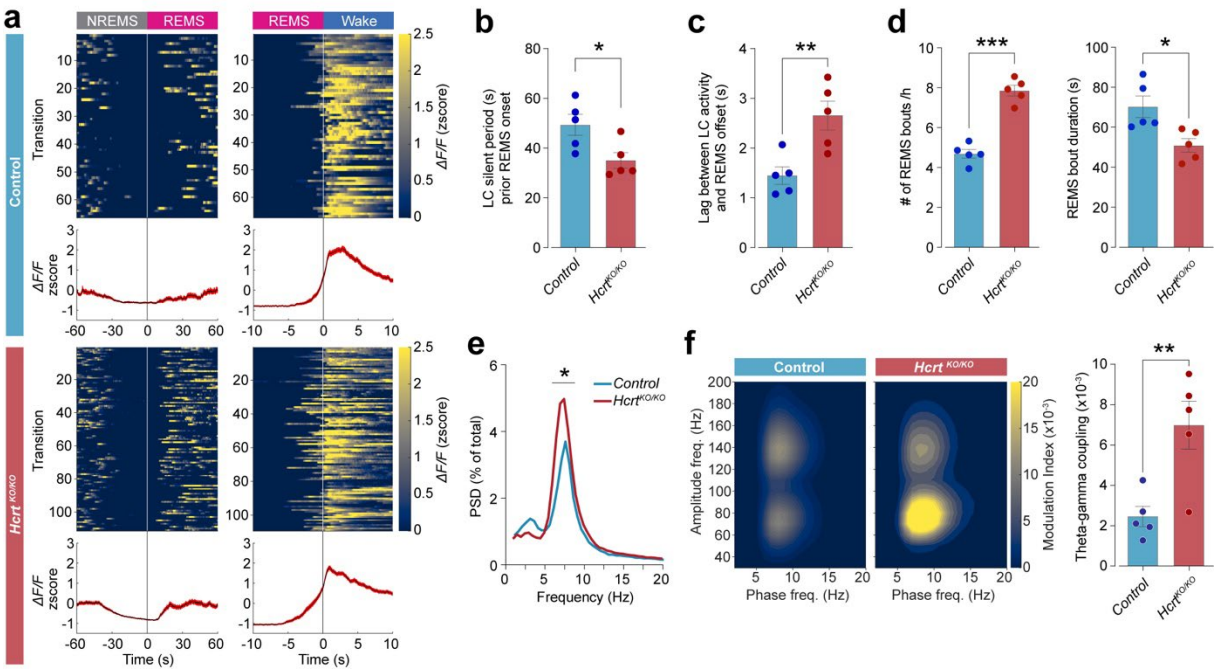
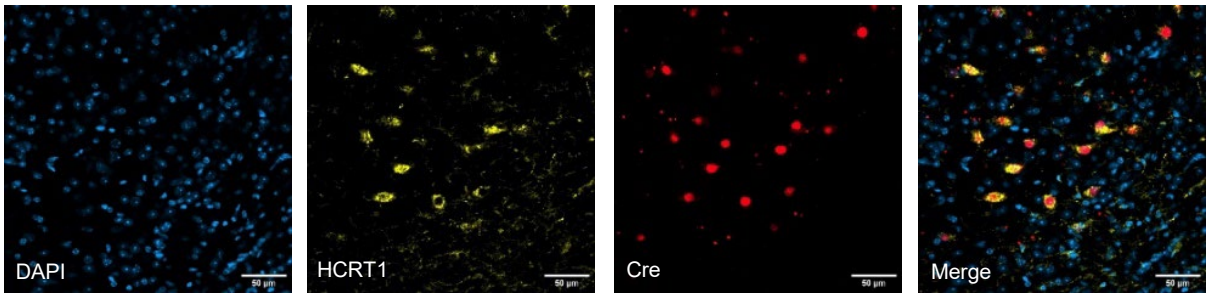


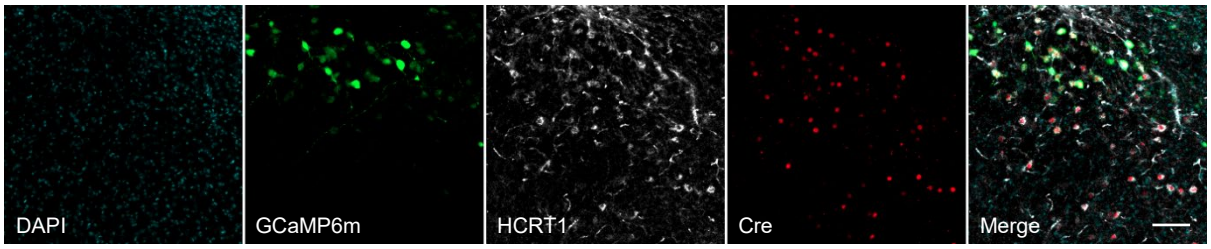
Fig. 3: Lack of hypocretin dysregulates noradrenergic activity at REMS onset and offset. **a)** Transition panel for REMS onset and offset in *Dbh-Cre* (top) and *Hcrt*$^{KO/KO}$*xDbh-Cre* mice (bottom). Normalized color-coded *ΔF/F* of photometry Ca^{2+} activity for individual transitions and averaged signal across all the transitions. Data represent transitions for 5 (*Dbh-Cre*) and 5 (*Hcrt*$^{KO/KO}$*xDbh-Cre*) mice. **b)** LC silent period prior to REMS onset (p = 0.0279, unpaired *t*-test). **c)** Lag between LC noradrenergic activity and REMS termination (p = 0.0077, unpaired *t*-test). **d)** Number of REMS bouts per hour (12:00-15:00; ZT4-7) (p < 0.0001, unpaired *t*-test) and mean duration of REMS bouts in the same recording session (p = 0.0162, unpaired *t*-test). **e)** Power spectral density (PSD) during REMS (two-way ANOVA, interaction: p < 0.0001, followed by Sidak test). **f)** Heatmaps show comodulogram analysis for phase-amplitude cross-frequency coupling, and bars indicate the theta-gamma coupling level during REMS (p = 0.0078, unpaired *t*-test). n = 5 mice per group.

Supplementary figures



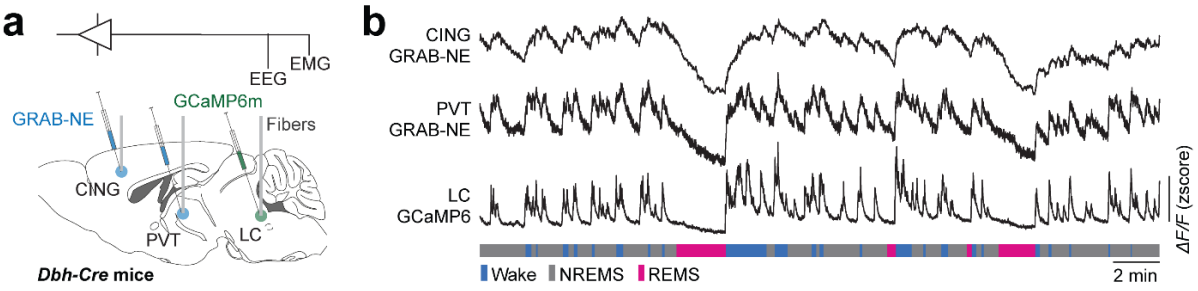
Supp. Fig. 1: Validation of the *Hcrt-IRES-Cre* mouse line.

Representative confocal acquisitions of a hypothalamic coronal section showing DAPI-, HCRT1- (Orexin-A), and Cre-stained neurons. Acquisitions merged show colocalization of HCRT1 and Cre. Scale bar: 50 µm.



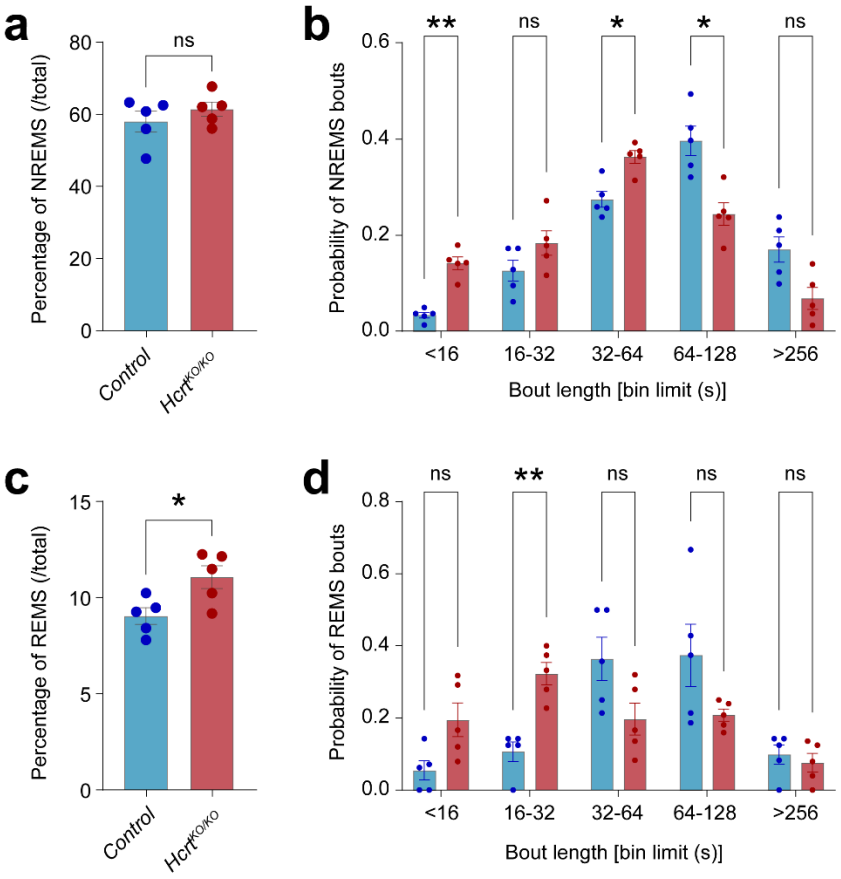
Supp. Fig. 2: Validation of GCaMP6m expression in hypocretin neurons.

Representative confocal acquisitions of a hypothalamic coronal section showing DAPI-, GCaMP6m-, HCRT1- (Orexin-A), and Cre-stained neurons. Acquisitions merged show colocalization of GCaMP6m-positive neurons and HCRT1/Cre neurons. Scale bar: 100 µm.



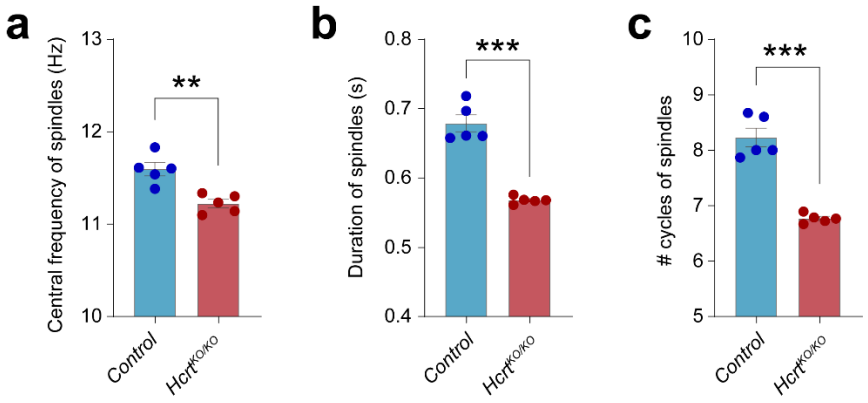
Supp. Fig. 3: Locus coeruleus noradrenergic activity correlates with the release of norepinephrine in the midline thalamus and prefrontal cortex.

a) Schematic of multisite fiber photometry of the locus coeruleus (LC), paraventricular thalamus (PVT), and cingulate cortex (CING). **b)** Multisite fiber photometry of LC noradrenergic activity and norepinephrine release in the midline thalamic and cortical regions in *Dbh-Cre* mice showed high synchronization between these signals across the vigilance states and their transitions. From bottom to top, hypnogram of vigilance states, *ΔF/F* of GCaMP6m in the LC, $GRAB_{NE}$ signal in the PVT, and $GRAB_{NE}$ signal in the CING.



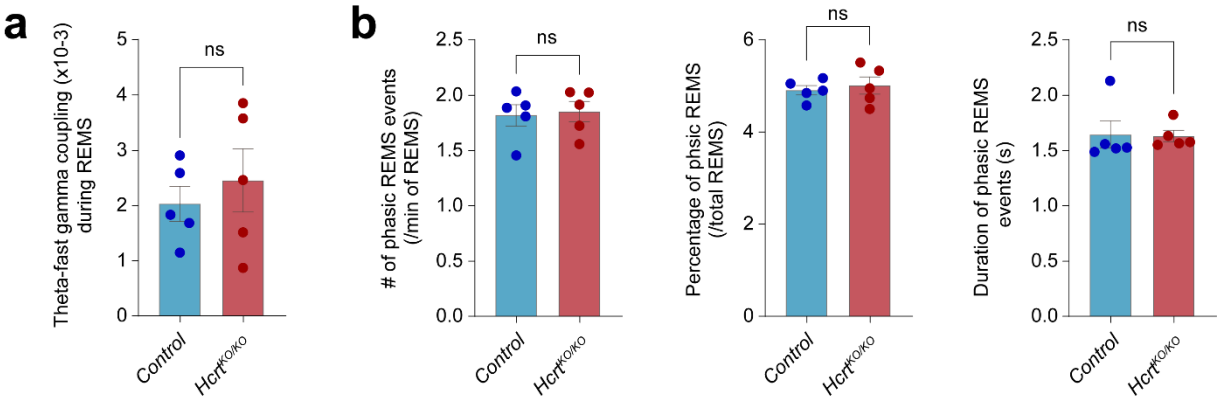
Supp. Fig. 4: Quantification of sleep bouts between *Hcrt*$^{KO/KO}$ and control mice.

a) Percentage of NREMS during the whole recording session (p = 0.3606, unpaired t-test). **b)** Distribution of NREMS bout durations indicates significantly shorter bouts in *Hcrt*$^{KO/KO}$ mice compared to controls (two-way ANOVA, interaction: $p < 0.0001$, followed by Sidak test). **c)** Percentage of REMS during the whole recording session (p = 0.0245, unpaired t-test). **d)** Distribution of REMS bout durations indicates significantly shorter bouts in *Hcrt*$^{KO/KO}$ mice compared to controls (two-way ANOVA, interaction: p = 0.0007, followed by Sidak test). n = 5 mice per group.



Supp. Fig. 5: Characterization of NREMS spindles in *Hcrt*$^{KO/KO}$ and control mice.

a) Central frequency of NREMS spindles is lower in *Hcrt*$^{KO/KO}$ mice compared to controls (p = 0.0024, unpaired t-test). **b)** Duration of NREMS spindles is shorter in *Hcrt*$^{KO/KO}$ mice compared to controls (p < 0.0001, unpaired t-test). **c)** Number of cycles per spindle is lower in *Hcrt*$^{KO/KO}$ mice compared to controls (p < 0.0001, unpaired t-test). n = 5 mice per group.



Supp. Fig. 6: Characterization of theta-fast gamma coupling and phasic REMS events in *Hcrt*$^{KO/KO}$ and control mice.

a) Theta-fast gamma coupling during REMS is not affected in the absence of hypocretin compared to controls (p = 0.5363, unpaired t-test). **b**) No significant differences were observed in the number (p = 0.8173, unpaired t-test), percentage (p = 0.6562, unpaired t-test) and duration (p = 0.9159, unpaired t-test) of phasic REMS events between *Hcrt*$^{KO/KO}$ and control mice. n = 5 mice per group.